Investigation of GLT-1d in Juvenile, Adult, and Pathological Mouse Brains

A thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

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Declaration of Authorship

I certify that except where due acknowledgment has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

Seán Hugo Klinkradt
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List of Abbreviations

AAALAC  Association for Assessment and Accreditation of Laboratory Animal Care International
ALS  Amyotrophic lateral sclerosis, also known as Lou Gehrig’s Disease and motor neurone disease
AMPA  \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA  Analysis of variance
ASCT(s)  Alanine-serine-cysteine-threonine transporter(s)
BDNF  Brain-derived neurotrophic factor
BSA  Bovine serum albumin
bp  Base pairs
c  Contralateral hemisphere
C57BL/6  An inbred strain of mouse that is widely used in research
CA  Cornu ammonis, fields of the hippocampus, i.e., CA1–CA3
cDNA  Complementary DNA
COS-7  African green monkey, Cercopithecus aethiops, kidney fibroblast-like cell line
CT  C-terminal of a protein, i.e., EAAT2c and EAAT2d C-terminals
DHK  Dihydrokainate
DNA  Deoxyribonucleic acid
dNTPs  Deoxynucleotide triphosphates
EAAC1  Excitatory amino acid carrier 1 (Humans = EAAT3)
EAAT  Excitatory amino acid transporter
EAAT2a  Human homolog of rodent GLT-1a
EAAT2c  Human homolog of rodent GLT-1c
EAAT2d  Human homolog of rodent GLT-1d
EAAT4  Excitatory amino acid transporter 4
EAAT5  Excitatory amino acid transporter 5
EAATs  Excitatory amino acid transporters
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
ESC  End-stage control
ESS  End-stage SOD1
Fab  Fragment antigen-binding, a limited proteolytic cleavage product of rabbit IgG cleaved by papain
FALS  Familial amyotrophic lateral sclerosis
Fc  Fragment that crystallizes, a limited proteolytic cleavage product of rabbit IgG cleaved by papain
FGF-2  Fibroblast growth factor 2
GABA  \(\gamma\)-amino butyric acid
GLAST  Glutamate aspartate transporter (Humans = EAAT1)
GLAST-1  Corresponds to GLAST in brain, but is found in the plasma membrane of osteoblasts and osteocytes
Gln  Glutamine
Glt\(\text{ph}\)  Aspartate transporter of Pyrococcus horikoshii, a glutamate transporter homologue
GLT-1  Glutamate transporter 1 (Humans = EAAT2)
GLT-1a  Glutamate transporter 1, splice variant a
GLT-1b  Glutamate transporter 1, splice variant b
GLT-1c  Glutamate transporter 1, splice variant c
GLT-1d  Glutamate transporter 1, splice variant d
<table>
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<th>Definition</th>
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<tr>
<td><strong>Glu</strong></td>
<td>Glutamate</td>
</tr>
<tr>
<td><strong>GPCRs</strong></td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td><strong>GRAVY</strong></td>
<td>Grand average of hydropathicity</td>
</tr>
<tr>
<td><strong>GS</strong></td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td><strong>hnRNP</strong>s</td>
<td>Heterogeneous nuclear ribonucleoproteins</td>
</tr>
<tr>
<td><strong>HPLC</strong></td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td><strong>i</strong></td>
<td>Ipsilateral hemisphere</td>
</tr>
<tr>
<td><strong>Ig</strong></td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td><strong>IUPHAR</strong></td>
<td>International Union of Basic and Clinical Pharmacology</td>
</tr>
<tr>
<td><strong>kb</strong></td>
<td>Kilobase, a length of nucleic acids that is equal to 1000 base pairs</td>
</tr>
<tr>
<td><strong>kDa</strong></td>
<td>Kilodalton</td>
</tr>
<tr>
<td><strong>KLH</strong></td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td><strong>MALDI-TOF</strong></td>
<td>Matrix assisted laser desorption/ionization-time of flight, an ionization technique used in mass spectrometry</td>
</tr>
<tr>
<td><strong>MCAO</strong></td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td><strong>MGlur</strong>(s)</td>
<td>Metabotropic glutamate receptor(s)</td>
</tr>
<tr>
<td><strong>MHC</strong></td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td><strong>MND</strong></td>
<td>Motor neurone disease</td>
</tr>
<tr>
<td><strong>mRNA</strong></td>
<td>Messenger RNA</td>
</tr>
<tr>
<td><strong>MSC</strong></td>
<td>Mid-stage control</td>
</tr>
<tr>
<td><strong>MSS</strong></td>
<td>Mid-stage SOD1</td>
</tr>
<tr>
<td><strong>MW</strong></td>
<td>Molecular weight markers</td>
</tr>
<tr>
<td><strong>NMDA</strong></td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td><strong>NP-40</strong></td>
<td>Nonyl phenoxypolyethoxyl ethanol</td>
</tr>
<tr>
<td><strong>OLAW</strong></td>
<td>Office of Laboratory Animal Welfare, a body responsible for oversight of the care and use of animals in research funded by the United States of America’s Public Health Service</td>
</tr>
<tr>
<td><strong>OSC</strong></td>
<td>Onset control</td>
</tr>
<tr>
<td><strong>OSS</strong></td>
<td>Onset SOD1</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>Postnatal day, e.g., P_{10} is postnatal day 10</td>
</tr>
<tr>
<td><strong>PACAP</strong></td>
<td>Pituitary adenylate cyclase-activating polypeptide</td>
</tr>
<tr>
<td><strong>PAG</strong></td>
<td>Phosphate-activated glutaminase</td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td><strong>PDZ</strong></td>
<td>A peptide domain originally found in three proteins that comprise the domain, i.e., post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (Zo-1)</td>
</tr>
<tr>
<td><strong>PICK1</strong></td>
<td>Protein Interacting with C Kinase - 1</td>
</tr>
<tr>
<td><strong>pre-mRNA</strong></td>
<td>Precursor messenger RNA</td>
</tr>
<tr>
<td><strong>PSC</strong></td>
<td>Pre-symptomatic control</td>
</tr>
<tr>
<td><strong>PSS</strong></td>
<td>Pre-symptomatic SOD1</td>
</tr>
<tr>
<td><strong>qPCR</strong></td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td><strong>Rb 1713</strong></td>
<td>Polyclonal antiserum raised in Rabbit 1713 or the rabbit itself depending on the context</td>
</tr>
<tr>
<td><strong>Rb 1714</strong></td>
<td>Polyclonal antiserum raised in Rabbit 1714 or the rabbit itself depending on the context</td>
</tr>
<tr>
<td><strong>Rb 1715</strong></td>
<td>Polyclonal antiserum raised in Rabbit 1715 or the rabbit itself depending on the context</td>
</tr>
<tr>
<td><strong>Rb 1716</strong></td>
<td>Polyclonal antiserum raised in Rabbit 1716 or the rabbit itself depending on the context</td>
</tr>
<tr>
<td><strong>RNA</strong></td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td><strong>SALS</strong></td>
<td>Sporadic amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td><strong>SDCT2</strong></td>
<td>Sodium-dependent dicarboxylate transporter number 2</td>
</tr>
<tr>
<td><strong>SDS</strong></td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td><strong>SDS-PAGE</strong></td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td><strong>SLC1</strong></td>
<td>Solute Carrier family 1, also known as the high-affinity glutamate and neutral amino acid transporter family</td>
</tr>
<tr>
<td><strong>SNAT</strong></td>
<td>Glutamine transporter</td>
</tr>
<tr>
<td><strong>SNAT1</strong></td>
<td>Glutamine transporter, isoform 1</td>
</tr>
<tr>
<td><strong>SNAT2</strong></td>
<td>Glutamine transporter, isoform 2</td>
</tr>
<tr>
<td><strong>SNAT3</strong></td>
<td>Glutamine transporter, isoform 3</td>
</tr>
<tr>
<td><strong>SNAT4</strong></td>
<td>Glutamine transporter, isoform 4</td>
</tr>
<tr>
<td><strong>SNAT5</strong></td>
<td>Glutamine transporter, isoform 5</td>
</tr>
<tr>
<td><strong>SOD1</strong></td>
<td>Cu/Zn superoxide dismutase or superoxide dismutase 1</td>
</tr>
<tr>
<td><strong>SR-proteins</strong></td>
<td>Serine/arginine-rich proteins</td>
</tr>
<tr>
<td><strong>TAE</strong></td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td><strong>TCA</strong></td>
<td>Tricarboxylic acid cycle</td>
</tr>
</tbody>
</table>
List of Abbreviations

**TEMED**
\(N,N,N',N'-\text{Tetramethylethylenediamine}\)

**TGF** Transforming growth factor

**TM** Preceding trade mark is unregistered in Australia, but is used to promote a brand or product

**tMCAO** Transient middle cerebral artery occlusion

**tMCAO (c)** Transient middle cerebral artery occlusion (contralateral hemisphere)

**tMCAO (i)** Transient middle cerebral artery occlusion (ipsilateral hemisphere)

**TNF** Tumor necrosis factor

**Tris** Tris(hydroxymethyl)aminomethane

**TrkB** Tyrosine kinase receptor-B

**UTR** Untranslated Region

**VGLUT** Vesicular glutamate transporter

**VGLUT1** Vesicular glutamate transporter, isoform 1

**VGLUT2** Vesicular glutamate transporter, isoform 2

**VGLUT3** Vesicular glutamate transporter, isoform 3
This thesis describes, for the first time, the expression, localisation, and potential relevance of GLT-1d, a newly discovered splice variant of the glutamate transporter GLT-1, in mouse brain. GLT-1d was identified in both rat and human brain during preliminary investigations. Importantly, during these preliminary investigations, GLT-1d was shown to be a functional and clinically relevant excitatory amino acid transporter that warranted further investigation. Since known GLT-1 splice variants are differentially expressed in terms of development, distribution, and disease, it was anticipated that GLT-1d may likewise be differentially expressed and distributed. Accordingly, investigation of the expression and distribution of GLT-1d in juvenile, adult, and pathological mouse brains was contemplated and ultimately led to the research for this thesis.

The aim of the research for this thesis was to investigate the expression and distribution of GLT-1d in juvenile, adult, and pathological mouse brains. In order to achieve this aim, the research for this thesis was carried out in four studies:

First (Chapter 4), antisera were produced using synthetic peptides that corresponded to the unique C-terminal amino acid sequences of rat and human GLT-1d. The antisera were then validated for use in the investigation of the expression and distribution of GLT-1d in mouse brain. This study established that one of the antisera produced, namely Rb 1713 antiserum, was a useful research tool to investigate GLT-1d in mouse brain. Interestingly, heating of samples prior to western blotting led to a \(~50 \text{kDa}\) downward shift in the apparent molecular weight of a higher molecular weight oligomer form of GLT-1d. It is plausible that this downward shift in apparent molecular weight of the higher molecular weight form of GLT-1d may be due to heat-induced dissociation of a protein partner, potentially PICK1.

Second (Chapter 5), the expression and regional and cellular distribution profile of GLT-1d was examined in juvenile mouse brain. This study demonstrated that GLT-1d exhibited a temporal expression and distribution profile in juvenile mouse brain that was
coordinated with the expression profiles of GLT-1a, GLT-1b, GLAST, glutamine synthetase, and VGLUT1, and that GLT-1d protein was expressed in astrocytes in juvenile mouse brain, a juvenile mouse being defined as postnatal day 1 (P1)–P21. GLT-1d expression increased most noticeably from P7–P14 in juvenile mouse brain, which suggested that there was an increasing demand for GLT-1d activity during mouse brain development and maturation. The increase in expression of GLT-1d was largely coincident with gliogenesis, synaptogenesis, a transition to mature forms and expression levels of glutamate receptors, and myelination. Based on the findings of this study, it is plausible that GLT-1d may be associated with the inception of and ensuing glutamatergic neurotransmission in juvenile mouse brain.

Third (Chapter 6), the expression, and regional and cellular distribution profile of GLT-1d was examined in adult mouse brain. This study established that GLT-1d mRNA and protein expression and distribution was highest in adult mouse forebrain, with enriched GLT-1d immunoreactivity observed in neuronal somata in layers IV–VI of the neocortex, CA1 and CA3 regions of the hippocampus, and the dentate gyrus, but also observed in astrocytes. The GLT-1d expression decreased in a rostrocaudal manner with the lowest level in the cerebellum. It is plausible that GLT-1d may play an important role in glutamate homeostasis in adult brain, particularly in the forebrain. Interestingly, the optic and auditory vestibular nerves and white matter of the cerebellum exhibited high levels of GLT-1d expression. The high level of GLT-1d expression in the white matter of the cerebellum, despite this region having the lowest level in general, suggested a possible role for GLT-1d protein in white matter tracts. The elevated GLT-1d expression in white matter tracts points to GLT-1d as a potential therapeutic target for neurodegenerative disease associated with white matter tracts, e.g., spinal injury and Alzheimer’s disease.

Finally (Chapter 7), the expression profile of GLT-1d in acute and chronic pathological adult mouse brains was examined in two mouse models. The mouse models were a transient focal cerebral ischaemic insult and reperfusion mouse model, i.e., the acute pathological condition, and an hSOD1G93A amyotrophic lateral sclerosis mouse model, i.e., the chronic pathological condition. The final study suggested that the modest reduction in GLT-1d expression observed in the contralateral hemisphere may have been due to dysfunction within/associated with the terminals of the intercortical glutamatergic neurons projecting into the contralateral hemisphere and/or changes in secretion by intercortical neurons projecting into the contralateral hemisphere of neurotrophic factors that are known to modulate GLT-1 expression. Intriguingly, no significant change was observed for GLT-1a mRNA in both the ipsilateral and contralateral hemispheres. One possible explanation for this finding may be the cellular distribution of GLT-1a and GLT-1d. GLT-1a, is predominantly expressed in astrocytes, whereas GLT-1d was expressed in both neurons and astrocytes in adult mouse brain. It is plausible, therefore,
given the substantial differences in sensitivity of neurons and astrocytes to glutamate that the neuronal and astrocytic GLT-1d may exhibit different kinetics in response to a mild cerebral ischaemic insult—as was the case in this study—relative to the predominantly astrocytic GLT-1a. Indeed, the sensitivity of GLT-1d to a mild cerebral ischaemic insult is an important finding. In the chronic pathological condition, the elevated GLT-1a and GLT-1d mRNA expression observed in the pre-symptomatic hSOD1G93A mouse motor cortex relative to the age-matched wildtype may be a compensatory phase during which neurons were more active in a hostile environment. The marked decline in GLT-1a and GLT-1d mRNA expression as the hSOD1G93A mouse motor cortex neurons approached the symptomatic stage, i.e., onset stage, may have been due to the neurons dying, which was followed by further compensatory phase during which the remaining neurons endeavoured to restore glutamate homeostasis in their immediate environment.

The characterisation of GLT-1d as set out in this thesis expanded the knowledge regarding GLT-1 and its association with the development, normal function, and pathological conditions of mouse brains. It is plausible that the knowledge derived here with respect to mouse brains may be useful in understanding the role of GLT-1d, and perhaps GLT-1 in general, in other mammalian brains.
GenBank Submissions, Presentations, Patent Applications, and Professional Affiliations

GenBank Submissions


Presentations


Provisional Patent Applications


Professional Affiliations

Seán Klinkradt is a Trans-Tasman Patent Attorney registered to practice in Australia and New Zealand, a Fellow of the Institute of Patent and Trade Mark Attorneys of Australia, and an Attorney of the High Court of South Africa.
Animal Ethics Committee Approval

Animal Ethics Committee approval was obtained for the collection of all animal tissues that were used in the experiments of this thesis.

Tissue collection was carried out under veterinary supervision in accordance with the following current Australian codes of practice and legislation:

1. The *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* 8th Edition 2013;

2. The *Code of Practice for the Housing and Care of Laboratory Mice, Rats, Guinea Pigs and Rabbits* made under the *Prevention of Cruelty to Animals Act 1986* and *Prevention of Cruelty to Animals Regulations 2008*; and


Best efforts were exercised during the design and conduct of the research for this thesis to minimize both the number of animals used and animal suffering.

Animal Ethics Committee Approval Letters are appended to this thesis as appendices C and D.
Part I

Context of the Present Studies
Motivation, Aim, and Research Questions

1.1 Motivation for the Research

A novel C-terminal splice variant of the excitatory amino acid transporter GLT-1, termed GLT-1d, was identified in both rat and human brain during preliminary investigations (set out in Chapter 3: Preliminary Study - Discovering GLT-1d of this thesis). Importantly, during these preliminary investigations, GLT-1d was shown to be a functional and clinically relevant excitatory amino acid transporter that warranted further investigation. Since known GLT-1 splice variants are differentially expressed in terms of development, distribution, and disease, it was anticipated that GLT-1d may likewise be differentially expressed and distributed. Accordingly, investigation of the expression and distribution of GLT-1d in juvenile, adult, and pathological mouse brains was contemplated and ultimately led to the research for this thesis.

1.2 Aim of the Research

The aim of the research for this thesis was to investigate the expression and distribution of GLT-1d in juvenile, adult, and pathological mouse brains.
Chapter 1: *Motivation, Aim, and Research Questions*

### 1.3 Research Questions

The questions underlying the research for this thesis were as follows:

1. Can synthetic peptides that correspond to the unique C-terminal amino acid sequences of rat and human GLT-1d be used to produce antisera that will be useful in the investigation of GLT-1d expression and distribution in mouse brain?

2. What is the temporal expression and distribution profile of GLT-1d in juvenile mouse brain?

3. What is the spatial expression and distribution profile of GLT-1d in adult mouse brain?

4. What is the pathological expression profile of GLT-1d in an acute pathological condition in mouse brain?

5. What is the pathological expression profile of GLT-1d in a chronic pathological condition in mouse brain?

The relationship between the research questions, experimental studies, and the relevant chapters of this thesis is set out in Table 1.1.

**Table 1.1:** Relationship between the research questions, experimental studies, and chapters of this thesis.

<table>
<thead>
<tr>
<th>Research Question</th>
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2

Background

2.1 Introduction

This chapter presents specific aspects of the background that underpinned the research completed for this thesis. The background is set out in four themes: The first theme relates to the excitatory amino acid glutamate. This theme also considers the synthesis and recycling of glutamate in mammalian brain. The second theme briefly covers glutamatergic neurotransmission and the role of glutamate receptors in mammalian brain. The third theme briefly reviews glutamate transporters with specific reference to the excitatory amino acid transporters and the vesicular glutamate transporters. The fourth theme relates to the excitatory amino acid transporter GLT-1 (EAAT2 in humans). This theme also considers alternative splicing, the known GLT-1 splice variants, and PDZ domain binding motifs.

Germane background relating to antibodies as research tools, and GLT-1 in juvenile, adult, and pathological brains is presented independently in Chapters 4, 5, 6, and 7 of this thesis.
Chapter 2: Background

Glutamate in Mammalian Brain

2.2 Glutamate, the Primary Excitatory Neurotransmitter

L-glutamate (glutamate) has a number of unique attributes that set it apart from other amino acids in the brain: its abundance, its role in numerous metabolic pathways and physiological processes, and its role as the primary excitatory neurotransmitter. The majority of the glutamate in the brain, 5–15 mmol/kg (5–15 mM) depending on the structure or region, is intracellular, with only ~1–2 µM found in the extracellular fluid. Such an abundance of glutamate is consistent with its participation in almost all aspects of normal function and pathological conditions of the brain. In terms of normal brain function, glutamate is at the crossroads of carbohydrate and amino acid metabolism, is a precursor of molecules such as γ-aminobutyric acid (GABA) and glutathione, and is associated with numerous physiological processes such as developmental plasticity and long-term potentiation, synapse induction and elimination, and migration and differentiation of cells within the brain. Importantly, of course, glutamate is the primary excitatory neurotransmitter in the brain. Approximately 40% of the synapses of the mammalian brain in general and 80–90% of the synapses of the neocortex use glutamate as the neurotransmitter. Not unexpectedly, therefore, glutamate dyshomeostasis is also implicated in a number of pathological conditions including excitotoxicity, cerebral ischaemia, and amyotrophic lateral sclerosis.

2.3 Glutamate Supply and Recycling

Glutamate in the brain is distributed into two pools: a neurotransmitter pool and a metabolic pool. The neurotransmitter pool consists of 35–45% of the total glutamate in the brain, with the remainder found in the metabolic pool. The metabolic pool provides glutamate that functions as, for example, a precursor for γ-aminobutyric acid (GABA), a building block in peptide and protein synthesis, and a metabolite in general metabolism. Exogenous glutamate is excluded from entering the brain by the blood-brain barrier. Accordingly, glutamate must be synthesized de novo in the brain. Glucose, the primary fuel in the brain, is metabolized in astrocytes to produce pyruvate. Pyruvate is converted into oxaloacetate by pyruvate carboxylase, i.e., an anaplerotic substrate. Pyruvate carboxylase is canonically considered an astrocytic enzyme, but has been reported in some neurons. De novo synthesis of glutamate from pyruvate does not, however, appear to be likely to occur in neurons at a physiologically relevant rate. In
astrocytes, oxaloacetate is converted into α-ketoglutarate via the citric acid cycle, and subsequently to glutamate by glutamate dehydrogenase,\textsuperscript{39} alanine transaminase,\textsuperscript{40,41} or branched chain aminotransferase.\textsuperscript{41–43} The astrocyte-specific enzyme glutamine synthetase converts glutamate into glutamine.\textsuperscript{44} Glutamine is transported into the extracellular space\textsuperscript{45,46} and then into a neuron\textsuperscript{47–50} where is it converted back into glutamate by phosphate-activated glutaminase.\textsuperscript{51–53} The glutamine transporter system comprises members of the $\text{Slc38}$ membrane transporter family which includes SNAT2, SNAT3, and SNAT5\textsuperscript{54–57} that transport glutamine out of astrocytes and SNAT1, SNAT2, and SNAT4\textsuperscript{54,55,57,58} that transport glutamine into neurons. Glutamine is neurophysiologically inert and can be released into the extracellular space and will not interact with either glutamate or γ-aminobutyric acid receptors.\textsuperscript{28}

Glutamatergic neurotransmission requires that the glutamate supply in presynaptic terminals be replenished to replace the glutamate that has been released into a synaptic cleft.\textsuperscript{15,38} Glutamate that has been released into the synaptic cleft can be transported by glutamate transporters, e.g., GLT-1 and GLAST (these two glutamate transporters are discussed further below), into astrocytes.\textsuperscript{14,59} As mentioned above, astrocyte-specific glutamine synthetase can convert glutamate into glutamine.\textsuperscript{44} Glutamine can then be transferred from the astrocyte into the extracellular space\textsuperscript{45,46} and then into a neuron\textsuperscript{47–50} where it can be converted back into glutamate by phosphate-activated glutaminase,\textsuperscript{51–53} i.e., completes the glutamate–glutamine cycle (Figure 2.1 on page 13).\textsuperscript{60–62} The glutamate produced by phosphate-activated glutaminase can be packaged into vesicles by the vesicular glutamate transporters (Section 2.6.2 - Vesicular Glutamate Transporters on page 18). The glutamate–glutamine cycle is an open cycle and does not operate in a stoichiometric manner.\textsuperscript{15,63} Accordingly, the synthesis of glutamine in astrocytes is necessary to maintain this cycle.\textsuperscript{15,63}

Glutamate that has been transported into astrocytes can also be converted into α-ketoglutarate by glutamate dehydrogenase.\textsuperscript{64} α-Ketoglutarate and metabolites derived from α-ketoglutarate, e.g., citrate and malate, can be transferred to neurons and provide glutamate precursors.\textsuperscript{65}

Transporting released glutamate back into a presynaptic terminal of an excitatory neuron would appear to be the most direct approach to recycle glutamate.\textsuperscript{38} The concentration of glutamate in the cytoplasm of excitatory neurons must, however, be high to drive the packaging of glutamate into vesicles by vesicular glutamate transporters.\textsuperscript{66} Accordingly, it would appear that this approach may likely be energetically unfavourable.\textsuperscript{38}

A detailed review of the glutamate-glutamine cycle is beyond the scope of this thesis. An exceptional treatment of this subject matter is available in a recent review by Marx \textit{et al.}\textsuperscript{38}
Chapter 2: Background

Figure 2.1: Schematic illustration of the glutamate–glutamine cycle depicting the transport of glutamate out of a synaptic cleft into an astrocyte. In the astrocyte, glutamate is converted into glutamine by glutamine synthetase. Glutamine is transported out of the astrocyte into the extracellular space and then into a presynaptic neuron. In the presynaptic neuron, glutamine is converted back into glutamate by phosphate-activated glutaminase. Abbreviations: glutamate (Glu), glutamine (Gln), glutamine synthetase (GS), ammonium ion (NH\(_4^+\)), phosphate-activated glutaminase (PAG), glutamine transporter isoforms 1–5 (SNAT1–5), and tricarboxylic acid (TCA). Modified from Figure 1 of Bak et al. with permission under copyright held by the authors Lasse K. Bak, Arne Schousboe, and Helle S. Waagepetersen.

Glutamatergic Neurotransmission and Glutamate Receptors in Mammalian Brain

2.4 Glutamatergic Neurotransmission

Glutamate, the primary excitatory neurotransmitter, acts on glutamate receptors that are located in the plasma membrane of both pre- and postsynaptic cells. The glutamate receptors are the predominant transducers and regulators of neurotransmission in the central nervous system. In addition to being closely associated with normal neurotransmission, abnormal glutamate receptor function is associated with the aetiology of numerous neurological diseases.
2.5 Glutamate Receptors

There are two main families of glutamate receptors: the ionotropic (ligand-gated ion channels) and the metabotropic (G-protein coupled) glutamate receptors. In addition to glutamate, both ionotropic and metabotropic glutamate receptors are responsive to alternative agonists, antagonists, and allosteric modulators. A detailed examination of such alternative agonists, antagonists, and allosteric modulators, and their effects on the ionotropic and metabotropic glutamate receptors is beyond the scope of this thesis.

It should be noted that the revised International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature for both ionotropic and metabotropic glutamate receptor subunits has been adopted for the discussion that follows.

2.5.1 Ionotropic Glutamate Receptors

The ionotropic glutamate receptor family comprises the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and δ receptor sub-families. The ionotropic family members, with the exception of the δ sub-family, were originally named for the selective agonist that activates the relevant sub-family members.

The NMDA receptors are widely distributed throughout the central nervous system and are heteromeric complexes consisting of combinations of subunits GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A, and GluN3B. The heteromeric complex formed by these subunits surround a calcium ion channel. Under resting potential conditions, however, the receptor channel is blocked by Mg$^{2+}$, thereby inhibiting the receptor. Splice variants of the NMDA subunits confer diverse physiological and pharmacological characteristics on the NMDA receptors. An important distinguishing characteristic of the NMDA receptors is the obligatory requirement for both glycine and glutamate for activation.

The AMPA receptors are heteromeric complexes composed of combinations of the GluA1, GluA2, GluA3, and GluA4 subunits. The heteromeric complexes define an ion channel that is Na$^+$, K$^+$, and, for some subunit combinations, Ca$^{2+}$ permeable. Splice variation of the subunits, particularly of the flip/flop exon, lead to a diverse range of functional AMPA receptors. The AMPA receptors mediate the majority of the fast excitatory neurotransmission. AMPA receptors are associated with normal brain functions such as learning and memory, and development, and perturbations are evident in neuropathologies such as Alzheimer’s disease and stroke.
Activation of the NMDA and AMPA receptors is associated with rapid synaptic excitation in the mammalian central nervous system. The role of each of these two receptor subtypes can be distinguished: the AMPA receptors are, under resting conditions, the primary mediators of rapid neurotransmission, whereas the NMDA detect the coincidence of glutamate release and postsynaptic depolarization.

The kainate and δ receptors are less well-characterised than the NMDA and AMPA receptors. The kainate receptors are complexes of the subunits GluK1, GluK2, GluK3, GluK4, and GluK5 and the δ receptors consist of GluD1 and GluD2. The kainate receptors form tetrameric complexes that are important mediators of both pre- and postsynaptic glutamate-associated effects, i.e., play subtle roles in the modulation of neurotransmission. In addition to their canonical ionotropic functions, kainate receptors have also been implicated in other functions that bear a resemblance to metabotropic signalling. GluD2 has been implicated in cerebellar function and presumably serves a receptor function.

### 2.5.2 Metabotropic Glutamate Receptors

The metabotropic glutamate receptors belong to family C of the G-protein coupled receptors (GPCRs) and can be divided into three groups based on their agonist pharmacology, sequence homology, and the relevant effector coupled to the G-protein: Group I (mGlu1 and mGlu5), Group II (mGlu2 and mGlu3) and Group III (mGlu4, mGlu6, mGlu7, and mGlu8). Unlike the ionotropic glutamate receptors, the metabotropic glutamate receptors do not comprise an ion channel. Stimulation of Group I receptors lead to the release of intracellular Ca²⁺ via the action of phospholipase C and inositol triphosphate production, whereas Group II and III receptors are associated with adenylate cyclase inhibition. The metabotropic glutamate receptors primarily play a modulatory role in the central nervous system and are, for example, associated with synaptic plasticity.

### Glutamate Transporters in Mammalian Brain

### 2.6 Glutamate Transporters

Glutamate transporters are expressed in a number of mammalian tissues including brain, bone, heart, intestine, kidney, liver, pancreas, skeletal muscle, and testis. In brain, glutamate transporters
primarily transport glutamate out of the extracellular space into cells to terminate glutamatergic signalling. On the other hand, glutamate transporters in tissues other than brain may fulfill different functions in addition to glutamate transport. Such different functions may, for example, be to perform as a glutamate-gated ion channel as in the case of GLAST-1 in bone. In keeping with the focus of this thesis, however, the discussion that follows is directed to glutamate transporters in mammalian brain.

Extracellular glutamate is transported into cells by glutamate transporters located in the plasma membrane. The plasma membrane glutamate transporters include the excitatory amino acid transporters (EAATs), a cystine/glutamate exchange transporter, and the sodium-dependent dicarboxylate transporter number 2 (SDCT2). Intracellular glutamate, on the other hand, is transported by glutamate transporters that include vesicular glutamate transporters (VGLUTs) and mitochondrial glutamate transporters. Interestingly, the EAATs and VGLUTs can be distinguished on the basis of functional properties such as substrate specificity and ion requirements. The excitatory amino acid transporters and vesicular glutamate transporters are considered further below.

2.6.1 Excitatory Amino Acid Transporters

The excitatory amino acid transporters (EAATs) belong to the solute carrier 1 (SLC1) family of transmembrane proteins. Five EAATs have been identified to date: GLT-1 (Slc1a2), GLAST (Slc1a3), EAAC1 (Slc1a1), EAAT4 (Slc1a6), and EAAT5 (Slc1a7). Rodent and human proteins and genes are designated in this thesis using standard nomenclatures. The EAATs are Na\(^+\)-dependent transporters of L-glutamate (glutamate), L-aspartate, and D-aspartate, but not D-glutamate. The human forms of GLT-1, GLAST, and EAAC1 are known as EAAT2, EAAT1, and EAAT3, respectively. In the discussion that follows, the relevant EAATs are generally referred to as GLT-1, GLAST, EAAC1, EAAT4, and EAAT5.

The EAATs are 500–600 amino acid residue proteins that exhibit \(\sim\)50% identity and \(\sim\)60% similarity within species. The transmembrane topology of an EAAT monomer consists of eight putative transmembrane domains, two putative hairpin loops, and N- and C-terminals located in the cytoplasm. Although the higher order structure of EAAT monomers is beginning to be defined, structural studies relating to archaeal and bacterial orthologs and other transporters have to date provided the best indication of a putative structure. The archaeal glutamate transporter Glt\(_{ph}\) of Pyrococcus horikoshii is bowl-shaped trimer. The bowl of Glt\(_{ph}\) faces the extracellular
environment and extends halfway into the membrane bilayer. The base of the bowl comprises three independent binding sites. Glt$_{PH}$ shares 37% amino acid identity with the human form of GLT-1, i.e., human excitatory amino acid transporter 2 (EAAT2).

The EAATs are all expressed in the brain, but exhibit specific distribution patterns depending on the region and particular cell type. GLT-1 is the primary glutamate transporter of the forebrain and predominantly an astrocytic glutamate transporter. The highest levels of expression of GLT-1 are seen in the hippocampus and neocortex. Interestingly, GLT-1 protein represents ~1% of the total protein in the brain and is responsible for ~95% of glutamate uptake in the forebrain. GLAST, also an astrocytic glutamate transporter, is the primary glutamate transporter found in the cerebellum, cochlea, circumventricular organs, and retina. EAAC1 is highly expressed in neurons of the hippocampus, cerebellum, and basal ganglia, but is widely distributed in neurons in the brain. EAAT4 is distributed throughout the brain, but is particularly abundant in the Purkinje neurons of the molecular layer of the cerebellum. EAAT5 is thought to be primarily a retinal glutamate transporter. EAAT4 and EAAT5 appear to act more like slow-gated glutamate receptors/a glutamate buffering system, as opposed to high-capacity glutamate transporters.

The primary function of the EAATs in the central nervous system is to maintain a low concentration of glutamate in the extracellular space. In addition to controlling the concentration of glutamate in the synapses, the EAATs also control the diffusion of glutamate between synapses. A low concentration of glutamate in the extracellular space mitigates the risk of chronic glutamate receptor activation/desensitization and/or excitotoxicity and ensures an appropriate signal-to-noise ratio for the purposes of glutamatergic neurotransmission. The EAATs, primarily GLT-1 and GLAST, stoichiometrically transport glutamate into cells by a secondary active, electrogenic process (Figure 2.2 on page 18). The electrogenic process comprises the co-transport of a glutamate molecule, 3 Na$^+$ ions, and a H$^+$ ion, and the counter-transport of a K$^+$ ion. As a consequence of such transport, the EAATs mediate an electrogenic inward-rectifying current that arises from the net transport of two positive charges with each glutamate molecule transported, and a thermodynamically uncoupled anion conductance current. The anion conductance current results from Cl$^-$ ion translocation that is activated by glutamate binding. The Cl$^-$ ion flux current of GLT-1, GLAST, and EAAC1 is low relative to the current associated with the ion-coupled co-transport. In contrast, the Cl$^-$ ion flux current of EAAT4 and EAAT5 is significantly higher than the ion-coupled co-transport.
Figure 2.2: Schematic illustration of the stoichiometric transport of glutamate and the uncoupled anion conductance of Cl\(^-\) by an excitatory amino acid transporter. Excitatory amino acid transporters stoichiometrically transport glutamate into cells by an electrogenic process. The electrogenic process comprises the co-transport of a glutamate molecule, 3 Na\(^+\), and a H\(^+\)\(^{177-180}\) and the counter-transport of a K\(^+\).\(^{180-183}\) The Cl\(^-\) translocation is activated by glutamate binding.\(^{106,107,186}\) Glutamate transporters exhibit two phase kinetics: fast binding and slow transport of glutamate.\(^{187}\) Abbreviations/Symbols: Cl\(^-\) (chloride ion), excitatory amino acid transporter (EAAT), Glu\(^-\) (L-glutamate), H\(^+\) (hydrogen ion or proton), K\(^+\) (potassium ion), and Na\(^+\) (sodium ion).

The EAATs can be distinguished on the basis of their functional properties and \(K_m\) values for glutamate transport. GLT-1, GLAST, and EAAC1 are relatively similar in terms of functional properties and glutamate affinity.\(^{102,116,177,179,188-192}\) Noticeably, amongst the EAATs, EAAT4 has the highest glutamate affinity,\(^{106,193-195}\) whereas EAAT5 has the lowest glutamate affinity.\(^{166,167}\)

A full discussion of the distribution and functional properties of GLAST, EAAC1, EAAT4, and EAAT5, which have been reviewed previously,\(^{14,187,196}\) is beyond the scope of this thesis. GLT-1, and its known splice variants, is considered in more detail below (Section 2.7 - GLT-1 on page 19).

2.6.2 Vesicular Glutamate Transporters

Vesicular glutamate transporters (VGLUTs) transport intracellular glutamate into synaptic vesicles.\(^{66,126,127}\) Three VGLUTs have been identified and functionally characterized: VGLUT1,\(^{197-199}\) VGLUT2,\(^{126,200-203}\) and VGLUT3.\(^{204-207}\) The VGLUTs are proteins of
~600 amino acid residues, which share ≥70% homology. The VG-LUTs exhibit distinct distribution patterns in the brain. VGLUTs 1 and 2 are expressed in glutamatergic presynaptic neurons, whereas VGLUT3 is expressed in GABAergic, cholinergic and monoaminergic neurons. The VGLUTs can be distinguished from the excitatory amino acids transporters in terms of their Na$^+$ and K$^+$ independence and lower glutamate affinity. In contrast to the excitatory amino acids transporters, the uptake of glutamate by the VGLUTs is dependent upon a proton electrochemical gradient generated by the vesicular proton ATPase.

**GLT-1, Alternative Splicing, and PDZ Domain Binding Motifs**

### 2.7 GLT-1

#### 2.7.1 GLT-1: Features and Function

GLT-1 is an integral membrane protein (Figure 2.3 on page 20) that, like the other excitatory amino acid transporters (EAATs), comprises eight putative transmembrane domains, two putative hairpin loops, and N- and C-terminals located in the cytoplasm. GLT-1 monomers associate into a trimeric quaternary structure. In the central nervous system, GLT-1 is highly expressed in adult neocortex, hippocampus, lateral septum, and striatum and is typically more abundant in perisynaptic regions. Such perisynaptic distribution supports the view that GLT-1 protein is trafficked to areas of high demand. GLT-1 protein is primarily, although not exclusively, an astrocytic protein that, with the exception of retina, has been considered elusive in neurons of adult and normal brain. In contrast, GLT-1 protein has been detected in neurons of juvenile and pathological brain and in neuronal cell culture. Interestingly, in contrast to GLT-1 protein, almost all cells in the brain express GLT-1 mRNA. As mentioned previously, approximately 95% of the glutamate transport in the forebrain is mediated by GLT-1. The importance of GLT-1 with respect to glutamate homeostasis is supported by the finding that deletion of GLT-1 eliminates almost all glutamate transport in forebrain. Peripherally, GLT-1 protein is expressed in glandular tissues and perivenous hepatocytes. GLT-1 protein is susceptible to oxidation and post-mortem proteolysis of the C-terminal. Oxidation of GLT-1 protein may lead to the formation of aggregates, i.e., dimers and trimers, whereas post-mortem proteolysis typically leads to a loss of C-terminal immunoreactivity.
Chapter 2: Background

Figure 2.3: Putative two-dimensional and three-dimensional representations of GLT-1 in the plasma membrane as predicted based on the putative structure of the archaeal glutamate transporter GltPh of *Pyrococcus horikoshii*. [A] Two-dimensional schematic representation of the putative structure of GLT-1 in the plasma membrane. [B] Three-dimensional schematic side-view representation of the putative structure of GLT-1 in the plasma membrane. Each monomer of the trimer set out in [B] is coloured to correspond with the rainbow pattern defined in [A]. Abbreviation: HP (hairpin loop). Modified from Figure 4 of Divito and Underhill\textsuperscript{135} with permission under copyright held by Elsevier.

2.7.2 Modulation of GLT-1 Expression and Activity

GLT-1 expression and activity is affected by several modulators (Table 2.1) including:

1. Biomolecules such as endothelins, BDNF, EGF, FGF-2, PACAP, TGF\(\alpha\), and TNF\(\alpha\).\textsuperscript{238–249}
2. β-lactam antibiotics, e.g., ceftriaxone;\textsuperscript{250}

3. other compounds,\textsuperscript{251–253} including arachidonic acid;\textsuperscript{254–256} and

4. unlike the other excitatory amino acid transporters GLAST, EAAC1, EAAT4, and EAAT5, is inhibited by dihydrokainate and kainate.\textsuperscript{102}

Table 2.1: Modulation of GLT-1 expression and activity by biomolecules, antibiotics, and inhibitors.

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<th>Modulator</th>
<th>Cell Type</th>
<th>In vitro/In vivo</th>
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<tr>
<td>BDNF</td>
<td>Astrocytes</td>
<td>\textit{In vivo}\textsuperscript{248,249}</td>
</tr>
<tr>
<td>EGF</td>
<td>Astrocytes</td>
<td>\textit{In vitro}\textsuperscript{239}</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Astrocytes</td>
<td>\textit{In vitro}\textsuperscript{242}</td>
</tr>
<tr>
<td>PACAP</td>
<td>Astrocytes</td>
<td>\textit{In vitro}\textsuperscript{238}</td>
</tr>
<tr>
<td>TGF\textalpha</td>
<td>Astrocytes</td>
<td>\textit{In vitro}\textsuperscript{239,242}</td>
</tr>
<tr>
<td>TNF\textalpha</td>
<td>Astrocytes</td>
<td>\textit{In vitro}\textsuperscript{242}</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>Astrocytes</td>
<td>\textit{In vitro/In vivo}\textsuperscript{250}</td>
</tr>
<tr>
<td>Arachidonic Acid</td>
<td>Astrocytes</td>
<td>\textit{In vitro}\textsuperscript{254–256}</td>
</tr>
<tr>
<td>Dihydrokainate/Kainate</td>
<td>COS-7 cells</td>
<td>\textit{In vitro}\textsuperscript{102}</td>
</tr>
</tbody>
</table>

2.7.3 Alternative Splicing and GLT-1

2.7.3.1 Alternative Splicing

Eukaryotes use alternative splicing of precursor mRNA (pre-mRNA) to generate protein diversity.\textsuperscript{257,258} The basic types of alternative splicing include: intron retention, cassette-exon skipping or inclusion, alternative 5′-end splicing, and alternative 3′-end splicing (Figure 2.4 on page 22).\textsuperscript{259} Pre-mRNA is edited by spliceosomes,\textsuperscript{260} which are large complexes that comprise small nuclear ribonucleoproteins and accessory proteins.\textsuperscript{260,261} Splice enhancing mRNA sequences (enhancers) and splice silencing mRNA sequences (silencers) are distributed in exons and introns within the pre-mRNA.\textsuperscript{260–262} Many enhancers associate with serine/arginine-rich proteins (SR-proteins)\textsuperscript{263,264} to influence splicing adjacent to where the SR-proteins bind to the pre-mRNA.\textsuperscript{259,263} On the other hand, a number of silencers associate with heterogeneous nuclear ribonucleoproteins (hnRNPs)\textsuperscript{263} to influence splicing adjacent to where the hnRNPs bind to the pre-mRNA.\textsuperscript{259,263} The combined influence of the enhancers, silencers, SR-proteins, and hnRNPs determine whether a specific exon or intron is included in the mature RNA.\textsuperscript{263} A detailed description of the mechanisms and regulation of alternative pre-mRNA splicing is beyond the scope of this thesis. Recent reviews\textsuperscript{265–268} provide informative treatments of this complex subject matter.
Chapter 2: Background

A Intron retention

B Cassette-exon skipping or inclusion

C 5′-end splicing

D 3′-end splicing

Figure 2.4: Schematic illustration of the basic types of alternative precursor messenger RNA (pre-mRNA) splicing: A. intron retention, B. cassette-exon skipping or inclusion, C. alternative 5′-end splicing, and D. alternative 3′-end splicing. The centrally located rectangles represent pre-mRNA. The black lines indicate the regions that can be alternatively spliced, i.e., the lines above each set of rectangles indicate the splicing that took place to provide the mature mRNA to the left, and the lines below each set of rectangles indicate the splicing that took place to provide the mature mRNA to the right. Modified from Figure 1 of Nilsen and Graveley with permission under copyright held by the Nature Publishing Group.

Alternative pre-mRNA splicing is known to be associated with development, differentiation, distribution, and disease. Important structural changes introduced by alternative splicing include changes to the primary structure of the encoded protein that may influence binding between proteins. Such changes include, for example, the introduction of a PDZ domain binding motif in the known splice variants GLT-1b and GLT-1c.

2.7.3.2 GLT-1 Gene and Transcript

The GLT-1 gene extends over more than 100 kb and comprises 11 exons. The GLT-1 transcript is 10-12 kb and contains an unusually long 3′-untranslated region of ~10 kb. It would appear that the 3′-untranslated region may comprise regulatory elements that affect differential expression of at least the C-terminal splice variants. The protein encoding region of the transcript is approximately 1.7 kb.
Chapter 2: Background

Figure 2.5: The genomic structure of GLT-1 and the known N-terminal and C-terminal splice variants detected in brain. [A] Schematic illustration of the genomic structure of GLT-1 comprising exons 1–11 and 5′ and 3′ untranslated regions (not to scale). Exon 1 represents the untranslated 5′-untranslated region and exons 2–11 are coding sequences (CDS) 1–10. Exons known to undergo alternative splicing are shown in grey. [B] Schematic illustration of the 5′-end sequence of known GLT-1 C-terminal splice variants with exon 1 and putative exons A–E (not to scale). Arrows and double lines represent upstream start and stop signals, respectively. Truncated forms of putative exon A are shown as A* and A**. [C] Schematic illustration of the 3′-end sequence of known GLT-1 C-terminal splice variants with exon 10 and 11 depicted for GLT-1a, and exon 10 and the unique exons depicted for GLT-1b and GLT-1c (not to scale). Each unique exon of GLT-1b and GLT-1c is depicted as a small rectangle. The solid lines represent the 3′-untranslated region and dotted lines intronic sequences. The 3′-untranslated region of GLT-1b and GLT-1c comprises exon 11 of GLT-1a. Modified from Figure 2 of Lee and Pow, Figure 1 Rozyczka et al., and Figure 1 of Lauriat and McInnes with permission under copyright held by Elsevier (Figures 2.5 A and 2.5 B) and Springer Nature (Figure 2.5 C).
2.7.3.3 C-Terminal Splice Variants and PDZ Domain Binding Motifs

Initially, the GLT-1 C-terminal splice variant nomenclature developed idiosyncratically. The nomenclature has now, however, been standardized. Three C-terminal splice variants have been identified (according to the standardized nomenclature): GLT-1a, GLT-1b, and GLT-1c. GLT-1a is encoded by the 11 exons of the GLT-1 gene, whereas GLT-1b and GLT-1c are each encoded by exons 1–10, but include a portion of the intron directly after exon 10. Each of these splice variants comprises a unique C-terminal amino acid sequence (Table 2.2).

<table>
<thead>
<tr>
<th>Splice Variant</th>
<th>Unique C-terminal Peptide</th>
</tr>
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<tbody>
<tr>
<td>GLT-1a</td>
<td>VTLAANGKSADCSVEEEPWKREK</td>
</tr>
<tr>
<td>GLT-1b</td>
<td>PFPFLDIETCI</td>
</tr>
<tr>
<td>GLT-1c</td>
<td>SLHYVEYQSWV</td>
</tr>
</tbody>
</table>

All three of the C-terminal splice variants have been detected in the central nervous system. GLT-1a is the most abundant GLT-1 splice variant in brain, with low levels of GLT-1b observed, and GLT-1c barely detectable. GLT-1a, GLT-1b, and GLT-1c exhibit differences relative to sub-cellular distribution. GLT-1a is typically distributed around synapses in astrocytic plasma membranes, whereas GLT-1b is typically located away from synapses and around astrocyte somata. GLT-1c, on the other hand, is predominantly located in the endfeet of astrocytes.

The C-terminal variants cannot be distinguished on the basis of glutamate transport or anion conductance. Accordingly, it would appear that the unique C-terminal amino acid sequences do not confer detectable functional properties. Notwithstanding the apparent functional similarities, it is interesting to note that GLT-1a is rapidly trafficked to and from the plasma membrane, thus providing a constitutively cycling glutamate transporter that can be mobilized rapidly.

In contrast to GLT-1a, the final four C-terminal amino acids of both GLT-1b and GLT-1c define a class I PDZ domain binding motif (Table 2.3). A class I PDZ domain binding motif comprises an X-S/T-X-Φ amino acid sequence, where X may be any amino acid and Φ is a hydrophobic amino acid. GLT-1b has been shown to interact with the PDZ proteins PICK1 and PSD95. To date, no PDZ proteins that interact with GLT-1c have been identified.
Table 2.3: PDZ domain binding motifs in GLT-1b and GLT-1c.

<table>
<thead>
<tr>
<th>Splice Variant</th>
<th>PDZ Domain Binding Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLT-1b</td>
<td>ETCI</td>
</tr>
<tr>
<td>GLT-1c</td>
<td>QSWV</td>
</tr>
</tbody>
</table>

2.7.3.4 N-terminal Splice Variants

Four GLT-1 N-terminal splice variants have been identified (Table 2.4). Three of these splice variants have been detected in brain and a fourth in liver.\textsuperscript{2,232} The brain N-terminal splice variants are MASTEG, MPK, and MKRPKEHSIQRS\textsuperscript{2,102,105,283,286,295} and the liver is MVS.\textsuperscript{2,232} The tissue specific distribution suggests different promoters in the brain and liver are associated with the expression of these splice variants.\textsuperscript{294}

Table 2.4: Known GLT-1 N-terminal splice variants.

<table>
<thead>
<tr>
<th>Unique N-terminal Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>MASTEG\textsuperscript{2,102,105,283,286,295}</td>
</tr>
<tr>
<td>MPK\textsuperscript{283,295}</td>
</tr>
<tr>
<td>MKRPKEHSIQRS\textsuperscript{283}</td>
</tr>
<tr>
<td>MVS\textsuperscript{2,232}</td>
</tr>
</tbody>
</table>

Interestingly, the GLT-1 N-terminal splice variants are not distinct in terms of functional and pharmacological properties when compared to the GLT-1 C-terminal splice variants (Section 2.7.3.3 - C-Terminal Splice Variants and PDZ Domain Binding Motifs on page 24).\textsuperscript{294} As such, it would appear that N- and C-terminal splice variation is more likely associated with expression and/or cellular localization of the splice variants rather than the regulation of glutamate transport.\textsuperscript{294}

2.7.3.5 Exon-Skipping and Intron Retention Splice Variants

Although at least six GLT-1 exon skipping (exon\(\Delta\)) splice variants have been identified (Table 2.5), not all of the corresponding proteins have yet been detected.\textsuperscript{294} Exon\(\Delta 4\) and exon\(\Delta 9\) splice variant protein has, however, been detected in rodent brain.\textsuperscript{281,282} It is unclear at this stage whether exon\(\Delta 4\) protein is intrinsically a functional transporter or affects the trafficking of full length GLT-1 to its functional location.\textsuperscript{282,296} Interestingly, exon\(\Delta 9\) protein is predominantly associated with white matter in the brain.\textsuperscript{281}

A GLT-1 intron retention splice variant that includes intron 7 has been identified.\textsuperscript{299} Initially, this intron retention splice variant was thought to be associated with amyotrophic lateral sclerosis,\textsuperscript{299} but this was subsequently shown not to be the case.\textsuperscript{300} In any event,
Table 2.5: Known GLT-1 exon-skipping (exonΔ) splice variants.

<table>
<thead>
<tr>
<th>Splice Variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>exonΔ4282</td>
</tr>
<tr>
<td>part exonΔ6/part Δ7271,297</td>
</tr>
<tr>
<td>exonΔ7298</td>
</tr>
<tr>
<td>exonΔ7Δ9298</td>
</tr>
<tr>
<td>part exonΔ7/part exonΔ11271,297</td>
</tr>
<tr>
<td>exonΔ9271,297</td>
</tr>
</tbody>
</table>

retained intron 7 introduces a premature termination codon that presumably results in nonsense-mediated decay of this splice variant.301

2.7.3.6 5′-UTR Splice Variants

GLT-1 5′-untranslated region (UTR) splice variants have been identified.283,295,302,303 Such splice variants appear to be widespread and functionally important.283,295,302 Alternative promoters contained in some of the 5′-UTR splice variants may facilitate cellular- and regionally specific expression of those variants.304,305 At least regional specificity is supported by the identification of tissue specific forms of GLT-1.2 5′-UTR splice variants that generate short upstream open reading frames (ORFs) tend to result in reduced expression of the GLT-1 protein.5 The reduced expression is thought to be a consequence of dissociation of the ribosome from the transcript.306 Interestingly, the translation of GLT-1 splice variants having a 5′-UTR longer than ~310 nucleotides is highly regulated by substances such as corticosterone, retinol, and β-lactam antibiotics.303

2.7.3.7 3′-UTR Splice Variants

GLT-1 splice variants with unusually long 3′-untranslated regions (UTRs) have been identified.307,308 These 3′-UTRs may contain regulatory elements that are associated with the control of mRNA localization, translation efficiency, mRNA stability, and post-transcriptional control.216,294,308 A conserved 3′-end sequence of the GLT-1a 3′-UTR was observed across species.308 Such conservation suggests that an important regulatory control function is associated with the conserved sequence.294 Interestingly, alignment of the 3′-UTRs of GLT-1a and GLT-1b indicate that these variants comprise unique elements in each respective 3′-UTR.294 Such unique elements suggest potential regulatory elements within the 3′-UTR may be associated with differential expression of these variants.294 The full 3′-UTR sequence of GLT-1c has yet to be sequenced, accordingly it is not yet known whether it contains similar potential regulatory elements as observed in GLT-1a and GLT-1b.294
Chapter 2: Background

2.7.4 Neuronal Expression of GLT-1

A so-called "conundrum" exists with respect to the neuronal expression of GLT-1. GLT-1 mRNA is widely expressed in neurons in the brain. On the other hand, both GLT-1 mRNA and protein expression has been detected in neuronal cell culture, mammalian retina, mouse developing spinal cord neurons, and post-hypoxic-ischaemic brain neurons. Furthermore, evidence exists for an unknown form of presynaptic GLT-1, in particular direct neuronal glutamate uptake. Notwithstanding a very active research effort in many laboratories around the world, the GLT-1 protein has, however, for the most part been considered elusive in neurons of adult and normal brain.

2.7.5 GLT-1: Juvenile, Adult, and Pathological Brains

The expression and distribution of GLT-1 in juvenile, adult, and pathological mouse brains is explored in Chapters 5–7 of this thesis.

2.7.6 Simplification of GLT-1 Nomenclature in the Literature

Holmseth et al. adopted a useful approach to simplify the classification of all published GLT-1 splice variants by reference only to the unique amino acid sequences of their C-terminals. This approach was adopted in the chapters of this thesis that follow. Briefly, the antigens that were used to raise the antibodies and/or polynucleotide sequences of primers/probes in cited publications were cross-referenced to the amino acid sequences set out in Table 2.6, i.e., the unique amino acid sequences of the C-terminals (set out here again for ease of reference). The relevant GLT-1 splice variant(s) were designated accordingly.

Table 2.6: Simplified nomenclature of known GLT-1 splice variants.

<table>
<thead>
<tr>
<th>C-Terminal Amino Acid Sequence</th>
<th>Common Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTLAANGKSADCSVEEEPWKREK²,102,105,270,285</td>
<td>GLT-1a</td>
</tr>
<tr>
<td>VPFPLDIFETCI²,285,286</td>
<td>GLT-1b</td>
</tr>
<tr>
<td>SLHYVEYQSWV²87</td>
<td>GLT-1c</td>
</tr>
</tbody>
</table>
3.1 Synopsis

_Aim_: This preliminary study investigated whether any potential novel GLT-1 C-terminal splice variants existed, and whether an identified novel GLT-1 C-terminal splice variant, termed GLT-1d, was functional and clinically relevant.

_Research Question_: Is the additional GLT-1 immunolabelling detected in human dentate gyrus relative to GLT-1a due to one or more novel, functional, and clinically relevant GLT-1 C-terminal splice variant(s)?

_Methods_: Immunohistochemistry, identification of potential donor splice sites in the human EAAT2 (human homolog of GLT-1) gene intron 10, PCR, polynucleotide sequencing, transfection, cell culture, functional transport of D-aspartate, N-terminal identification and tissue distribution, and global cerebral ischaemia experiments were used to investigate potential novel GLT-1 C-terminal splice variant(s).

_Results_: Extensive immunolabelling of human dentate gyrus using a common GLT-1 epitope antiserum relative to GLT-1a antiserum did not accord with the expected label for GLT-1b and GLT-1c in view of the accepted ratio for GLT-1a, GLT-1b, and GLT-1c, i.e., 90%, 6%, and 1%, respectively. Potential donor splice sites were identified
in the human EAAT2 (human homolog of GLT-1) gene intron 10. The full putative GLT-1d (and EAAT2d) encoding polynucleotide sequence was cloned, sequenced, and transfected into COS-7 cells. The transfected COS-7 cells exhibited functional transport of D-aspartate that was dihydrokainate sensitive. Two predicted N-terminal sequences were identified for GLT-1d: MASTEG and MVS, with tissue specific distribution. Severe global hypoxia-ischaemia significantly reduced GLT-1d expression in neonatal pig brain.

**Inferences:** The novel GLT-1 splice variant GLT-1d identified in these preliminary investigations is a functional transporter that has at least two predicted N-terminal amino acid sequences that appear to exhibit tissue specific distribution. The expression of GLT-1d was sensitive to severe global hypoxia-ischaemia, which suggested that this splice variant may be clinically significant.

**Significance:** GLT-1d is a novel GLT-1 splice variant that is a functional transporter and may be clinically significant.

### 3.2 Introduction

The preliminary investigations that led to the research for this thesis were initiated by the identification of anomalous immunolabelling for GLT-1a and a common epitope for GLT-1 in human dentate gyrus. The anomaly in the immunolabelling prompted Prof. David Pow and Dr Aven Lee to investigate further. The inceptive immunohistochemistry experiment in human dentate gyrus and the ensuing preliminary experiments that ultimately led to the identification of GLT-1d are set out below.

### 3.3 GLT-1a/GLT-1 and Human Dentate Gyrus

Sequential serial sections of human dentate gyrus were immunolabelled for either GLT-1a or a common GLT-1 epitope (Figure 3.1 on page 30). The antisera for GLT-1a was raised against a GLT-1a specific epitope (amino acids 558–568: KSADCSVEEEP) and the antisera for the common epitope against an amino acid sequence common to all GLT-1 splice variants (amino acids 12–26: KQVEVRMHDSHELSE). The accepted ratio of the known GLT-1 C-terminal splice variants was thought to be 90%, 6%, and 1% for GLT-1a, GLT-1b, and GLT-1c, respectively. The disparity in signal observed between Figures 3.1 A and 3.1 B was intriguing. The difference in the signal detected for the common epitope and that for GLT-1a was unlikely to be solely...
Chapter 3: Preliminary Study - Discovering GLT-1d

A GLT-1a  B Common GLT-1 epitope

Figure 3.1: Immunolabelled serial sections of human dentate gyrus labelled for [A] GLT-1a and [B] a common GLT-1 epitope. The labelling for the common GLT-1 epitope was more extensive than that of GLT-1a. In effect, more astrocytes are labelled for the common GLT-1 epitope than GLT-1a. This suggested the existence of alternative C-terminal splice variants. The accepted ratio for GLT-1a, GLT-1b, and GLT-1c was thought to be 90%, 6%, and 1%, respectively.\textsuperscript{318} It is clear that the extensive labelling for the common GLT-1 epitope observed in [B] does not accord with the residual amount of label, i.e., for GLT-1b and GLT-1c, that would be expected relative to the label for GLT-1a observed in [A]. In effect, there appeared to be some missing labelling. The GLT-1a epitope was amino acids 558–568: KSADCSVEEP of the GLT-1a splice variant and the common GLT-1 epitope was amino acids 12–26: KQVEVRMHDSHLSSE of all GLT-1 splice variants.\textsuperscript{141,272} Scale bar: 100 µM. Permission to use the photomicrographs to prepare this figure was granted by Prof. David Pow of the Centre for Clinical Research, University of Queensland.

contributed by GLT-1b and/or GLT-1c given the accepted ratio for the known GLT-1 C-terminal splice variants. Accordingly, a number of hypotheses were advanced to explain this disparity:

1. The expression of GLT-1b and/or GLT-1c is higher than the accepted ratio.

2. Cleavage of the C-terminal of GLT-1a results in the loss of the GLT-1a specific epitope, but retention of the common epitope.

3. One or more additional GLT-1 C-terminal splice variants exist.

Probing for GLT-1b and GLT-1c confirmed that the additional common-epitope label observed (Figure 3.1 B) relative to GLT-1a (Figure 3.1 A) was not contributed solely by GLT-1b and/or GLT-1c. In fact, immunohistochemistry established that only low GLT-1b signal was detectable and GLT-1c was barely detectable. Cleavage of the C-terminal of GLT-1a is known to occur post mortem,\textsuperscript{235,319} which could support the loss of the GLT-1a specific epitope. Such cleavage appears to be associated with activated caspase 3,\textsuperscript{319} but this has not been reported for non-pathological conditions. Furthermore, cleaved proteins detectable as smaller bands were not observed by western blotting using the antiserum to the common GLT-1 epitope. This left the third hypothesis to account
for the additional signal detected by the antiserum to the common GLT-1 epitope. To test this hypothesis, further investigation to identify putative novel C-terminal GLT-1 splice variants was performed as set out below.

### 3.4 Identification of Putative Novel GLT-1/EAAT2 Splice Variant(s)

Analysis of the EAAT2 (human homolog of GLT-1) gene intron 10 sequence using the NetGene2 server\(^{320}\) identified the expected donor splice site at the 3′-end of the intron that corresponded to the donor site for a previously cloned GLT-1 splice variant, i.e., GLT-1c.\(^{287}\) A donor splice site is a splice site at the 5′ end of an intronic sequence, whereas an acceptor site is at the 3′ end of an intronic sequence.\(^{321–325}\) Interestingly, this analysis also revealed a series of potential donor splice sites upstream of the GLT-1c donor splice site. The presence of potential donor splice sites supported the contention that novel GLT-1 C-terminal splice variants exist. To identify such novel GLT-1 splice variants, primers were designed to anneal to a region downstream to the GLT-1c consensus splice site and a region in the EAAT2 gene exon 8 (Table 3.1).

**Table 3.1:** Primers designed to anneal to a region downstream to the GLT-1c consensus splice site and a region in the EAAT2 gene exon 8 used to identify novel GLT-1 C-terminal splice variants.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward EAAT2 (EXON8)</td>
<td>5′-ACTTTTGCCCTGTCACCTTCCGTGC-3′</td>
</tr>
<tr>
<td>Reverse EAAT2c</td>
<td>5′-TAAACCCAGGATTGATATCCACA-3′</td>
</tr>
</tbody>
</table>

This combination of primers was selected to encompass the GLT-1c donor splice site and the series of potential donor splice sites. PCR of human cerebral cortex cDNA according to a protocol outlined previously\(^{326}\) generated two amplification products (Figure 3.2 on page 32).

The two amplification products were excised from the gel and cloned according to a protocol outlined previously.\(^{326}\) Positive clones were sequenced by the Australian Genome Research Facility as described previously.\(^{326}\) The two amplification products corresponded to polynucleotide sequences that encode the C-terminals of GLT-1c, i.e., the human form EAAT2c-CT, and a potential novel C-terminal variant, tentatively named EAAT2d-CT (Figure 3.2 on page 32).
Chapter 3: Preliminary Study - Discovering GLT-1d

Figure 3.2: RT-PCR amplification of two amplification products from human cerebral cortex. Cloning and sequence analysis of the amplification products revealed the identification of polynucleotide sequences encoding C-terminals of EAAT2c (EAAT2c-CT) and a potential novel splice variant EAAT2d (the human homolog of GLT-1d)(EAAT2d-CT). The size of selected bands in the ladder are indicated to the left in base pairs. Permission to use the blot image to prepare this figure was granted by Dr Aven Lee of the Centre for Clinical Research, University of Queensland.

3.5 Generation of EAAT2d Polynucleotide Sequence

The polynucleotide sequence EAAT2d-CT exhibited high sequence similarity with GLT-1c (GenBank: AY578981.1), but comprised a 3′-end sequence that encoded a different predicted C-terminal peptide (Table 3.2).

Table 3.2: Predicted C-terminal peptides of EAAT2d-CT and GLT-1c (GenBank: AY578981.1).

<table>
<thead>
<tr>
<th>Partial 3′-End Polynucleotide Sequence</th>
<th>Predicted C-terminal Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAT2d-CT</td>
<td>DECKHVVCGHDACISLPLG</td>
</tr>
<tr>
<td>GLT-1c</td>
<td>DECKSLOYVEYQSWV</td>
</tr>
</tbody>
</table>

To generate the full EAAT2d polynucleotide sequence, primers were designed to anneal to a region corresponding to the start codon of EAAT2 and an EAAT2d specific region (Table 3.3 on page 33). In effect, these primers encompassed the full EAAT2d polynucleotide sequence (Figure 3.3 on page 33).

The full EAAT2d polynucleotide sequence was generated by PCR of human cerebral cortex cDNA using the primers set out in Table 3.3 using a previously outlined protocol.\textsuperscript{326}
Table 3.3: Primers used to generate the full EAAT2d polynucleotide sequence. ATG is the start site of exon 1 of EAAT2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward EAAT2 (MASTEG)</td>
<td>5′-ACCATGGCATCTACGGAAGGTGCCAA-3′</td>
</tr>
<tr>
<td>Reverse EAAT2d (STOP)</td>
<td>5′-TTAAACCCACGACTGATATTCCACA-3′</td>
</tr>
</tbody>
</table>

Figure 3.3: Schematic illustration of the EAAT2d-specific reverse primer and the EAAT2-common forward primer relative to the full EAAT2d polynucleotide sequence.

The amplification product (Figure 3.4) was excised from the gel and cloned according to a previously outlined protocol. Positive clones were sequenced by the Australian Genome Research Facility as described previously.

Figure 3.4: RT-PCR amplification of the full coding sequence of EAAT2d from human cerebral cortex. The size of selected bands in the ladder are indicated to the left in base pairs. Permission to use the blot image to prepare this figure was granted by Dr Aven Lee of the Centre for Clinical Research, University of Queensland.

3.6 Generation of GLT-1d Polynucleotide Sequence

The full GLT-1d polynucleotide sequence was generated from rat cerebral cortex cDNA essentially as set out in Section 3.5 - Generation of EAAT2d Polynucleotide Sequence on page 32, except the primers set out in Table 3.4 on page 34 were used.
3.7 GenBank Submission of Polynucleotide and Predicted Amino Acid Sequences

The EAAT2d and GLT-1d polynucleotide and predicted amino acid sequences were deposited under GenBank Accession Numbers KP966086.1 (Appendix A) and GenBank: KP966087.1 (Appendix B), respectively.

3.8 Expression of EAAT2d, GLT-1d, and GLT-1a in COS-7 Cells

The full EAAT2d and GLT-1d polynucleotide sequences were independently inserted into the mammalian expression vector pcDNA3.3-ToPo as set out previously. The mammalian expression vector GLT1a:pBK-CMV comprising the entire coding region of rat GLT-1a was a generous gift from Prof. Philip Poronnik of the School of Medical Sciences, University of Sydney. COS-7 cells were grown, maintained, and independently transfected with pcDNA3.3-ToPo [EAAT2d], pcDNA3.3-ToPo [GLT-1d], or GLT1a:pBK-CMV as set out previously.

Effective transfection and a high level of expression of EAAT2d, GLT-1d, and GLT-1a was confirmed by western blotting using an antibody directed to the common GLT-1 epitope, i.e., amino acids 12–26: KQVEVRMHDSHLSSE of all GLT-1 splice variants.

3.9 EAAT2d and GLT-1d: Functional Transporters

The transfected COS-7 cells of Section 3.8 - Expression of EAAT2d, GLT-1d, and GLT-1a in COS-7 Cells above were used to investigate whether EAAT2d and GLT-1d were effective transporters using a previously outlined protocol. Both EAAT2d and GLT-1d effectively transported the non-metabolizable glutamate analog D-aspartate. Dihydrokainate, a GLT-1/EAAT2 specific inhibitor, inhibited D-aspartate uptake (Figure 3.5 on page 35). In effect, the effective transport of D-aspartate by EAAT2d and GLT-1d confirmed that both EAAT2d and GLT-1d were functional transporters.
Chapter 3: Preliminary Study - Discovering GLT-1d

that localized to the plasma membrane and exhibited transport characteristics similar to GLT-1a, i.e., sensitivity to dihydrokainate.\(^{329}\)

![Graph showing transport of D-aspartate by transfected cells](image)

**Figure 3.5:** Transport of the non-metabolizable glutamate analog D-aspartate by COS-7 cells transfected with GLT-1d, EAAT2d, and GLT-1a was investigated using a previously outlined protocol.\(^{326}\) Such transport established that EAAT2d and GLT-1d are effective transporters comparable to GLT-1a. Dihydrokainate, a GLT-1/EAAT2 specific inhibitor, significantly inhibited the D-aspartate transport. Permission to use the data to prepare this graph was granted by Dr Aven Lee of the Centre for Clinical Research, University of Queensland.

### 3.10 N-Terminal GLT-1d Splice Variant Expression and Distribution

Known GLT-1 C-terminal variants have been described with at least two N-terminal splice variants, i.e., MASTEG\(^2,102,105,277,285,286\) and MVS\(^2,277\) N-terminal amino acid sequences. In addition to retina\(^{153,221,287}\) and brain,\(^{14,147}\) GLT-1 has been detected in a number of tissues including liver\(^{287,288}\) and testis.\(^{282}\) Accordingly, the expression of the GLT-1 MASTEG and MVS N-terminal splice variants was investigated in retina, brain, liver, and testis using the primers set out in Table 3.5 according to a protocol as set out previously.\(^{326}\)

The MASTEG N-terminal GLT-1d splice variant was detected in retina, brain, and testis, whereas the MVS N-terminal GLT-1d splice variant was only detected in liver (Figure 3.6).
Table 3.5: Primers used to investigate the expression of GLT-1 MASTEG and the MVS N-terminal splice variants in selected rat tissues.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward GLT-1 (MASTEG)</td>
<td>5′-ATGGCATCAACCGAGGGT-3′</td>
</tr>
<tr>
<td>Forward GLT-1 (MVS)</td>
<td>5′-TCCTGTAGATAAGAGAATGGGTCA-3′</td>
</tr>
<tr>
<td>Reverse GLT-1d (3′-UTR)</td>
<td>5′-GGTGCCCTTGCACTCATCTA-3′</td>
</tr>
</tbody>
</table>

Figure 3.6: Distribution of MASTEG and MVS N-terminal GLT-1d splice variants in selected rat tissues. The size of selected bands in the ladder are indicated to the left in base pairs. Permission to use the blot image to prepare this figure was granted by Dr Aven Lee of the Centre for Clinical Research, University of Queensland.

3.11 Effect of Hypoxia-Ischaemia on GLT-1d mRNA Expression

PCR was performed on cDNA extracted from hypoxic-ischaemic neonatal pig cerebral cortex using the primers set out in Table 3.6 according to a previously described protocol. The hypoxic-ischaemic neonatal pig cerebral cortex was generated using a protocol as set out previously.

Table 3.6: Primers used to investigate the effect of cerebral cortex hypoxia-ischaemia on GLT-1d mRNA expression in neonatal pig cerebral cortex.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward GLT-1 (EXON8)</td>
<td>5′-ACTTTGCTGCTGACCTTCGTTGC-3′</td>
</tr>
<tr>
<td>Reverse GLT-1d (3′-UTR)</td>
<td>5′-GGTGCCCTTGCACTCATCTA-3′</td>
</tr>
<tr>
<td>Forward β-Actin (369)</td>
<td>5′-GTGTGAGACCTTCACACACCCAG-3′</td>
</tr>
<tr>
<td>Reverse β-Actin (1080)</td>
<td>5′-CTGCTTGCTGATCACCATCTGC- 3′</td>
</tr>
</tbody>
</table>
Figure 3.7 shows that in response to a severe global hypoxic-ischaemic insult there is a significant reduction in expression of GLT-1d mRNA in neonatal pig cerebral cortex. Such a reduction is of clinical importance and supported further investigation of GLT-1d.

**Figure 3.7:** Effect of hypoxia-ischaemia on GLT-1d expression in neonatal pig cerebral cortex. [A] RT-PCR gel showing GLT-1d and β-Actin mRNA expression in hypoxic-ischaemic neonatal pig cortex was significantly decreased relative to control. [B] Graphical illustration of the reduction in GLT-1d mRNA expression in hypoxic-ischaemic neonatal pig cerebral cortex relative to control. Permission to use the gel image and data to prepare this figure was granted by Dr Aven Lee of the Centre for Clinical Research, University of Queensland.

### 3.12 Significance Statement

These preliminary investigations established that GLT-1d was a novel GLT-1 splice variant that was a functional transporter that may be clinically significant.

### 3.13 Key Points

- The accepted ratio of the known GLT-1 C-terminal splice variants, i.e., 90% (GLT-1a), 6% (GLT-1b), and 1% (GLT-1c)\(^{318}\) did not appear to accurately reflect the population of GLT-1 splice variants.

- The present preliminary study identified a novel GLT-1 splice variant, termed GLT-1d.
GLT-1d is a functional transporter of the non-metabolizable glutamate analog D-aspartate.

GLT-1d has at least two predicted N-terminal amino acid sequences: MASTEG and MVS, which appeared to exhibit tissue specific distribution.

GLT-1d was sensitive to global hypoxia-ischaemia in neonatal pig cerebral cortex and, as such, was considered potentially clinically significant.

Further studies were contemplated to investigate the expression and distribution profiles of GLT-1d in juvenile, adult, and pathological mouse brains.
Part II

Antisera as Research Tools
4

Production and Validation of Antisera to Detect GLT-1d in Mouse Brain

4.1 Synopsis

Aim: The aim of this study was to produce antisera using synthetic peptides that corresponded to the unique C-terminal amino acid sequences of rat and human GLT-1d, and to validate the antisera for use in the investigation of the expression and distribution of GLT-1d in mouse brain.

Research Question: Can synthetic peptides that correspond to the unique C-terminal amino acid sequences of rat and human GLT-1d be used to produce antisera that will be useful in the investigation of GLT-1d expression and distribution in mouse brain?

Methods: Synthetic peptides that corresponded to unique C-terminal amino acid sequences of rat GLT-1d and human EAAT2d were conjugated to keyhole limpet hemocyanin. The synthetic peptide-keyhole limpet hemocyanin conjugates were used to produce polyclonal antisera in New Zealand White rabbits. The synthetic peptides were also conjugated to bovine serum albumin to produce synthetic peptide-bovine serum albumin conjugates. The synthetic peptide-bovine serum albumin conjugates were used as
positive controls in dot- and western blots. Preliminary western blot experiments were performed to optimise the western blotting conditions for use in subsequent studies.

**Results:** The antisera that were produced—designated Rb 1713, Rb 1714, Rb 1715, and Rb 1716—detected their target synthetic peptide-BSA conjugates by dot- and western blotting, whereas the corresponding pre-immune sera did not. Two prominent bands were detected by western blotting in adult mouse whole brain lysate using Rb 1713 antiserum, presumably these bands were a monomer and a higher molecular weight oligomer form of GLT-1d. Detection of the GLT-1d monomer in adult mouse whole brain lysate by western blotting using Rb 1713 antiserum was not affected when either the strong anionic detergent sodium dodecyl sulfate (SDS) or the mild non-ionic detergent, nonyl phenoxypolyethoxyethanol (NP-40) were used in lysis buffers. Importantly, heating the samples at 85°C for 10 minutes before western blotting led to a downward shift of ~50 kDa in the apparent molecular weight of the higher molecular weight oligomer form of GLT-1d and the monomer form of GLT-1d migrating at a slightly lower apparent molecular weight. Detection of GLT-1d in adult mouse whole brain lysate by western blotting using Rb 1713 antiserum was optimal in 0.25% bovine serum albumin, 20 mM Tris Base, and 200 mM NaCl (pH 7.3) buffer.

**Inferences:** One of the antisera produced, namely Rb 1713 antiserum, at 1:100,000 was sensitive and detected 10 ng of the rat synthetic peptide-bovine serum albumin conjugate and GLT-1d in 2.5 μg of adult mouse whole brain protein by western blotting. The two prominent bands detected by western blotting in adult mouse whole brain lysate using Rb 1713 antiserum were likely monomer and higher molecular weight oligomer forms of GLT-1d. The ~50 kDa downward shift in the apparent molecular weight of the higher molecular weight oligomer form of GLT-1d detected in heated samples may be due to heat-induced dissociation of PICK1. The slight downward shift in the apparent molecular weight of the monomer form of GLT-1d on heating may be due to increased sodium dodecyl sulfate solvation of the monomer form of GLT-1d.

**Significance:** This study established that the Rb 1713 antiserum was a useful research tool to investigate GLT-1d in mouse brain. The ~50 kDa downward shift in the apparent molecular weight of the prominent higher molecular weight oligomer form of GLT-1d—and possible heat-induced dissociation of PICK1—detected in heated samples was an important finding that warrants further investigation.
4.2 Introduction

As set out in Chapter 3, GLT-1d was a newly discovered C-terminal splice variant of the excitatory amino acid transporter GLT-1. The mature mRNA that encodes GLT-1d results in unique rat and human C-terminal amino acid sequences compared to known C-terminal splice variants (Table 2.2 on page 24). The unique C-terminal amino acid sequences are amino acid residues 551–572 and 552–566 of the rat and human sequences, respectively (Figure 4.1 on page 43). Importantly, GLT-1d had been shown to be a functional and clinically significant glutamate transporter (Section 3.11 - Effect of Hypoxia-Ischaemia on GLT-1d mRNA Expression on page 36). As such, the expression and distribution of GLT-1d warranted further investigation.

Since known GLT-1 splice variants are differentially expressed in terms of development,\(^1\) distribution,\(^2,3\) and disease\(^4–6\) in mammalian brain, the aim of the research for this thesis was to investigate the expression and distribution of GLT-1d in juvenile, adult, and pathological mouse brains. To achieve the aim, at least in part, GLT-1d-specific antibodies were required. Accordingly, given the novelty of GLT-1d, the aim of the study underlying this chapter was to produce antisera using synthetic peptides that corresponded to the unique C-terminal amino acid sequences of rat and human GLT-1d, and to validate the antisera for use in the investigation of the expression and distribution of GLT-1d in mouse brain.

This chapter addresses four topics: First, a brief review of antibodies, antibody structure and classes, and antibody production is provided. This brief review is intended to provide context for the production of antisera and their use as research tools. Next, the production of antisera in rabbits using synthetic peptides is examined. In the third topic, the validation of the produced antisera is considered. In the final topic, the importance of the optimization of the conditions for western blotting is explored.

4.2.1 Antibodies

Antibodies, or immunoglobulins,\(^331–333\) are serum glycoproteins that are produced by plasma cells of the B lymphocyte lineage in response to antigens.\(^332,334\) Antibodies are exquisitely specific and able to bind antigens with a high degree of affinity.\(^332,334,335\) Antigens, on the other hand, are molecules that are recognized by the immune system and can be bound by antibodies, major histocompatibility complex (MHC) molecules, and T-cell receptors.\(^333\)
Chapter 4: Antisera - Research Tools to Investigate GLT-1d

Rat MASTEGANNM PKQVEVRMHD SHLSSEEPKH RNLGMRMCDK LGKNNLLSIT VFGVILGAVC
Human MASTEGANNM PKQVEVRMHD SHLGSLEEPKH RHLLGRLCDK LGKNNLLLIT VFGVILGAVC

70  80  90  100  110  120
Rat GGLRLASPI HPDVVMLIAF PGDILMRMLK MILPLISS LITGGLSDLDA KASGRGLTRA
Human GGLRLASPI HPDVVMLIAF PGDILMRMLK MILPLISS LITGGLSDLDA KASGRGLTRA

130  140  150  160  170  180
Rat MVYYSTTTII AAVLGVLVL ALHPGPKNLK KQLGPGKSKN EVSSLDFADL LIRNLFPPLN
Human MVYYSTTTII AAVLGVLVL AIHPGPKNLK KQLGPGKSKN EVSSLDFADL LIRNLFPPLN

190  200  210  220  230  240
Rat VQACFQQIQT VTKKVLVAPP SEEANTKAV ISQILNETMNE APEETKVIVK KGLEFKDMN
Human VQACFQQIQT VTKKVLVAPP PDEEANATS AWSLLNETVT EVPVEETKIVI KGLEFKDMN

250  260  270  280  290  300
Rat VLGIIGFMIA FGIAMGKMGE QAKLMVEFFN ILNEIVMKVL MIMIWYSPGL IAICLICGKII
Human VLGIIGFMIA FGIAMGKMGE QAKLMVEFFN ILNEIVMKVL MIMIWYSPGL IAICLICGKII

310  320  330  340  350  360
Rat AIKDFLEVVAR QLGMYMITV LITILAVGLP TEDISLLVAVD WLLDRMRTSV
Human AIKDFLEVVAR QLGMYMITV LITILAVGLP TEDISLLVAVD WLLDRMRTSV

370  380  390  400  410  420
Rat ASSAGTLPVT FRCRENLGL DKKRTVRFVL VGATINMDGT AYEAVAAIA IAQCMGVYL
Human ASSAGTLPVT FRCRENLGL DKKRTVRFVL VGATINMDGT AYEAVAAIA IAQCMGVYL

430  440  450  460  470  480
Rat GGQIVTVSLQ ATLASIGAAS IPSAGLVTML LILTAVGLP TEDISLLVAVD WLLDRMRTSV
Human GGQIVTVSLQ ATLASIGAAS IPSAGLVTML LILTAVGLP TEDISLLVAVD WLLDRMRTSV

490  500  510  520  530  540
Rat NVVGDSFGAG IVYHLISKGSE DTIDSQHHRM EDEMTKQTS YYDDTKHRE SNSNCVYAA
Human NVVGDSFGAG IVYHLISKGSE DTIDSQHHRM EDEMTKQTS YYDDTKHRE SNSNCVYAA

550  560  570
Rat HNSVVIDECK GTWFSVPML HVLACISFP LG
Human HNSVVIDECK KHVVCBDAC ISLPLG

Figure 4.1: Predicted amino acid sequence of rat (GenBank: AMD11602.1) and human (GenBank: AMD11601.1) GLT-1d protein. Alternative splicing of the 3′-end of rat and human GLT-1-coding polynucleotide sequences encodes unique C-terminal amino acid sequences. The unique amino acid sequences are the highlighted amino acid residues 551–572 and 552–566 of the rat and human amino acid sequences, respectively.

If antibodies are to be used effectively as research tools, it is important that one understand their structure and how they are produced. What follows is a brief description of antibody classes, structure, and production.

For clarity purposes, the terms antiserum and antisera as used in this thesis should be understood to encompass the terms antibody and antibodies. These terms are used interchangeably, as appropriate.

4.2.2 Antibody Classes and Structure

Antibodies can be classified into five classes, i.e., IgA, IgD, IgE, IgG, and IgM. This classification is based on the type of heavy chain in the antibody.334 While there are five
different types of heavy chains (α, δ, ε, γ, and µ), there are only two types of light chains (κ and λ).\textsuperscript{334} IgG (Table 4.1) is the dominant antibody class\textsuperscript{334} and, consequently, the most common class of antibody used as a research tool.\textsuperscript{336} Rabbit IgG antibodies were produced for the research reported in this thesis.

**Table 4.1:** The IgG class of antibodies. Modified from Sarantopoulos\textsuperscript{334} with permission under copyright held by Cold Spring Harbor Laboratory Press.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy chain</td>
<td>γ</td>
</tr>
<tr>
<td>Light chain</td>
<td>κ or λ</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>γ_2κ_2 or γ_2λ_2</td>
</tr>
<tr>
<td>Structure</td>
<td>Y</td>
</tr>
<tr>
<td>Valency</td>
<td>2</td>
</tr>
<tr>
<td>Concentration in Serum</td>
<td>8–16 mg/mL</td>
</tr>
<tr>
<td>Function</td>
<td>Secondary response</td>
</tr>
</tbody>
</table>

The IgG molecule consists of four polypeptide chains: two identical heavy chains (V\textsubscript{H}) and two identical light chains (V\textsubscript{L}) arranged in a characteristic Y-shape (Figure 4.2 on page 45). The heavy chains and light chains are held together by a combination of covalent disulfide bridges and non-covalent bonds.\textsuperscript{333}

Limited proteolytic cleavage of rabbit IgG with papain divides the molecule into two identical Fab (F\textsubscript{r}agment a\textsubscript{n}tigen-b\textsubscript{b}inding) fragments that form the arms and the Fc (F\textsubscript{r}agment that c\textsubscript{r}ystallizes) fragment that forms the tail of the Y-shaped molecule (Figure 4.2 on page 45).\textsuperscript{334}

The Fab fragments include a series of homologous globular motifs known as Ig domains.\textsuperscript{333} Variable regions within some of these Ig domains at the N-terminals of the heavy and light chains form the antigen-binding sites\textsuperscript{333,334} or paratopes.\textsuperscript{336} Each IgG molecule comprises two antigen-binding sites and is consequently bivalent.\textsuperscript{334}

The Fc fragment comprises the constant region, which can be used to detect the antibody via labelled binding partners, e.g., labelled secondary antibodies, labelled Protein A, or labelled Protein G.\textsuperscript{336}

### 4.2.3 Antibody Production

While there are many factors that influence the production of antibodies for research tools,\textsuperscript{337–339} three specific factors, namely, immunogen selection, carrier proteins, and adjuvants are discussed briefly below.
4.2.3.1 Immunogen Selection

Pure antigens are generally considered the best form of immunogen to use to raise antibodies.\textsuperscript{340} Purified GLT-1d proteins were not appropriate for raising antibodies against the rat and human GLT-1d. As mentioned previously, only amino acid residues 551–572 and 552–566 of the rat and human GLT-1d sequences (Figure 4.1 on page 43), respectively, are unique as compared to known GLT-1 C-terminal splice variants (Table 2.2 on page 24). Accordingly, these unique sequences had to be used to raise the antibodies. The option used was to prepare synthetic peptides that corresponded to the unique sequences and use those synthetic peptides to raise the antibodies. In support of this option, antibodies raised against synthetic peptides are known to produce target-specific
Chapter 4: Antisera - Research Tools to Investigate GLT-1d

results in western blotting,\textsuperscript{341} which was one of the methods used to study GLT-1d for this thesis.

4.2.3.2 Synthetic Peptide Design

Synthetic peptides that corresponded to amino acid residues 550–572 of the rat and 551–566 of the human GLT-1d sequences (Figure 4.1 on page 43) were generated (see Table F.1 in Appendix F: Suppliers of Animals, Chemicals, Equipment, and Services on page 232). While amino acid residues 551–572 of the rat and 552–566 of the human GLT-1d sequences are unique (compare Figure 4.1 on page 43 and Table 2.2 on page 24), the common lysine residue was purposely included in the synthetic peptides to enable efficient conjugation using formaldehyde (Table 4.2). Importantly, where a synthetic peptide is designed to represent the C-terminal of a protein, it is preferable to couple that synthetic peptide via the N-terminal and thereby more accurately reflect the C-terminal of the protein.\textsuperscript{342}

\textbf{Table 4.2:} Synthetic peptides designed to correspond with amino acid residues 550–572 of the rat and 551–566 of the human GLT-1d sequences.

<table>
<thead>
<tr>
<th>Synthetic Peptide</th>
<th>Animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-KGTWFSVPSMLHVLDACISFPLG-OH</td>
<td>Rat</td>
</tr>
<tr>
<td>Ac-KHVVCGHDACISLPLG-OH</td>
<td>Human</td>
</tr>
</tbody>
</table>

4.2.3.3 Carrier Proteins

The conjugation reaction for preparing the immunogens was based on the method most often used by members of the Pow Laboratory, i.e., conjugation to a carrier protein via an amino group using formaldehyde.\textsuperscript{342} While conjugation using formaldehyde may also occur at the N-terminal amino group, cysteine, and histidine residues of the synthetic peptides,\textsuperscript{343} this conjugation method is known to favour the \(\epsilon\)-amino group of lysine residues.\textsuperscript{342}

The synthetic peptides (Table 4.2) that were used to raise the antibodies are \(\sim2\) kDa in size. Synthetic peptides of this size are haptens, i.e., exhibit low immunogenicity.\textsuperscript{340,344,345} The immunogenicity of haptens can be improved by conjugating them to a carrier protein, such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin, porcine thyroglobulin, \textit{Mycobacterium tuberculosis}, or purified protein derivative of tuberculin.\textsuperscript{340} Carrier proteins are necessary when using synthetic peptides to raise antibodies as the carrier proteins provide the major histocompatibility complex (MHC) class II binding sites essential for antibody production.\textsuperscript{340,345}
KLH is one of the most commonly used carrier proteins. The decision to use KLH as the carrier protein to raise the antibodies was based on the following factors:

1. KLH is divergent from known mammalian proteins, which reduces the risk of anti-KLH antibodies cross-reacting with mammalian proteins. Conversely, mammalian proteins, such as BSA and thyroglobulin, are immunogenic so using them as carrier proteins would yield anti-BSA and anti-thyroglobulin antibodies. Such antibodies may cross-react with other mammalian proteins.

2. BSA is a good immunogen, so using it as the carrier protein would likely result in predominant production of anti-BSA antibodies.

3. The possibility that anti-BSA antibodies may detect mouse serum albumin in brain tissue and lysate was a concern for the following reasons:
   
   (a) Mouse serum albumin (GenBank: AAH49971.1):
      
      i. ~70% identical to BSA (GenBank: AAA51411.1), as determined using the EMBL-EBI EMBOSS Needle Pairwise Sequence Alignment (PROTEIN) server.
      
      ii. Predicted molecular weight of ~69 kDa of mouse serum albumin determined in silico using the Protein Identification and Analysis Tools on the ExPASy ProtParam server. As such, mouse serum albumin would run close to the predicted ~65 kDa monomer form of GLT-1 normally observed in western blotting for GLT-1.

   (b) Albumin is 35–50% of total serum protein in the blood of animals. Brain tissue and other tissue lysates contain blood, and hence albumin.

   (c) Healthy cerebrospinal fluid contains albumin.

   (d) Transient focal cerebral ischaemia increases albumin in the extracellular space and the cerebrospinal fluid (via the brain interstitial fluid). A transient focal cerebral ischaemic insult was used to study the effect of transient ischaemia on the expression of GLT-1d in mouse brain (Chapter 7: GLT-1d in Pathological Mouse Brains, a Preliminary Study).

4. BSA is a common blocking agent used in immunological techniques, whereas KLH is not. Anti-BSA antibodies would likely detect BSA if it were used as the blocking agent, i.e., cause undesirable background.
4.2.3.4 Adjuvant

As mentioned previously, the synthetic peptides that were used to raise the antibodies are haptens, i.e., have low inherent immunogenicity. In addition to this low inherent immunogenicity, the synthetic peptides are hydrophobic, i.e., poorly soluble in water, which reduces accessibility. These characteristics strongly suggested that the synthetic peptides may be poor immunogens. Because the synthetic peptides may be poor immunogens, Freund’s adjuvant (Table 4.3) was used to help raise the antibodies.

Freund’s adjuvant, the so-called ‘gold standard’ adjuvant, was selected because it stimulates a strong and sustained response that leads to the production of high-titre antibodies. This strong and sustained response is due to the following factors:

1. The immunogen is trapped by the adjuvant at the administration site followed by slow release, i.e., a depot of the immunogen is formed.

2. The adjuvant causes an increase in secretion of lymphokines. Lymphokines induce a chemotactic effect that increases the number of macrophages at the administration site, which in turn increases the level of phagocytosis.

3. The adjuvant enhances the response to the immunogen by providing a Toll-like receptor signal. The Toll-like receptor signal promotes the differentiation of dividing B cells into antibody-secreting plasma cells and memory cells.

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Components Include</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete Freund’s Adjuvant (CFA)</td>
<td>Water, non-metabolizable oil, and killed <em>Mycobacterium tuberculosis</em></td>
<td>Increased surface area for antigen presentation, and excellent immune stimulation</td>
<td>Chronic inflammation, and can increase granulomas and abscesses*</td>
</tr>
<tr>
<td>Incomplete Freund’s Adjuvant (IFA)</td>
<td>Water and non-metabolizable oil</td>
<td>Increased surface area for antigen presentation, and excellent immune stimulation (but less effective than CFA)</td>
<td>Chronic inflammation, and can increase granulomas and abscesses*</td>
</tr>
</tbody>
</table>

* Abscesses are typically associated with poor immunization technique and back-tracking of material along the needle track (Prof. David Pow, personal communication).
4.3 Experimental Methods

All chemicals used in the experiments of this chapter were of analytical grade and purchased from commercial suppliers. Specific details of the suppliers of the animals, chemicals, equipment, and services cited in this chapter are set out in Table F.1 in Appendix F: Suppliers of Animals, Chemicals, Equipment, and Services on page 232.

4.3.1 Synthetic Peptides and Antisera Production

The majority of the practical work reported in this thesis was performed at RMIT University, which has limited animal house facilities. Accordingly, New England Peptide, Inc. was instructed to produce the synthetic peptides, synthetic peptide-keyhole limpet hemocyanin conjugates, and antisera used for this thesis. New England Peptide, Inc. is a commercial service provider that produces synthetic peptides, synthetic peptide conjugates, and antisera to order (Table F.1 in Appendix F: Suppliers of Animals, Chemicals, Equipment, and Services on page 232).

4.3.1.1 Synthetic Peptide Production

The synthetic peptides (Section 4.2.3.2 - Synthetic Peptide Design on page 46) were synthesized using solid phase synthesis.\textsuperscript{354,355} The purification of the peptides was performed using reverse-phase high performance liquid chromatography. Purity (>85\%) of the synthetic peptides was confirmed by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Synthetic peptides of \( \geq 75\% \) purity—in some cases lower—can be used to produce effective polyclonal antibodies.\textsuperscript{356}

The quality control information of the synthetic peptides is set out in Appendix E: Synthetic Peptides - Quality Control Information on page 223.

4.3.1.2 Synthetic Peptide-Keyhole Limpet Hemocyanin Conjugates

The synthetic peptides (Section 4.2.3.2 - Synthetic Peptide Design on page 46) were conjugated with keyhole limpet hemocyanin according to a standard Pow Laboratory protocol.\textsuperscript{342} Briefly, formaldehyde was used to conjugate the synthetic peptides to keyhole limpet hemocyanin. Conjugation using formaldehyde occurs at the amino group of the N-terminal amino acid residue and the side-chain of arginine, cysteine, histidine, and lysine residues.\textsuperscript{343} As mentioned previously, this conjugation reaction is known to occur preferentially at the \( \epsilon \)-amino group of the lysine residue.\textsuperscript{342}
Antisera Production

Antisera to investigate GLT-1d were produced according to a standard Pow Laboratory protocol. Briefly, four New Zealand White rabbits—designated Rb 1713, Rb 1714, Rb 1715, and Rb 1716—were immunized with the respective synthetic peptide-keyhole limpet hemocyanin conjugate (Table 4.4 on page 50). The animal facility of New England Peptide, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), assured under the auspices of the Office of Laboratory Animal Welfare (OLAW), a body responsible for oversight of the care and use of animals in research funded by the United States of America’s Public Health Service, and licensed by the United States of America’s Department of Agriculture (USDA). Such oversight, assurance, and licensure ensured that New England Peptide, Inc. undertook all proper care in the treatment of the rabbits used to produce the antisera for the research studies underlying this thesis.

Table 4.4: Identity of the rabbits and the synthetic peptides used to produce antisera against rat GLT-1d and human EAAT2d.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Synthetic Peptide</th>
<th>Animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb 1713</td>
<td>Ac-KGTWFSSVPSMLHVLDACISFPLG</td>
<td>Rat</td>
</tr>
<tr>
<td>Rb 1714</td>
<td>Rat</td>
<td></td>
</tr>
<tr>
<td>Rb 1715</td>
<td>Ac-KHVVCGHDACISLPLG</td>
<td>Human</td>
</tr>
<tr>
<td>Rb 1716</td>
<td>Human</td>
<td></td>
</tr>
</tbody>
</table>

The rabbits were female and three months old at the start of the immunization procedure (Table 4.5). The immunization procedure consisted of three subcutaneous injections according to a standard regimen. The primary immunization comprised a dose of 400 µg synthetic peptide-KLH conjugate in Complete Freund’s Adjuvant, while the second and third immunization each comprised a dose of 200 µg synthetic peptide-KLH conjugate in Incomplete Freund’s Adjuvant, respectively. The advantages and disadvantages associated with the use of Freund’s adjuvant have been discussed briefly above (Section 4.2.3.4 - Adjuvant on page 48).

Table 4.5: Immunization and production bleed strategy for the production of the antisera.

<table>
<thead>
<tr>
<th>Day</th>
<th>Bleed and/or Immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Pre-Immune Bleed + Primary Immunization</td>
</tr>
<tr>
<td>14</td>
<td>Second Immunization</td>
</tr>
<tr>
<td>28</td>
<td>Third Immunization</td>
</tr>
<tr>
<td>35</td>
<td>Production Bleed #1</td>
</tr>
<tr>
<td>40</td>
<td>Production Bleed #2</td>
</tr>
</tbody>
</table>
Female New Zealand White rabbits are the most commonly used host animal and respond well to immunization protocols. New Zealand White rabbits typically produce high titre antibodies in a relatively short period of time. Young adult rabbits were used since young adults tend to respond to immunization better than older rabbits. The more effective response by young adult rabbits is due to their limited exposure to agents in their environment that may have stimulated an immune response, i.e., young rabbits are more immuno-naïve than older rabbits. There was no scientific reason for using female rabbits; female rabbits are simply more docile and consequently can be housed together.

Pre-immune sera were taken to investigate whether any of the rabbits produced antibodies before the immunization that may interfere with the detection of the synthetic peptides and/or GLT-1d protein.

4.3.2 Synthetic Peptides- and Glycine Bovine Serum Albumin Conjugates

Short peptides, such as the synthetic peptides (~2 kDa), do not bind in a stable manner to nitrocellulose membrane; whereas larger proteins are strongly adsorbed to nitrocellulose. Accordingly, short peptides have to be conjugated to a larger protein in order to facilitate binding to the nitrocellulose membrane thereby enabling their use as positive controls in dot- and western blotting. Conjugation of a synthetic peptide to a larger protein minimizes the risk of false negatives that may occur if the peptide is used on its own. The synthetic peptides were conjugated to bovine serum albumin (BSA), which is a very soluble protein, for the following reasons:

1. To evaluate the antisera for reactivity with the synthetic peptides.

2. As targets for the antisera in dot- and western blotting to determine the sensitivity of the antibodies.

3. As positive controls for dot- and western blotting.

4. The synthetic peptide-BSA conjugates and GLT-1 proteins would run at similar molecular weights on western blots, i.e., ~66 kDa and ~65 kDa, respectively. As such, the synthetic peptide-BSA conjugates would serve as a useful method control, particularly as a transfer control.

Similarly, glycine was conjugated to BSA for the following reasons:
1. To evaluate the antisera for reactivity with the aldehyde cross-link (methylen
bridge) formed by the conjugation reaction between the synthetic peptides and
keyhole limpet hemocyanin, i.e., in the synthetic peptide-keyhole limpet hemo-
cyanin conjugates used to raise the antibodies.

2. As a probable negative control for dot- and western blotting.

The solutions that were used in the conjugation reaction were prepared as follows:

1. 0.2 M phosphate buffer (pH 7.2) was prepared from 0.2 M stock solutions of
   NaH$_2$PO$_4$ and Na$_2$HPO$_4$. The pH of 100 mL of 0.2 M Na$_2$HPO$_4$ was adjusted to
   pH 7.2 by adding 0.2 M NaH$_2$PO$_4$.

2. 4% formaldehyde in 0.1 M phosphate buffer solution (pH 7.2) was prepared using
   a previously described method. Briefly, 4 g of paraformaldehyde was added to
   25 mL of ultrapure water and heated to 60°C. 5 M NaOH solution was added
   dropwise until the paraformaldehyde depolymerized to form a formaldehyde so-
   lution. Ultrapure water was added to the formaldehyde solution to make up the
   volume to 50 mL. 50 mL of 0.2 M phosphate buffer (pH 7.2) was added to the
   diluted formaldehyde solution to provide freshly prepared 4% formaldehyde in 0.1
   M phosphate buffer (pH 7.2). Preparation of the 4% formaldehyde solution was
   carried out in a fume hood.

3. 30 mg BSA was dissolved in 3 mL freshly prepared 4% formaldehyde/0.1 M phos-
   phate buffer (pH 7.2) to provide a 10 mg BSA/mL 4% formaldehyde solution.

The synthetic peptides were conjugated with BSA using an adaptation of a previously
described method. Briefly, for each conjugate, 1 mg of the synthetic peptide was
added to 1 mL freshly prepared 10 mg BSA/4% formaldehyde/0.1 M phosphate buffer
solution (pH 7.2). Conjugation of the synthetic peptides to BSA was allowed to proceed
on a laboratory shaker overnight at room temperature. The synthetic peptide-BSA
conjugates were aliquoted out and stored at -80°C until required.

Similarly, glycine was conjugated with BSA. Briefly, 1 mg of glycine was added to 1 mL
freshly prepared 10 mg BSA/4% formaldehyde/0.1 M phosphate buffer solution (pH 7.2).
Conjugation of glycine to BSA was allowed to proceed on a laboratory shaker overnight
at room temperature. The glycine-BSA conjugate was aliquoted out and stored at -80°C
until required.
4.3.3 Animal Tissue Collection

The animal tissues for the experiments of this chapter were obtained from adult male mice of a common inbred strain of laboratory mouse, the C57BL/6 mouse strain.

Mice were housed under standard temperature- and light-controlled conditions, i.e., \(\sim 22^\circ C\) and 12 hours light/12 hours dark, with free access to food and water prior to the collection of tissues.

Adverse welfare impacts on the mice were minimized insofar as possible.

The mice were terminally anaesthetized by intraperitoneal injection of sodium pentobarbital (150 mg/kg). Once the foot withdrawal reflex was absent, the deeply anaesthetized animals were decapitated using a pair of surgical scissors. Each brain was rapidly dissected from the cranial vault according to a previously described method, divided into the forebrain, midbrain, hindbrain, and cerebellum, immediately frozen in liquid nitrogen, and stored at -80\(^\circ C\) until required.

For the purposes of this study, the forebrain, midbrain, and hindbrain were defined as comprising the following brain structures:

1. Forebrain: cerebral cortex, basal ganglia, thalamus, hypothalamus, subthalamus, epithalamus, pretectum, and associated white matter;
2. Midbrain: superior and inferior colliculi, midbrain tegmentum, crus cerebri, and substantia nigra; and
3. Hindbrain: medulla and pons.

The hindbrain is generally understood to include the cerebellum, but for the purposes of this study the cerebellum was treated as an independent structure.

4.3.4 Protein Extraction

Frozen brain tissue was crushed in a mortar and pestle under liquid nitrogen and then homogenized at 4\(^\circ C\) in 10x (m/v) ice cold 2x Laemmli buffer (125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 10% \(\beta\)-mercaptoethanol, 20% glycerol) excluding bromophenol blue or in 10x (m/v) ice cold NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris Base (pH 8.0)) using a T10 basic Ultra Turrax\textsuperscript{TM} homogenizer on setting 1 (2 x 10 seconds followed by 2 minutes incubation on ice; repeated 2-3 times). Bromophenol blue was omitted from the 2x Laemmli buffer because it is not compatible
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with the bicinchoninic acid assay\textsuperscript{366} used in this thesis (see Appendix G: ThermoScientific Protein Assay Compatibility Table on page 237). Both lysis buffers contained a protease inhibitor cocktail (see Appendix I: Roche cOmplete\textsuperscript{TM} Ultra Protease Inhibitor Cocktail on page 250) and were cooled to 4°C to reduce proteolysis. Homogenates were incubated on a laboratory roller for 3 hours at 4°C. After incubation on the roller, the homogenates were centrifuged at 14,000 x g for 30 minutes at 4°C. The supernatant fractions were collected, aliquoted out, and stored at -80°C until required.

4.3.5 Analytical Procedures

The analytical procedures of this chapter consisted of the antibody titre determination, \textit{in silico} determination of the physico-chemical properties of the synthetic peptides, protein assays, and antibody-based assays. The antibody-based assays were enzyme-linked immunosorbent assays, dot blots, and western blots.

4.3.5.1 Antibody Titre

The antibody titre for production bleed #1 of each rabbit was determined by New England Peptide, Inc. according to their standard protocol. Briefly, the synthetic peptides were solubilized in dimethyl sulfoxide. The solubilized synthetic peptides were diluted to 10 \(\mu\)g/mL in carbonate-bicarbonate buffer (pH 9.5).\textsuperscript{361} 50 \(\mu\)L aliquots of the diluted synthetic peptides were added to test wells of microtitre plates to coat the plates. The plates were covered and set aside to incubate overnight at 4°C. After incubation, the plates were washed 3x in phosphate buffered saline (pH 7.3)/0.05% Tween-20, 30 seconds/wash. 2% bovine serum albumin (BSA)/phosphate buffered saline (pH 7.3) was added to the wells of the microtitre plates to block the wells. The plates were covered and incubated for 1 hour at 37°C. After incubation, the plates were washed 3x in phosphate buffered saline (pH 7.3)/0.05% Tween-20, 30 seconds/wash. Antibodies were serially diluted in 2% BSA/phosphate buffered saline (pH 7.3). 100 \(\mu\)L of each of the diluted antibodies was added to each of the relevant wells of the microtitre plate. The plates were covered and incubated for 1 hour at 37°C. The plates were washed 3x in phosphate buffered saline (pH 7.3)/0.05% Tween-20, 30 seconds/wash. Antibodies were serially diluted in 2% BSA/phosphate buffered saline (pH 7.3). 100 \(\mu\)L of each of the diluted antibodies was added to each of the relevant wells of the microtitre plate. The plates were covered and incubated for 1 hour at 37°C. The plates were washed 3x in phosphate buffered saline (pH 7.3)/0.05% Tween-20, 30 seconds/wash. Antibodies were serially diluted in 2% BSA/phosphate buffered saline (pH 7.3). 100 \(\mu\)L of each of the diluted antibodies was added to each of the relevant wells of the microtitre plate. The plates were covered and incubated for 1 hour at 37°C. The plates were washed 3x in phosphate buffered saline (pH 7.3)/0.05% Tween-20, 30 seconds/wash. 50 \(\mu\)L of freshly prepared 3,3’,5,5’-tetramethylbenzidine was added to each of the relevant wells of the microtitre plate. The plates were covered and set aside to incubate for 10–15 minutes at
room temperature. The horseradish peroxidase activity was stopped by adding 50 µL 0.5 M H$_2$SO$_4$/well. Signal detection at 450 nm was performed using a SpectraMax™ Plus 384 microplate reader.

4.3.5.2 Physico-Chemical Properties of the Synthetic Peptides

The physico-chemical properties of the synthetic peptides were determined \textit{in silico} using the Protein Identification and Analysis Tools on the ExPASy ProtParam server.\textsuperscript{347}

4.3.5.3 Protein Assay

The protein concentration of the supernatant fractions (Section 4.3.4 - Protein Extraction on page 53) was determined using a bicinchoninic acid assay\textsuperscript{366} kit according to the manufacturer’s instructions (see Appendix H: Pierce\textsuperscript{TM} BCA\textsuperscript{TM} Protein Assay MicroTitre Plate Reader Protocol on page 241). Bovine serum albumin was used as the protein standard. Signal detection was performed using a BMG LABTECH CLARIOstar\textsuperscript{TM} microplate reader and data were analyzed using BMG LABTECH MARS Data Analysis software.

4.3.5.4 Protein Sample Preparation

Protein samples were prepared after determining the protein concentration of the supernatant fractions (Section 4.3.5.3 - Protein Assay). Supernatant fractions were diluted in 2x Laemmli buffer (125 mM Tris-HCl, 4% sodium dodecyl sulfate, 10% β-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue, pH 6.8)\textsuperscript{364} to provide the desired concentration of the protein samples.

4.3.5.5 Dot Blotting

Initial evaluation of the antibody reactivity was performed using an adaptation of a previously described method.\textsuperscript{342} Dot blots were used for the initial evaluation because this technique is a quick way to identify if a target peptide or protein can be detected by antibodies.\textsuperscript{342} It should be understood that dot blotting does not normally identify non-specific antibody binding activity,\textsuperscript{342} unless a range of off-target conjugates are also evaluated.

The peroxidase-sensitive chromogenic substrate used in this experiment was prepared as follows:
1. 100 mg 3,3’-diamino-benzidine (DAB) was dissolved in 10 mL with ultrapure H$_2$O.

2. 150–250 µL concentrated HCl was added to the dissolved DAB, which formed a DAB-HCl stock solution.

3. The DAB-HCl stock solution was gently mixed using a vortex mixer until the DAB-HCl had completely dissolved.

4. The DAB-HCl stock solution was aliquoted and stored at -20°C until required.

5. Immediately prior to use, 750 µL of the DAB-HCl stock solution was mixed with 750 µL 0.3% H$_2$O$_2$, and made up to 15 mL with 0.1 M phosphate buffer (pH 7.2) (phosphate buffer) yielding a solution containing 0.05% DAB-HCl and 0.015% H$_2$O$_2$, termed the peroxidase-sensitive chromogenic substrate where appropriate.

0.5 µL dots of a range of concentrations of the respective synthetic peptide-bovine serum albumin (BSA) conjugates in 3% BSA in phosphate buffer were applied to 0.45 µm nitrocellulose membrane strips (3 cm x 0.15-0.25 mm). The benefits of using BSA as a conjugate protein partner have been discussed previously (Section 4.3.2 - Synthetic Peptides- and Glycine Bovine Serum Albumin Conjugates on page 51).

A range of dilutions of the antisera and pre-immune sera were used to probe the dotted strips. The dotted strips were incubated in the antisera or pre-immune sera on a laboratory rocker overnight at 4°C. After a brief wash in phosphate buffer, the dotted strips were incubated for 1 hour in 1:500 biotinylated anti-rabbit secondary antibody in 5% BSA/0.1 M phosphate buffer at room temperature. Following a brief wash in phosphate buffer, the dotted strips were incubated in 1:500 streptavidin horseradish peroxidase complex in 5% BSA/phosphate buffer at room temperature. After a brief wash in phosphate buffer, the dotted strips were incubated in the peroxidase-sensitive chromogenic substrate.

The phosphate buffer used for dot blotting experiments was prepared as set out previously (Section 4.3.2 - Synthetic Peptides- and Glycine Bovine Serum Albumin Conjugates on page 51).

The membrane strips were marked for orientation purposes by clipping the upper right hand corner.

4.3.5.6 Western Blotting

1.5 mm thick 8% polyacrylamide separating gels (Table 4.6 on page 57) were cast using a Bio-Rad Mini-PROTEAN™ Tetra Handcast System. 250 µL isopropanol was overlaid
on each separating gel to remove bubbles, even the surface, and minimize exposure of
the gel solution to atmospheric oxygen while the gel underwent initial polymerization.\textsuperscript{368}
The separating gels were allowed to polymerize for 30 minutes at room temperature.
After 30 minutes, the isopropanol was carefully removed and the gel surface flushed
with 1.5 M Tris (pH 8.8), i.e., the separating gel buffer. 5% polyacrylamide stacking
gel solution (Table 4.6) was poured onto each separating gel and the comb carefully
inserted. Cast gels were sealed in a container with water-moistened paper towel to
maintain humidity and set aside to allow polymerization to proceed fully overnight at
room temperature.\textsuperscript{368–370} Typically, initial polymerization of the gels takes between 30
minutes to 1 hour to occur, but polymerization has not completed at that stage and
continues for several hours.\textsuperscript{370} Incompletely polymerized gels can disrupt separation of
the proteins.\textsuperscript{370}

Table 4.6: Volumes of the solution components for preparing 5\% stacking and 8\%
separating gels for SDS-PAGE. Modified from Roskams & Rodgers\textsuperscript{364} with permission
under copyright held by Cold Spring Harbor Laboratory Press.

<table>
<thead>
<tr>
<th>Solution Components</th>
<th>5% Stacking Gel (mL)</th>
<th>8% Separating Gel (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrapure H\textsubscript{2}O</td>
<td>6.8</td>
<td>11.5</td>
</tr>
<tr>
<td>30% Acrylamide/Bis-Acrylamide, 29:1</td>
<td>1.7</td>
<td>6.7</td>
</tr>
<tr>
<td>1.0 M Tris (pH 6.8)</td>
<td>1.25</td>
<td>-</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>-</td>
<td>6.3</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>10% Ammonium Persulfate</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Immediately before loading the gels, the samples were allowed to reach room tem-
perature, mixed gently in a vortex mixer, and then centrifuged for 2 minutes at 14,000
x g to remove insoluble material.\textsuperscript{370} Failure to remove insoluble material can lead to
streaking.\textsuperscript{370}

\textbf{Note:} While the heating of samples is typically included as a step prior to sample loading
for SDS-PAGE, the effect of heating on the separation of GLT-1d was investigated and
is discussed below.

Sample proteins were separated electrophoretically in running buffer (25 mM Tris Base,
192 mM glycine, and 0.1\% SDS running buffer)\textsuperscript{371–373} using a Bio-Rad Mini-PROTEAN\textsuperscript{TM}
Tetra Cell 1-D Vertical Gel Electrophoresis System (Mini-PROTEAN\textsuperscript{TM} electrophoresis
system). During electrophoresis, the voltage was kept constant at 100V and the tempera-
ture was 4\degree C. After electrophoresis, the gels were removed from the Mini-PROTEAN\textsuperscript{TM}
electrophoresis system and incubated for 10 minutes in transfer buffer (25 mM Tris Base,
192 mM glycine, 20\% methanol) at room temperature to allow the gels to equilibrate
with the transfer buffer. At the same time, 0.45 \( \mu \)m pore nitrocellulose membranes were also equilibrated for at least 10 minutes in transfer buffer.

After equilibration, the gels and membranes were assembled into transfer cassettes of a Bio-Rad Mini-Trans-Blot\textsuperscript{TM} Electrophoretic Transfer Cell System. The proteins were electrophoretically wet-transferred to the nitrocellulose membranes in transfer buffer at a constant voltage of 90V for 90 minutes at 4\(^\circ\)C.

Since both monomer and higher molecular weight oligomer forms of GLT-1d were investigated, consistency of the protein loaded per lane and transfer efficiency was evaluated by Ponceau S staining the membranes immediately after transfer.\textsuperscript{374–376} Quantitation of Ponceau S staining has an advantage over single protein normalization/loading controls as quantitation of Ponceau S staining allows a wide range of sizes of proteins to be evaluated (big proteins or oligomers of proteins transfer more slowly than small ones).

A series of preliminary western blot experiments was performed to optimize western blotting conditions and validate the antisera generated for this thesis. The preliminary western blot experiments included:

**Pre-Immune Sera Reactivity** The pre-immune sera were evaluated for reactivity with the synthetic peptide-bovine serum albumin conjugates and adult mouse whole brain lysate. This validation step was carried out to confirm that the pre-immune sera were not reacting with either the synthetic peptide-bovine serum albumin conjugates or adult mouse whole brain lysate.

**Secondary Antibody Cross-Reactivity** The secondary antibody was evaluated for cross-reactivity with the synthetic peptide-bovine serum albumin conjugates and adult mouse whole brain lysate. This validation step was carried out to confirm that the secondary antibody was not cross-reacting with either the synthetic peptide-bovine serum albumin conjugates or adult mouse whole brain lysate.

**Pre-Absorption of Antisera** Each antiserum was pre-absorbed with the synthetic peptide-bovine serum albumin conjugate that contained the same synthetic peptide as the immunogen used to raise the relevant antiserum. An excess (final concentration \( \sim 200 \) \( \mu \)g/mL) of each synthetic peptide in the form of the synthetic peptide-bovine serum albumin conjugate was added to independent aliquots of the antisera in 0.25% BSA/20 mM Tris Base, 150 mM NaCl and 0.1% Tween-20 (pH 7.2)/0.1% thimerasol and incubated on a laboratory roller overnight at 4\(^\circ\)C. This validation step was carried out to confirm that the antisera detected the synthetic peptides.
Determination of Dynamic Range The dynamic ranges of the antisera were determined using the synthetic peptide-bovine serum albumin conjugates at a range of concentrations. This allowed an approximate determination of dilutions of antisera to be generated for use in western blotting and an indication of the detection threshold of the protein to be estimated.

Preliminary Western Blotting of Adult Mouse Whole Brain Lysate Each antiserum was evaluated to investigate whether it detected a predicted prominent band for the monomeric form and higher molecular weight oligomeric forms of GLT-1d, given that other forms of GLT-1 present as monomer and higher molecular weight oligomer bands on western blotting.\(^{146,212,213}\)

Effect of Detergent in Lysis Buffer Lysates were prepared using a strong anionic detergent, sodium dodecyl sulfate (SDS),\(^{377}\) and a mild non-ionic detergent, nonyl phenoxypolyethoxyl ethanol (NP-40),\(^{377}\) in the lysis buffers to determine their effect on the detection of the GLT-1d monomeric and higher molecular weight oligomer bands. A mild detergent was predicted to allow GLT-1d to remain in a native oligomer form, such as the trimeric form of other forms of GLT-1 that is thought to represent a functional transporter.\(^{146,212}\)

Effect of Sample Heating Standard protocols for western blotting typically comprise heating the samples before SDS-PAGE to denature the proteins.\(^{341,368}\) GLT-1 proteins are membrane proteins.\(^{14}\) Membrane bound proteins are known to be susceptible to aggregation during heating.\(^{368}\) Accordingly, the effect of heating of the samples on the detection of the monomeric and higher molecular weight oligomer forms of GLT-1d was investigated. Samples that were heated to 85°C for 10 minutes were run alongside unheated samples.

Effect of Blocking Agent Antibody binding is known to be affected by blocking agents.\(^{378–380}\) Accordingly, the effect of the blocking agent was investigated. The blocking agents used were commercial (supermarket) fat-free milk powder, bovine serum albumin, goat serum, and horse serum. The blocking agents were made up in 20 mM Tris Base, 150 mM NaCl and 0.1% Tween-20 (pH 7.3).

Effect of pH and Ionic Strength Antibody-antigen binding is predominantly due to non-covalent interactions such as electrostatic forces, hydrogen bonds, Van der Waals forces, hydrophobic interactions, and cation-pi interactions.\(^{381}\) Both pH and ionic
strength are known to influence non-covalent interactions and thus antibody binding.\textsuperscript{382} Accordingly, the effect of both pH and ionic strength on the detection of the monomer and higher molecular weight oligomer forms of GLT-1d was investigated. Membranes were blocked and incubated in buffers containing 20 mM Tris Base and 0.1% Tween-20, with the pH or the NaCl concentration varied as required.

**Effect of Primary Antiserum Dilution**  It is important to establish the optimal primary antiserum dilution when optimizing western blotting conditions for a new primary antiserum to obtain the best signal-to-noise ratio.\textsuperscript{365} Accordingly, a series of dilutions of each antiserum was evaluated to investigate the effects on the detection of the monomer and higher molecular weight oligomer forms of GLT-1d.

**Effect of Total Protein Load**  It is also important to establish the optimal protein load when performing western blots to ensure the signal detected is within the dynamic range of the detection system employed.\textsuperscript{383} Accordingly, the effect of total protein loaded on signal detection was investigated.

**Optimized Western Blotting Method**  An optimized western blotting method was developed based on the results of the optimization and validation steps described above. The optimized method included the SDS-PAGE, wet transfer, and Ponceau S staining steps set out on pages 56–58 above and the following steps:

1. Incubation in blocking buffer, i.e., 0.25% bovine serum albumin in 20 mM Tris Base, 200 mM NaCl and 0.1% Tween-20 (pH 7.3) (TBST), on a laboratory rocker for 1 hour at room temperature.
2. Incubation overnight with primary antiserum in fresh blocking buffer on a laboratory rocker at 4°C.
3. Wash in TBST on a laboratory rocker at room temperature (6x 10 minutes/wash).
4. Incubation with anti-rabbit secondary antibody horseradish peroxidase complex (1:20,000) in fresh blocking buffer on a laboratory rocker for 2 hours at room temperature.
5. Wash in TBST on a laboratory rocker at room temperature (6x 10 minutes/wash).

Detection was revealed using an enhanced chemiluminescence substrate. Images of the chemiluminescent signal generated were taken using a Bio-Rad ChemiDoc\textsuperscript{TM} XRS+ system. Densitometry was performed using Bio-Rad Image Lab\textsuperscript{TM} software.
The predicted molecular weight of the separated proteins was determined by the simultaneous separation of molecular weight standards on each western blot. Image Lab™ software includes a merge feature that enables the combination of a colorimetric image with the corresponding chemiluminescent image. Western blot images in this thesis were prepared using this feature, which ensured accurate registration of molecular weight markers with the detected bands.

4.3.5.7 Neonatal Mouse Brain Lysate as Negative Control

If possible, antibody specificity should be confirmed by using lysate from knockout mice as negative controls. Although GLT-1 knockout mice exist, access to brain tissue or lysate of these mice could not be obtained. Neonatal mice and rats are known to express GLT-1 at very low/undetectable levels. Accordingly, neonatal mice represented a meaningful negative control.

The expression of GLT-1d and GLT-1a, the dominant GLT-1 variant, in neonatal and adult mouse brains was investigated using Rb 1713 antiserum and previously characterized GLT-1a antibodies, respectively. GLT-1a was selected for comparative purposes as GLT-1a is the most abundant known GLT-1 splice variant.

4.3.5.8 Pan-GLT-1 antiserum

Western blots of adult mouse brain lysate were probed for GLT-1d and GLT-1 in general using Rb 1713 antiserum and a pan-GLT-1 antiserum, respectively. The blots were evaluated for correspondence of the bands detected.

4.3.5.9 Identification of Putative Off-Target Linear B-Cell Epitopes

Off-target proteins comprising linear B-cell epitopes that correspond to sequences within the synthetic peptides (Section 4.2.3.2 - Synthetic Peptide Design on page 46) were identified by BLAST searches. The predicted molecular weights of the identified proteins were determined in silico using the Protein Identification and Analysis Tools on the ExPASy ProtParam server.

The predicted molecular weights were useful for the interpretation of western blots, i.e., to provide an indication of the possible identity of any anomalous bands that might be detected by western blotting.
4.4 Results

4.4.1 Physico-Chemical Properties of the Synthetic Peptides

Physico-chemical properties, i.e., theoretical pIs and grand average of hydropathicity (GRAVY) values, of the synthetic peptides were determined in silico using the Protein Identification and Analysis Tools on the ExPASy ProtParam server (Table 4.7). The GRAVY scores were determined by finding the sum of the hydropathy values of the amino acid residues of each synthetic peptide, and dividing the result by the relevant sequence length. A positive GRAVY score on a scale of -2 to +2 represents hydrophobicity. These values were useful to understand antibody binding and informed the optimization of western blotting conditions, i.e., to refine the pH and ionic strength of the buffers.

Table 4.7: Physico-chemical properties of the rat and human synthetic peptides determined in silico using the Protein Identification and Analysis Tools on the ExPASy ProtParam server.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical pI</td>
<td>6.73</td>
<td>6.9</td>
</tr>
<tr>
<td>Grand Average of Hydropathicity (GRAVY)</td>
<td>0.761</td>
<td>0.644</td>
</tr>
</tbody>
</table>

4.4.2 Antibody Production

The rabbits—designated Rb 1713, Rb 1714, Rb 1715, and Rb 1716—responded to the immunization and produced antibodies against the relevant synthetic peptides. Co-administering Freund’s Adjuvant with the immunogenic synthetic peptide-keyhole limpet hemocyanin conjugates resulted in the generation of high titre antibodies (Table 4.8 on page 63).

The antibody titre for Production Bleed #1, i.e., day 35 of the immunization procedure (Table 4.5 on page 50), was determined to be the dilution that gave a signal, i.e., absorbance at 450 nm, that was approximately 2.5x higher than the background reading. The antibody titres of Rb 1715 and Rb 1716 antisera were substantially higher than Rb 1713 and Rb 1714 antisera (Table 4.8).

4.4.3 Dot Blots

The antisera reactivity was initially evaluated by dot blotting for the relevant synthetic peptide-bovine serum albumin conjugates using 0, 5, 50, and 500 ng of the synthetic
Table 4.8: ELISA antibody titres of New Zealand White rabbits immunized using synthetic peptide-keyhole limpet hemocyanin conjugates as immunogens.

<table>
<thead>
<tr>
<th>Synthetic Peptide</th>
<th>Rabbit</th>
<th>ELISA Titre</th>
<th>Animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>KGTWFSVPSMHVL-DAcisFPLG</td>
<td>Rb 1713</td>
<td>795,100</td>
<td>Rat</td>
</tr>
<tr>
<td></td>
<td>Rb 1714</td>
<td>300,600</td>
<td></td>
</tr>
<tr>
<td>KHVVCGHDACISLPLG</td>
<td>Rb 1715</td>
<td>6,677,700</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>Rb 1716</td>
<td>10,007,900</td>
<td></td>
</tr>
</tbody>
</table>

peptide in conjugate form loaded as a 0.5 µL dot. 10 mg BSA/4% formaldehyde/0.1 M phosphate buffer solution (pH 7.2) was defined as 0 ng synthetic peptide/negative control.

Rb 1713 antiserum at 1:50,000 (Figure 4.3 A) detected the rat synthetic peptide across the concentration range suggesting an initial bracketing of the Rb 1713 antiserum around this dilution for western blots, i.e., 1:40,000 to 1:60,000. Rb 1714 antiserum at 1:50,000 (Figure 4.3 B) exhibited good distinction across the dynamic range, but with a higher signal-to-noise ratio at 1:10,000 (Figure 4.3 C) suggesting initial use of Rb 1714 antiserum at dilutions of 1:20,000 to 1:40,000 for western blots. Rb 1715 antiserum was able to detect the human synthetic peptide differentially, whereas Rb 1716 antiserum detected both rat and human synthetic peptides reflecting sequence homology between the rat and human synthetic peptides (data not shown).

None of the antisera detected 0 ng synthetic peptide/negative control (Figures 4.3 and 4.4) or the cross-link (methylene bridge) formed by the conjugation reaction, i.e., the glycine-bovine serum albumin conjugate (data not shown).

Figure 4.3: Representative dot blots of the synthetic peptide KGTWFSVPSMHVL-DAcisFPLG (rat) probed with Rb 1713 and Rb 1714 antisera. A range of concentrations of Rb 1713 and Rb 1714 antisera (1:10,000–1:200,000) was used to probe dot blots at a range of concentrations of the synthetic peptide KGTWFSVPSMHVL-DAcisFPLG, i.e., 5–500 ng synthetic peptide/0.5 µL dots. Rb 1713 antiserum (1:50,000) detected the synthetic peptide across the range of synthetic peptide concentrations, whereas Rb 1714 antiserum (1:50,000) exhibited good distinction across the range of synthetic peptide concentrations, but with an optimum signal-to-noise ratio at 1:10,000.
None of the pre-immune sera detected a negative control, the cross-link (methylene bridge), or the synthetic peptide-BSA conjugates (data not shown).

4.4.4 Western Blots

4.4.4.1 Pre-Immune Sera Reactivity

The pre-immune sera did not react with either the synthetic peptide-bovine serum albumin conjugates or adult mouse whole brain lysate (data not shown). The lack of reactivity confirmed that reactivity detected by the Rb 1713–Rb 1716 antisera was not due to pre-existing reactivity.

4.4.4.2 Secondary Antibody Cross-Reactivity

The secondary antibody conjugates used in these studies did not cross-react with either the synthetic peptide-bovine serum albumin conjugates or adult mouse whole brain lysate (data not shown). The lack of cross-reactivity confirmed that the signal detected using the primary antisera was due to reactivity of the primary antisera with either the synthetic peptide-bovine serum albumin conjugates or antigens in the adult mouse whole brain lysate.

4.4.4.3 Pre-Absorption of Antisera

Pre-absorbing the antisera with an excess of the relevant synthetic peptide-bovine serum albumin conjugate resulted in a dramatic reduction in the signal detection by all the antisera. Western blots using normal antisera detected a prominent band at ∼60 kDa.
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(Figure 4.5 B) for the synthetic peptide-conjugates, i.e., close to the expected ~66 kDa. A faint band was just detected when the pre-absorbed antisera were exposed for the same time, i.e., 1 second, as the normal antibodies. For example, when the exposure time of the pre-absorbed antisera of rabbit Rb 1713 was extended to 30 seconds a faint band was detected (Figure 4.5 D).

![Figure 4.5: Representative western blots demonstrating the effect of pre-absorption on the detection of the KGTWFSVPDMSVLSDACISFPLG (rat) synthetic peptide by Rb 1713 antiserum (1:50,000). The antiserum was pre-absorbed with excess KGTWFSVPDMSVLSDACISFPLG-bovineserumalbuminconjugate(finalpeptideconcentration~200 µg/mL) overnight at 4°C. The exposure time for the Rb 1713 antiserum (1:50,000) was 1 second [B], pre-absorbed Rb 1713 antiserum was 1 second [C] and 30 seconds [D] (hence the artefactual darker background of the image [D]), indicating that pre-absorption dramatically reduced signal detection by the Rb 1713 antiserum. [A] Molecular weights (MW) are indicated to the left of the blots (kDa). Samples lanes = 100 ng synthetic peptide/lane.](image)

**4.4.4.4 Determination of Dynamic Range**

The antisera were able to detect the synthetic peptide-bovine serum albumin across a wide range, i.e., from 10–100 ng. Detection of the synthetic peptide at nanogram levels was demonstrable at, for example, dilutions of 1:100,000 of Rb 1713 antiserum (Figure 4.6 on page 66), indicating a high level of sensitivity of each primary antiserum for the relevant synthetic peptide.

**4.4.4.5 Selection of Rb 1713 Antiserum for Further Assessment**

On the basis of the dot blot and dynamic range western blot experiments, Rb 1713 antiserum was selected for further assessment.
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Figure 4.6: Representative western blot of the KGTWFSVPSMHVLDACISFPLG (rat) synthetic peptide-bovine serum albumin conjugate detected with Rb 1713 antiserum (1:100,000). 10–100 ng of the synthetic peptide was detected indicating high level of sensitivity of the antiserum. Molecular weights are indicated to the left of the blot (kDa).

4.4.4.6 Preliminary Western Blotting of Adult Mouse Whole Brain Lysate

Western blot experiments performed using a standard protocol\textsuperscript{341} demonstrated that Rb 1713 antiserum detected a prominent broad band at \( \sim 65 \) kDa and a less prominent higher molecular weight band in adult mouse whole brain lysate. As mentioned previously, these bands are normally thought to represent monomer and oligomeric forms of GLT-1, at least for other known forms of GLT-1.\textsuperscript{146,212} Pre-immune Rb 1713 serum, on the other hand, did not detect either band.

4.4.4.7 Effect of Detergent in Lysis Buffer

No detectable difference in the signal was observed for the prominent broad monomer band at \( \sim 65 \) kDa as detected by Rb 1713 antiserum when either the strong anionic detergent sodium dodecyl sulfate or the mild non-ionic detergent, nonyl phenoxy(polyethoxyl) ethanol (NP-40) were used in the lysis buffers (Figure 4.7).

Figure 4.7: Representative western blot showing the effect of the detergent in the lysis buffer on the detection of GLT-1d using Rb 1713 antiserum (1:100,000). Lysis buffers containing either a strong anionic detergent, sodium dodecyl sulfate (SDS), or a mild non-ionic detergent, nonyl phenoxy(polyethoxyl) ethanol (NP-40), were used to prepare mouse whole brain lysates. 10 \( \mu \)g and 1.0 \( \mu \)g whole brain protein was loaded for each detergent. There was no observable difference in the signal detected by Rb 1713 antiserum for the two detergents. Molecular weights are indicated to the left of the blot (kDa).
4.4.4.8 Effect of Sample Heating

Heating the samples at 85°C for 10 minutes was associated with a shift in the apparent molecular mass of the prominent broad and the less prominent higher molecular weight bands (Figure 4.8). After heating, the monomer form of GLT-1d ran at an apparent molecular weight of ~62 kDa and the higher molecular weight form of GLT-1d at ~150 kDa. The downward shift in apparent molecular weight for the monomer form was ~3 kDa and the higher molecular weight oligomer form was ~50 kDa.

![Figure 4.8: Representative western blot showing the effect of heating the samples on the detection of GLT-1d by Rb 1713 antiserum (1:50,000) in mouse forebrain, midbrain, hindbrain, and cerebellum. Both the monomeric (~65 kDa) and higher molecular weight oligomer forms of GLT-1d underwent a shift in apparent molecular weight when samples were heated at 85°C for 10 minutes. Prior to immunodetection, identity of the amount of protein loaded per lane and transfer efficiency was evaluated by Ponceau S staining. Molecular weights (MW) are indicated to the left of the blot (kDa). Sample lanes = 5 µg protein/lane.]

4.4.4.9 Effect of Blocking Agent, pH, and Ionic Strength

The optimal blocking agent (Figure 4.9), pH (Figure 4.10), and ionic strength (Figure 4.11), i.e., NaCl concentration, was 0.25% bovine serum albumin, pH 7.3, and 200 mM NaCl, respectively, in a 20 mM Tris Base/0.1% Tween-20 buffer solution. The GLT-1d monomeric and higher molecular weight oligomeric bands were not detected when commercial (supermarket) fat-free milk was used as the blocking agent.
4.4.4.10 Effect of Primary Antiserum Dilution

High generalised signal was observed when using Rb 1713 antiserum at dilutions below 1:50,000. An optimal dilution of 1:100,000 (Figure 4.12) was determined for the detection of GLT-1d in adult mouse whole brain protein lysate.
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**Figure 4.11:** Representative western blot illustrating the effect of ionic strength on GLT-1d detection. Sample loaded = 2.5 µg total adult mouse brain protein. Molecular weights (MW) are indicated to the left of the blots (kDa).

**Figure 4.12:** Representative western blots illustrating the effect of primary antibody dilution on GLT-1d detection. Sample loaded = 2.5 µg total adult mouse brain protein. Molecular weights (MW) are indicated to the left of the blots (kDa).

### 4.4.4.11 Effect of Total Protein Load

The optimal protein load for the detection of GLT-1d was ∼2.5–5.0 µg adult mouse whole brain protein/lane, and saturation of signal was determined to be ≥ 15 µg adult mouse whole brain protein/lane.

### 4.4.4.12 Neonatal Mouse Brain Lysate as Negative Controls

GLT-1a and GLT-1d were undetectable in postnatal day one (P₁) mouse brain, whereas both were detected in the adult mouse brain (Figure 4.14).
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4.4.4.13 Pan-GLT-1 antiserum

The pan-GLT-1 antiserum is directed to an N-terminal region of GLT-1 spanning amino acids 12–26. This region is constant across all of the known GLT-1 variants. This antiserum detected a more intense and wider band at ~65 kDa than Rb 1713 antiserum (Figure 4.15), presumably because it collectively detects all forms of GLT-1 that contain the above-mentioned amino acids 12–26, including GLT-1a, b, c, and d.

Figure 4.13: Representative western blot illustrating the effect of total protein loaded on GLT-1d detection. Molecular weights (MW) are indicated to the left of the blots (kDa).

Figure 4.14: Representative western blots for GLT-1a and GLT-1d in postnatal day one (P1) and adult mouse brain. Both GLT-1a and GLT-1d were undetectable in P1 brains. Primary antisera dilutions = 1:100,000. Samples lanes = 2.5 µg whole brain protein/lane. Molecular weights (MW) are indicated to the left of the blots (kDa).
Figure 4.15: Representative western blots for GLT-1d and pan-GLT-1 in adult mouse forebrain. The ∼65 kDa band was more prominent for the pan-GLT-1 antiserum than for GLT-1d antiserum. Pan-GLT-1 antiserum was raised against a synthetic peptide that corresponds to an N-terminal amino acid sequence of GLT-1, which is common to most GLT-1 variants including GLT-1a–d. Accordingly, the pan-GLT-1 antiserum will detect all GLT-1 variants that contain that N-terminal amino acid sequence. Antisera dilutions = 1:100,000. Sample lanes = 2.5 µg forebrain protein/lane. Molecular weights are indicated to the left of the blots (kDa).

4.4.5 Putative Off-Target Linear B-Cell Epitopes

Proteins comprising putative linear B-cell epitopes that correspond with sequences within the rat synthetic peptide were identified by BLAST searches (Appendix J: Proteins with Putative B-Cell Epitopes on page 253) and the relevant proteins, including putative proteins, are summarised in Table 4.9 on page 72. The molecular weight of each of these identified proteins was determined using the Protein Identification and Analysis Tools on the ExPASy ProtParam server.

4.5 Discussion

The present study investigated whether synthetic peptides that corresponded to unique C-terminal amino acid sequences of rat and human GLT-1d could be used to produce antisera and, if so, whether the produced antisera were useful in the investigation of GLT-1d expression and distribution in mouse brain.
Table 4.9: Potential off-target proteins that are expressed in the mouse brain were identified by BLAST searches. These proteins contain sequences that correspond to putative linear B-cell epitopes in the synthetic peptide KGTWSVPMLHVLDACISFPLG. The molecular weight of each identified protein was determined using the Protein Identification and Analysis Tools on the ExPaSy ProtParam server.347

<table>
<thead>
<tr>
<th>Protein/Peptide</th>
<th>Molecular weight (kDa)</th>
<th>Sequence</th>
<th>Protein: Distribution and Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Synthetic Peptide</td>
<td>KGTWSVPMLHVLDACISFPLG</td>
<td>79.5</td>
<td>Expressed during brain development and in adult mouse brain, implicated in synapse formation and maintenance and adult brain function.396</td>
</tr>
<tr>
<td>2610204M08Rik protein (AAI15423.2)</td>
<td>79.5</td>
<td>FSVPSSMM</td>
<td>Expressed during brain development and in adult mouse brain, implicated in synapse formation and maintenance and adult brain function.396</td>
</tr>
<tr>
<td>pre-mRNA cleavage complex 2 protein Pcf11 (NP_083354.3)</td>
<td>173</td>
<td>SVPSKLHV</td>
<td>Various foetal and adult mouse tissues including brain.97</td>
</tr>
<tr>
<td>PREDICTED: pre-mRNA cleavage complex 2 protein Pcf11 (XP_006508335.1)</td>
<td>188</td>
<td>SVPSKLHV</td>
<td>Various foetal and adult mouse tissues including brain.97</td>
</tr>
</tbody>
</table>

4.5.1 Production of Antisera

Four New Zealand White rabbits—designated as Rb 1713, Rb 1714, Rb 1715, and Rb 1716—were immunized using synthetic peptide-keyhole limpet hemocyanin conjugates as immunogens. All four rabbits responded to the immunization and produced high titre antisera. Interestingly, the titre of the antisera produced against the synthetic peptide that corresponded to the human unique C-terminal amino acid sequence (Rb 1715 and Rb 1716) was ~15-fold higher than that produced against the synthetic peptide that corresponded to the rat unique C-terminal amino acid sequence (Rb 1713 and Rb 1714) (Table 4.8 on page 63). The production of the present antisera was complicated because only small hydrophobic regions, i.e., the unique sequence of amino acid residues 551–572 (rat) and 552–566 (human) (Figure 4.1 on page 43) could be used to produce the antisera. Typically, hydrophilic regions are more advantageous for producing antisera,340 presumably because hydrophilicity increases solubility and accessibility of the antigen during the production of antibodies in vivo and subsequently during detection. Alternatively, or in addition, it is possible that, during antibody production in rabbits 1715 and 1716, the synthetic peptide that corresponded to the unique C-terminal amino acid sequence of human GLT-1d may have been more efficiently processed for antigen presentation332,345 and, consequently, produced antisera having a higher antibody titre. One possible explanation for the higher antibody titre detected for the antisera directed against the human unique sequence of amino acid residues may be that the predicted
hydrophobicity of this peptide was $\sim 15\%$ lower than that predicted for the peptide comprising the rat unique sequence of amino acid residues (Table 4.7 on page 62 regarding the hydrophobicity of the synthetic peptides). Accordingly, it is possible that the peptide having the human unique sequence of amino acid residues was more hydrophilic in solution than the peptide having the rat unique sequence of amino acid residues.

The limited animal house facilities at RMIT University and the costs associated with having the antibodies produced by New England Peptide, Inc. necessarily restricted the number of rabbits used and antisera produced.

4.5.2 Validation of Antisera

The SLC1 high-affinity glutamate and neutral amino acid transporters include the EAAT (Excitatory Amino Acid Transporters) and ASCT (Alanine-Serine-Cysteine-Threonine Transporters) transporters. As mentioned previously, GLT-1 is a member of the EAAT family. The primary amino acid sequences of the EAATs exhibit 50–60\% homology within the EAATs and 30–40\% homology with the ASCTs. Such high levels of homology complicate the production of antisera for use as research tools to study members of the SLC1 transporter family.

The specificity of the antisera produced against the synthetic peptides was examined by dot- and western blotting. Dot blotting confirmed that the antisera were able to detect the relevant synthetic peptides used to produce them. On the other hand, none of the antisera and pre-immune sera detected bovine serum albumin or the cross-link (methylene bridge) formed by the conjugation reaction. Western blotting confirmed that each antiserum was highly sensitive to the relevant synthetic peptide used to produce the antiserum. No signal was observed in western blotting when either synthetic peptide or adult whole brain lysate were probed only using secondary antibody. Furthermore, when each antiserum was pre-absorbed using the relevant synthetic peptide used to produce the antiserum, the signal was undetectable in western blotting under the same conditions as for the corresponding normal antiserum. Signal for pre-absorbed antisera was only detected when the exposure time was substantially extended, i.e., 30x longer than the exposure time for the normal antisera (Figure 4.5 on page 65). The lack of signal for both the secondary antibody and the pre-absorbed antisera confirmed that the signal observed could be attributed to the antisera. Unlike the other antisera, using dot blotting Rb 1716 antiserum was able to detect both synthetic peptides. The last nine amino acids at the C-terminal of the rat and human synthetic peptides (Table 4.10) only differ by one amino acid residue, i.e., phenylalanine (F) and leucine (L), which is a highly conserved amino acid substitution according to the BLOSUM 90
substitution matrix. One explanation for this cross-reactivity is that the Rb 1716 antiserum contains antibodies that recognize both these sequences. The ability of Rb 1716 antiserum to detect both human and mouse GLT-1d indicates a potential use to detect GLT-1d of other mammalian species as well. This antibody will be used in future cross-species studies to investigate the expression of GLT-1d.

Table 4.10: C-terminal amino acid residues of the synthetic peptides.

<table>
<thead>
<tr>
<th>C-Terminal Amino Acids</th>
<th>Synthetic Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>DACISFPLG</td>
<td>Rat</td>
</tr>
<tr>
<td>DACISLPLG</td>
<td>Human</td>
</tr>
</tbody>
</table>

Western blots of GLT-1 are typically characterized by a prominent broad band at ~65 kDa and less prominent higher molecular weight oligomeric band(s). As mentioned previously, these bands are normally thought to represent monomeric and oligomeric forms of GLT-1, at least for other known forms of GLT-1. Rb 1713 antiserum detected a prominent band at ~65 kDa and a less prominent higher molecular weight band in adult mouse whole brain lysate. A similar distribution has been shown when GLT-1 was solubilised in an SDS buffer, similar to the lysis buffer used here. Interestingly, monomer bands typically arise on their own only when transporters are solubilised in an SDS-containing buffer and then immediately separated by SDS-PAGE. When Rb 1713 and pan-GLT-1 antisera were used to probe adult mouse whole brain lysate, they detected similar bands by western blotting, suggesting they were detecting similar targets. The ultimate validation of the Rb 1713 antiserum would have been to investigate whether this antiserum detected any signal in a GLT-1 knockout mouse. As mentioned previously, GLT-1 knockout mice exist, but lack of access precluded this option. Neonatal mice and rats express GLT-1 at very low/undetectable levels. Accordingly, neonatal mice were therefore used as a substitute. Western blots for GLT-1d and GLT-1a of adult mouse brain exhibited a prominent band at ~65 kDa, this band was undetectable in neonatal mouse brains (Figure 4.14 on page 70). The validation steps above, particularly the absence of signal observed in the neonatal mouse brain and the correspondence of the signal detected by the pan-GLT-1 and Rb 1713 antisera, support the contention that the Rb 1713 antiserum detects GLT-1d.

**4.5.3 Optimization of Western Blotting Conditions**

The optimization of western blotting conditions can dramatically improve the sensitivity and specificity of the results. Consequently, the effect of the detergent in the lysis buffers, heating of the samples before electrophoresis, blocking agent, pH, ionic strength,
primary antiserum dilution, and total protein load on the detection of GLT-1d by western blotting of adult whole brain lysate using the GLT-1d antibodies was investigated.

### 4.5.3.1 Lysis Buffer Detergent

GLT-1 proteins are membrane proteins,\(^\text{14}\) which are known to be resistant to detergents.\(^\text{377}\) No difference was observed in the detection of the monomeric band at ~65 kDa by western blot when either SDS or NP-40 were used in the lysis buffers (Figure 4.7 on page 66). Since both SDS and NP-40 are capable of breaking up the cellular membrane,\(^\text{365}\) it possible that once lysate samples were prepared for western blotting, the 2x Laemmli buffer used in the sample preparation further dissociated the GLT-1d protein into monomer form.

### 4.5.3.2 Heating of Western Blotting Samples

When the samples were heated at 85°C for 10 minutes a decrease in the apparent molecular weight at which the monomeric and higher molecular weight oligomeric forms of GLT-1d ran on western blot was observed (Figure 4.8 on page 67). One explanation for the downward shift observed for the higher molecular weight oligomeric form of GLT-1d is heat-induced dissociation of a putative ~50 kDa protein partner. Both rat and human GLT-1d contain a putative class II PDZ motif (Table 4.11), i.e., \(-X-\Phi-X-\Phi\), where X is any amino acid residue and \(\Phi\) is a hydrophobic amino acid residue.\(^\text{291}\)

**Table 4.11:** Putative class II PDZ motif in the C-terminal of rat and human GLT-1d.

<table>
<thead>
<tr>
<th>GLT-1d</th>
<th>Putative Class II PDZ Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>DACISFPLG</td>
</tr>
<tr>
<td>Human</td>
<td>DACISLPLG</td>
</tr>
</tbody>
</table>

One such protein partner that interacts with another GLT-1 C-terminal splice variant, i.e., GLT-1b, is the PDZ domain protein PICK1,\(^\text{292}\) which has a molecular weight of ~50 kDa.\(^\text{401}\) While GLT-1b contains a class I PDZ motif (Table 2.3 on page 25), PICK1 is known to interact promiscuously with all three classes of PDZ motifs.\(^\text{402}\) It is plausible, therefore, that PICK1 may interact with GLT-1d. Indeed, support for PICK1 being a protein that interacts with GLT-1d can be found in glutathione S-transferase-PICK1 fusion protein pull-down experiments (Dr Aven Lee, personal communication). These pull-down experiments demonstrated that rat GLT-1b and GLT-1d interacted, at least \textit{in vitro}, with PICK1 (Figure 4.16 on page 76). The mouse genome comprises ~928 PDZ domains distributed amongst ~328 proteins.\(^\text{403,404}\) Accordingly, future studies are required to identify protein partners that bind to GLT-1d via the PDZ motif.
Chapter 4: *Antisera - Research Tools to Investigate GLT-1d*

Figure 4.16: Representative western blots of pull-down experiments using a glutathione S-transferase-PICK1 fusion protein to illustrate PICK1 interaction with rat GLT-1b and GLT-1d. Molecular weights are indicated to the left of the blots (kDa). Abbreviations: glutathione S-transferase (GST), and protein interacting with C kinase - 1 (PICK1). Permission to use the blot images to prepare this figure was granted by Dr Aven Lee of the Centre for Clinical Research, University of Queensland.

Heating also induced a shift for the monomeric form of GLT-1d such that it ran as a broad band at \(\sim 62\) kDa. This shift may in part be due to so-called "gel shifting", which is common to membrane proteins.\textsuperscript{405} The heating may have increased the SDS solvation of the GLT-1d protein, resulting in the shift in apparent molecular weight.\textsuperscript{405} As mentioned previously, membrane bound proteins are known to be susceptible to aggregation during heating.\textsuperscript{368} When the heating experiment was repeated, but the stacking gel was retained in place during protein transfer to the nitrocellulose membrane, signal was detected in the wells, indicating a likelihood of aggregation. As such, aggregation may explain reduction in the intensity of relevant bands for the heated samples. Such changes in the apparent molecular weight and intensity of bands due to the heating of samples is an important finding with respect to GLT-1 investigations.

4.5.3.3 Blocking Agent

No signal was detected when commercial (supermarket) fat-free milk powder was used as blocking agent. It will be appreciated that commercial (supermarket) fat-free milk is notionally fat free. Accordingly, it is plausible that the hydrophobic C-terminal of GLT-1d interacted with fats found in the commercial (supermarket) fat-free milk powder, which is notionally fat-free. Such interaction might have masked the epitope on the C-terminal of GLT-1d protein that is detected by Rb 1713 antiserum. In contrast to the lack of signal detected here, fat-free milk is commonly used as blocking agent.\textsuperscript{219,288,290}
Chapter 4: Antisera - Research Tools to Investigate GLT-1d

with no apparent untoward effect on the detection of GLT-1, which may be due to epitope accessibility. Since 0.25% BSA was an efficient blocking agent, and surpassed both goat and horse serum at similar concentrations, the underlying reason for the lack of GLT-1d signal when commercial (supermarket) fat-free milk powder was used as blocking agent was not pursued further at this stage.

4.5.3.4 pH and Ionic Strength of Buffers

Antibody-antigen binding relies on non-covalent bonds, such as hydrogen bonds, van der Waals forces, Coulombic interactions, and hydrophobic interactions.\cite{335} Changes in these bonds due to pH and ionic strength\cite{382} can substantially reduce the strength of interaction between the antibody and antigen.\cite{335} Accordingly, the strength of these non-covalent bonds determine how useful the antibody will be as a research tool.\cite{406} The optimal pH and NaCl concentration for the GLT-1d antibodies were pH 7.3 and 200 mM NaCl, respectively. The optimal pH is physiological for rabbit serum, whilst the optimal NaCl concentration at 200 mM NaCl for western blotting is approximately isotonic if all serum ions are taken into consideration.\cite{407}

4.5.3.5 Primary Antiserum Dilution and Sample Loading

Primary antiserum dilution and sample loading are two factors that are important when optimizing western blotting conditions.\cite{383} The antibody titre for the Rb 1713–Rb 1716 antisera was high (Table 4.8 on page 63). Typically, high antibody titre antisera result in blots with high background and an increased detection of non-specific bands,\cite{341} unless used in a highly diluted state. Consequently, establishing the optimal GLT-1d antiserum dilution was critical if the best signal-to-noise ratio was to be obtained for western blotting.\cite{341} Similarly, it was important to establish the optimal protein load to ensure that signal detection was within the dynamic range of the primary antiserum.\cite{383} A good signal-to-noise ratio for the GLT-1d antiserum was obtained for a dilution of 1:100,000 and a total protein load of 2.5 µg/lane, which was indicative of a highly sensitive antiserum.

4.5.4 Putative Off-Target Linear B-Cell Epitopes

During antibody production in the rabbit the synthetic peptides would have been processed for antigen presentation.\cite{332,345} As such, it was considered possible that off-target proteins, i.e., proteins that are not monomer or higher molecular weight oligomer forms of GLT-1d, comprise epitopes that correspond to putative linear B-cell epitopes in the
synthetic peptides of the immunogens used to produce the antibodies. Accordingly, the possibility of non-specific cross-reactivity with non-target proteins could not be discounted. Predicted linear B-cell epitopes were identified based on the hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns or a combination of these properties for each of the synthetic peptides used to produce the antisera using the BcePred Prediction Server. Since Rb 1713 was selected for use in the further investigations of this thesis, attention was directed to the synthetic peptide that corresponded to the unique C-terminal amino acid sequence of rat GLT-1d.

Potential off-target proteins comprising linear B-cell epitopes that correspond to putative linear B-cell epitopes in the synthetic peptide KGTWFSVPSMLHVDACISFPLG were identified by BLAST searches. The predicted molecular weights of the identified proteins were determined in silico using the Protein Identification and Analysis Tools on the ExPASy ProtParam server. Selected potential off-target proteins that are expressed in the brain and were considered of particular importance with respect to the subsequent studies of this thesis are listed in Table 4.9.

The proteins set out in Table 4.9 were considered of particular importance with respect to the subsequent studies of this thesis, given these proteins are expressed or potentially expressed in the mouse brain. 2610204M08Rik protein (AAI15423.2) and 2610204M08Rik protein (AAI15424.2) are expressed during brain development and in adult mouse brain, are implicated in synapse formation and maintenance, and associated with adult brain function. A band was not detected at the predicted molecular weight for these proteins, i.e., 79.5 kDa. Pre-mRNA cleavage complex 2 protein Pcf11 (NP_083354.3) and the predicted protein pre-mRNA cleavage complex 2 protein Pcf11 (XP_006508335.1) were cloned from foetal and adult mouse tissues including brain. The predicted molecular weight of these proteins is 173 kDa and 188 kDa, respectively. Pre-mRNA cleavage complex 2 protein Pcf11 (NP_083354.3) and the predicted protein pre-mRNA cleavage complex 2 protein Pcf11 (XP_006508335.1) may, if present, consolidate into the higher molecular weight band detected by GLT-1d antiserum on western blotting. Since Rb 1716 antiserum detected both rat and human synthetic peptides, it would appear that the C-terminal sequences of the rat (DACISFPLG) and human (DACISLPLG) synthetic peptides may represent a common and dominant GLT-1d epitope. A future study will produce antibodies against the synthetic peptides DACISFPLG and DACISLPLG. Such antibodies should, accordingly, not detect 2610204M08Rik protein (AAI15423.2), 2610204M08Rik protein (AAI15424.2), Pre-mRNA cleavage complex 2 protein Pcf11 (NP_083354.3), or the predicted protein pre-mRNA cleavage complex 2 protein Pcf11 (XP_006508335.1).
4.6 Significance Statement

This study established that the Rb 1713 antiserum was a useful research tool to investigate GLT-1d in mouse brain. The study suggested that the $\sim 50$ kDa downward shift in the apparent molecular weight of the higher molecular weight oligomer form of GLT-1d detected in heated western blotting samples was possibly due to the heat-induced dissociation of a protein partner, PICK1.

4.7 Further Observations

Observations regarding this study, and its integration into achieving the aim of the research for this thesis, are set out in Chapter 8.

4.8 Key Points

This study was undertaken to produce antisera using synthetic peptides that corresponded to the unique C-terminal amino acid sequences of rat and human GLT-1d, and to validate the antisera for use in the investigation of the expression and distribution of GLT-1d in mouse brain. In the course of this study, the following key points were revealed:

* Synthetic peptides corresponding to the unique GLT-1d C-terminals of rat and human GLT-1d protein produced high titre antisera.

* All the antisera produced, i.e., Rb 1713–Rb 1716, were able to detect the relevant synthetic peptides by dot- and western blotting.

* Pre-immune sera did not detect the synthetic peptides by dot- and western blotting.

* Detection of a prominent band, possibly a GLT-1d monomer, at $\sim 65$ kDa by western blotting was not affected when either the strong anionic detergent (SDS) or the mild non-ionic detergent, nonyl phenoxypolyethoxyl ethanol (NP-40) were used in lysis buffers.

* Heating the samples at 85°C for 10 minutes before western blotting led to a downward shift in the apparent molecular weight of a higher molecular weight oligomer form of GLT-1d detected by Rb 1713 antiserum, such shift may be due to heat-induced dissociation of PICK1.
Heating the samples at 85°C for 10 minutes before western blotting led to the broad prominent band migrating at \( \sim 62 \text{ kDa} \), which may be due to increased SDS solvation. Aggregation in the wells of the stacking gel may explain the reduction in intensity of the relevant bands detected for the heated samples relevant to the corresponding bands of the unheated samples.

Rb1713 antiserum detected a prominent band at \( \sim 65 \text{ kDa} \) by western blotting that corresponds in size to the similar broad band detected by pan-GLT-1 antiserum. Detection of GLT-1d by western blotting was optimal in 0.25% bovine serum albumin, 20 mM Tris Base, and 200 mM NaCl at pH 7.3 buffer solution.

Rb 1713 antiserum was a useful research tool to investigate the temporal, spatial, and pathological expression profiles of GLT-1d in mouse brain.
Part III

GLT-1d in Juvenile, Adult, and Pathological Mouse Brains
GLT-1d in Juvenile Mouse Brain

5.1 Synopsis

Aim: The aim of this study was to examine the expression and regional and cellular distribution profile of GLT-1d in juvenile, defined here as postnatal day 1 (P1)–P21, mouse brain.

Research Question: What is the temporal expression and distribution profile in juvenile mouse brain?

Methods: Quantitative PCR (qPCR) primers were designed to examine expression of target mRNA, i.e., GLT-1a–d, VGLUT1–3, and glutamine synthetase. Before investigating the expression of the relevant target mRNA, the qPCR primers were examined in silico for autodimerization and hairpin formation, and specificity by melt curve analysis. The specificity of the GLT-1d-specific qPCR primers was also examined by electrophoresis of the PCR amplified products. Selected primer pairs were used to investigate the expression of target mRNA. Western blotting was used to investigate the expression of GLT-1a–d, GLAST, and glutamine synthetase protein. The distribution of GLT-1d was investigated using immunohistochemistry.
Results: *In silico* evaluation indicated that neither autodimerization nor hairpin formation was predicted for any of the qPCR primers. Melt curve analysis enabled selection of specific qPCR primers for the target mRNA. A preferred GLT-1d-specific qPCR primer pair was identified using electrophoresis of PCR amplified products. The study demonstrated GLT-1d mRNA increased in expression from P₁–P₂₈, then declined to adult. Similarly, western blotting showed that GLT-1d expression increased dramatically from P₇–P₁₄. Immunohistochemistry demonstrated that GLT-1d was expressed in astrocytes, but not observed in neurons in juvenile mouse brain. Consistent with quantitative PCR and western blotting results of this study, GLT-1d immunoreactivity increased most noticeably from P₇–P₁₄.

Inferences: GLT-1d expression exhibited a temporal expression profile that was coordinated with other key members of the glutamate homeostasis system in juvenile mouse brain. The coordinated expression suggested that there was an increasing demand for GLT-1d activity during mouse brain development and maturation. In juvenile mouse brain, GLT-1d protein was expressed in astrocytes, but not observed in neurons.

Significance: In juvenile mouse brain, GLT-1d protein was expressed in astrocytes, but not observed in neurons, and plausibly may be associated with the inception of and ensuing glutamatergic neurotransmission in juvenile mouse brain.

5.2 Introduction

The study of Chapter 4 of this thesis related to antisera and their production for use as research tools. Antisera comprising antibodies directed against synthetic peptides that corresponded to the unique C-terminal sequences of rat and human GLT-1d were produced and validated in that study. The ultimate purpose of the produced antisera was to provide research tools for use in the investigation of GLT-1d in mouse brain. One such use of these antisera included the investigation of the temporal expression and regional and cellular distribution profile of GLT-1d in juvenile mouse brain.

During brain development, extracellular glutamate acts on ionotrophic and metabotropic glutamate receptors to modulate cell proliferation and migration, differentiation, and synapse formation.¹⁰³,⁴¹⁰–⁴¹⁵ The extracellular level of glutamate is mediated by glutamate transporters¹⁴ including GLT-1, the most abundant glutamate transporter in the mature mammalian brain.¹⁴,¹⁴₄,¹⁴₆–¹⁴₈ In view of the known differential expression of GLT-1 in developing mammalian brains,¹⁰³,²₂₃,³₈₉,³₉₁,⁴₁₆ it was hypothesised that GLT-1d is differentially expressed and distributed in juvenile mouse brain.

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The aim of this study was to investigate the expression and regional and cellular distribution profiles of GLT-1d in juvenile mouse brain. To achieve this aim, the expression profiles of GLT-1a–d, GLAST, VGLUT1–3, and glutamine synthetase were investigated. It would be helpful to recall at this stage that glutamatergic neurotransmission comprises packaging of glutamate into vesicles by vesicular glutamate transporters (VGLUT1–3), exocytotic release, uptake predominantly into astrocytes by glutamate transporters located in plasma membranes (primarily GLT-1 and GLAST), and the catalytic conversion of glutamate into glutamine (glutamine synthetase) for transfer to neurons. The immunohistochemical distribution profile of GLT-1d was investigated to establish the regional and cellular distribution of GLT-1d in juvenile mouse brain.

5.3 GLT-1 in Developing Mammalian Brain

Glutamate, in its role as an excitatory amino acid neurotransmitter, is associated with a number of important developmental events in mammalian brain. Such events include cell proliferation, cell migration and differentiation, cell death, axon and dendrite outgrowth, the establishment, pruning, and maintenance of synapses, and the regulation of gene expression. The extracellular level of glutamate in mammalian brain is predominantly regulated by glutamate transporters located in the cell membranes, in particular the excitatory amino acid transporters. As mentioned previously, the excitatory amino acid transporters include GLT-1, which is the most abundant glutamate transporter in mature mammalian brain. GLT-1 is expressed widely in developing mammalian brain.

In rodents, during embryogenesis GLT-1 mRNA is expressed at low levels in the hippocampus, thalamus, telencephalon, brain stem, and spinal cord. Conversely, during this stage of development, GLT-1 protein expression is focused in the spinal cord. During postnatal rodent brain development, GLT-1 mRNA expression increases widely throughout the neuraxis, and is enhanced in the neocortex, hippocampus–specifically the CA3 pyramidal neurons–and spinal cord grey matter. At a subcellular level, in the juvenile rodent brain during development, the distribution of GLT-1 mRNA noticeably shifts from the proximal processes of astrocytes to the somata. Such distribution of GLT-1 mRNA in the astrocyte processes may be associated with local translation that occurs proximal to the forming synapses during synaptogenesis in the developing brain. In effect, local translation of GLT-1 may enable astrocytes to respond rapidly to changes in glutamate levels in the developing brain. Intriguingly, during the early stages of postnatal mammalian brain development, transient GLT-1 protein expression largely ceases in specific subsets of neurons where it was
expressed\textsuperscript{142,223,416} and becomes focused in astrocytes,\textsuperscript{141,142,213,214,223,312,391,416} albeit with the retention of neuronal GLT-1 mRNA expression.\textsuperscript{214,426} In rodents, GLT-1 protein expression progressively increases throughout the neuraxis to reach adult levels at postnatal day \(\sim\)10–14.\textsuperscript{103,142,312,389,391} This dramatic increase in GLT-1 protein expression is coincident with gliogenesis,\textsuperscript{427,428} synaptogenesis,\textsuperscript{429–431} a transition to mature forms and expression levels of glutamate receptors,\textsuperscript{432–434} and the onset of myelination\textsuperscript{435} in the developing mouse brain. The developing human foetal brain exhibits a similar distribution of GLT-1 protein expression as observed in the postnatal developing rodent brain.\textsuperscript{104} An important role of GLT-1 in developing mammalian brain may be to protect vulnerable white matter from excitotoxic damage.\textsuperscript{103,142,223,416} In support of such a role, GLT-1 mRNA is widely distributed along white-matter tracts in developing mammalian brain, whereas in mature mammalian brain expression is focused in the cell bodies in these tracts.\textsuperscript{103,416} Similarly, GLT-1 protein is abundantly and transiently expressed in white-matter tracts of developing rat brain,\textsuperscript{142} which corresponds to the GLT-1 protein distribution detected in white-matter tracts in developing sheep brain.\textsuperscript{416}

The expression of GLT-1 mRNA and/or protein in at least subsets of neurons, e.g. the subplate and layer VI neurons of the parietal cortex, CA3–CA4 neurons of the hippocampus, spinal cord, Purkinje neurons, and associated white matter\textsuperscript{103,142,223,416} during mammalian brain development suggests a transient neuronal requirement, i.e., GLT-1 may transport glutamate into neurons during mammalian brain development.\textsuperscript{416}

5.4 Experimental Methods

All chemicals used in the experiments of this chapter were of analytical grade and purchased from commercial suppliers. Specific details of the suppliers of the animals, chemicals, and equipment cited in this chapter are set out in Table F.1 in Appendix F: Suppliers of Animals, Chemicals, Equipment, and Services on page 232.

5.4.1 Animal Tissue Collection and Treatment

The experiments of this chapter were performed on tissues of female and male mice of a common inbred strain of laboratory mouse, the C57BL/6 mouse strain. The mice were at different stages of postnatal development, i.e, postnatal day 1 (P\(_1\)) (day of birth), P\(_7\), P\(_{14}\), P\(_{21}\), P\(_{28}\), and adult (\(\sim\)3 months). The P\(_1\)–P\(_{28}\) mice were obtained from timed pregnant dams.
Mice were housed under standard temperature- and light-controlled conditions, i.e., ∼22°C and 12 hours light/12 hours dark, with free access to their lactating dams and/or food and water (as appropriate) prior to the collection of tissues.

P₁ mice were decapitated using a pair of surgical scissors, whilst other mice (P₇–adult) were terminally anaesthetised by intraperitoneal injection of sodium pentobarbital (150 mg/kg). Once the foot withdrawal reflex was absent, the deeply anaesthetised animals were decapitated using a pair of surgical scissors. Each brain was rapidly dissected from the cranial vault according to a previously described method and, depending on the proposed use, treated as follows:

1. RNA extraction or protein extraction: Brains were immediately frozen in liquid nitrogen and stored at -80 °C until required.

2. Immunohistochemistry: Each brain was sagitally separated into hemispheres, including bisecting the fourth ventricle and thereby exposing the choroid plexus of the fourth ventricle. Each hemisphere was then treated according to a standard Pow Laboratory protocol. Briefly, tissue was fixed with 4% freshly prepared formaldehyde/0.1 M sodium phosphate buffer (pH 7.2) (phosphate buffer) for 2 hours at room temperature. The preparation of phosphate buffer and 4% formaldehyde/phosphate buffer has been discussed previously (Section 4.3.2 - Synthetic Peptides- and Glycine Bovine Serum Albumin Conjugates on page 51). The fixed tissue was dehydrated by passing it through a graded series of ethanol solutions, i.e., 50% ethanol/50% ultrapure H₂O (10 minutes) and 100% ethanol (10 minutes) and cleared in eucalyptus oil. Eucalyptus oil is a less toxic organic solvent alternative to xylene. The dehydrated tissue was embedded in paraffin wax. The embedded tissue was set aside until required.

5.4.2 Total RNA Extraction

Total RNA was extracted using an adapted Qiagen RNeasy Mini Kits protocol (Appendix K: Qiagen MiniKit Purification of Total RNA Protocol on page 258). The adapted protocol, which comprised a TRIzol™ reagent extraction step, is discussed briefly below.

Frozen brain tissue (n = 5 animals/age cohort) was crushed in a mortar and pestle under liquid nitrogen. The crushed tissue was sonicated in 1:10 (w/v) ice-cold TRIzol™ reagent (6 x 5 seconds sonication at 23% output, 15 seconds intervals) using a Branson Ultrasonics Sonifier™ to provide a homogenate. The homogenate was incubated at room temperature for 5 minutes. After incubation, 200 µL chloroform was added per
mL homogenate. The chloroform/homogenate solution was shaken vigorously for 15 seconds and then centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the upper aqueous phase was transferred to a new tube. Care was taken not to disturb or transfer the interphase. One volume of 70% ethanol was added to the aqueous phase and gently mixed by pipetting up and down. The combined ethanol/aqueous phase was not centrifuged. Once at this stage, i.e., the combined ethanol/aqueous phase stage, the protocol set out in steps 6–12 of the Qiagen MiniKit Purification of Total RNA Protocol on page 258 (Appendix K) was followed.

The RNA eluates were aliquoted out and stored at -80°C until required.

### 5.4.3 RNA Concentration and Integrity

RNA concentration and integrity was determined using a NanoDrop™ 2000 spectrophotometer according to the manufacturer’s instructions (Appendix M: ThermoFisher Scientific NanoDrop™ 2000 Spectrophotometer Operating Instructions on page 266).

### 5.4.4 Reverse Transcription

Reverse transcription of RNA was performed using a High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions (Appendix L: Applied Biosystems™ High-Capacity cDNA Reverse Transcription Protocol on page 263). Briefly, the appropriate volume of 2X RT master mix was prepared and set aside at 4°C until required. The 2X RT master mix included an RNase inhibitor. RNA samples were diluted to provide 1 µg RNA in 10 µL ultrapure/RNase-free H₂O. 10 µL of each RNA sample was combined with a 10 µL aliquot of 2X RT master mix and gently mixed by pipetting up and down. The mixed RNA sample/2X RT master mix solution was thermal cycled using a Multi-Gene™ OptiMax Thermal Cycler according to the High-Capacity cDNA Reverse Transcription Kit manufacturer’s instructions.

cDNA samples were aliquoted out and stored at -80°C until required.

### 5.4.5 Quantitative PCR Primer Design

The quantitative PCR (qPCR) primers (Table 5.1 on page 88) were designed using Oligo Explorer 1.2 and the PrimerBank: PCR Primers for Gene Expression Detection and Quantification server or were based on previously reported qPCR primers. The design of the qPCR primers could not, in all cases, accommodate intron-spanning due to sequence homology within the GLT-1 variants and VGLUT variants.
### Table 5.1: Quantitative PCR Primers for 18S, GLT-1a–d, glutamine synthetase (GS), and VGLUT1–3.

<table>
<thead>
<tr>
<th>Target and Amplicon Size (bp)</th>
<th>Primer Pair</th>
<th>Primer ID</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S† [187]</td>
<td>18S rRNA FWR (5' to 3')</td>
<td>CGGCTACCCACATCCCAAGGAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18S rRNA REV (5' to 3')</td>
<td>GCTGGAATTACCGCGGCACT</td>
<td></td>
</tr>
<tr>
<td>GLT-1a♦ [302]</td>
<td>F-m/rGLT1 (Universal 2)</td>
<td>GATTGTCTATCACCTTTCCAAGTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-GLT1a (REV)</td>
<td>CATAAGATACGCTGGGGAGTT</td>
<td></td>
</tr>
<tr>
<td>GLT-1b♦ [183]</td>
<td>F-m/rGLT1 (Universal 1)</td>
<td>CACCAATGACTCCCAAACCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-GLT1b (REV)</td>
<td>ATGCAAGGCTCTGATATCAC</td>
<td></td>
</tr>
<tr>
<td>GLT-1c♦ [216]</td>
<td>F-m/rGLT1 (Universal 2)</td>
<td>GATTGTCTATCACCTTTCAAAGTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-GLT1c (REV)</td>
<td>TAAACCACGAGTATATTTTACA</td>
<td></td>
</tr>
<tr>
<td>GLT-1d [140]</td>
<td>GLT-1d: PP1</td>
<td>F-mGLT1 (1703)</td>
<td>CAACCCAGAAGGAGAAC</td>
</tr>
<tr>
<td></td>
<td>R-m/rGLT1d (R1)</td>
<td>GAACAGAGAACCAGGTCCT</td>
<td></td>
</tr>
<tr>
<td>GLT-1d [250]</td>
<td>GLT-1d: PP2</td>
<td>F-m/rGLT1 (1595/1524)</td>
<td>TGGACTGGCTGATTGATAGAA</td>
</tr>
<tr>
<td></td>
<td>R-m/rGLT1d (R1)</td>
<td>GAACAGAAGAACCAGGTCCT</td>
<td></td>
</tr>
<tr>
<td>GLT-1d [150]</td>
<td>GLT-1d: PP3</td>
<td>For GLT-1d (R1)(Mouse/Rat)</td>
<td>AGGGCCACTGTGTTCTGTC</td>
</tr>
<tr>
<td></td>
<td>Rev GLT-1d (R2)(Mouse/Rat)</td>
<td>ATGAAGAGACTGGATTTATGGAAGA</td>
<td></td>
</tr>
<tr>
<td>GS [118]</td>
<td>F-GS (207)</td>
<td>CCAGGGCGAAAGAAGGTCCT</td>
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<tr>
<td></td>
<td>R-GS (325)</td>
<td>TCCACTAGCCTATCTTCCACA</td>
<td></td>
</tr>
<tr>
<td>VGLUT1 [166]</td>
<td>VGLUT1: PP1</td>
<td>VGLUT1 FWD 452</td>
<td>TTGTGGCTACCTCCACTAA</td>
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<tr>
<td></td>
<td>VGLUT1 REV 618</td>
<td>CAGCGACCCTGTTCAC</td>
<td></td>
</tr>
<tr>
<td>VGLUT1 [164]</td>
<td>VGLUT1: PP2</td>
<td>VGLUT1 FWD 617</td>
<td>TGGCAGCAAGCAGCCTTGG</td>
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<td></td>
<td>VGLUT1 REV 781</td>
<td>CCAGGAGCTAACATTTGAGG</td>
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</tr>
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<td>VGLUT1 [80]</td>
<td>VGLUT1: PP3</td>
<td>VGLUT1 FWD 805</td>
<td>GAGGAAGCAGAATTTGAGG</td>
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<td></td>
<td>VGLUT1 REV 885</td>
<td>CCAGGGGTTGTTAAAATCTG</td>
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</tr>
<tr>
<td>VGLUT1 [196]</td>
<td>VGLUT1: PP4</td>
<td>VGLUT1 FWD 543</td>
<td>GGTGGAGGGGGGCTGCACTAC</td>
</tr>
<tr>
<td></td>
<td>VGLUT1 REV 739</td>
<td>AGATCCCGAGAAGTCCATAG</td>
<td></td>
</tr>
<tr>
<td>VGLUT2 [90]</td>
<td>VGLUT2: PP1</td>
<td>VGLUT2 FWD 65</td>
<td>TGGAAAATCCCTGAGGAGGCTCCT</td>
</tr>
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<td></td>
<td>VGLUT2 REV 150</td>
<td>TGGAAAATCCCTGAGGAGGCTCCT</td>
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</tr>
<tr>
<td>VGLUT2 [102]</td>
<td>VGLUT2: PP2</td>
<td>VGLUT2 FWD 158</td>
<td>CTGAGAAGAAGGCTTCCT</td>
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<tr>
<td></td>
<td>VGLUT2 REV 256</td>
<td>ATGGCAGGAGATATGCAAGAG</td>
<td></td>
</tr>
<tr>
<td>VGLUT2 [148]</td>
<td>VGLUT2: PP3</td>
<td>VGLUT2 FWD 252</td>
<td>CTTCGGAGCTACGGGATGAAAA</td>
</tr>
<tr>
<td></td>
<td>VGLUT2 REV 400</td>
<td>CCCAGGAAGACATCTGAGG</td>
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<tr>
<td>VGLUT2 [119]</td>
<td>VGLUT2: PP4</td>
<td>VGLUT2 FWD 60</td>
<td>TGGAAAATCCCTGAGGAGGCTCCT</td>
</tr>
<tr>
<td></td>
<td>VGLUT2 REV 179</td>
<td>CATACGCGAGAAGCTTTCCT</td>
<td></td>
</tr>
<tr>
<td>VGLUT3 [196]</td>
<td>VGLUT3: PP1</td>
<td>VGLUT3 FWD 25</td>
<td>CATCGGCTGCGATCTTACA</td>
</tr>
<tr>
<td></td>
<td>VGLUT3 REV 79</td>
<td>GCGGTGGAGGGGGCTGCACTAC</td>
<td></td>
</tr>
<tr>
<td>VGLUT3 [225]</td>
<td>VGLUT3: PP2</td>
<td>VGLUT3 REV 257</td>
<td>GCAGATGCAGATCGGATGGGG</td>
</tr>
<tr>
<td></td>
<td>VGLUT3 FWD 258</td>
<td>CAAACGCGATACATGCTT</td>
<td></td>
</tr>
<tr>
<td>VGLUT3 [118]</td>
<td>VGLUT3: PP3</td>
<td>VGLUT3 FWD 473</td>
<td>CCTCCGGTGGCTCTATTCAA</td>
</tr>
<tr>
<td></td>
<td>VGLUT3 REV 591</td>
<td>AACAGCTGATGCGACCTTCA</td>
<td></td>
</tr>
<tr>
<td>VGLUT3 [174]</td>
<td>VGLUT3: PP4</td>
<td>VGLUT3 FWD 65</td>
<td>AGAATGGCGGTGGGAGGAGA</td>
</tr>
<tr>
<td></td>
<td>VGLUT3 REV 239</td>
<td>CAGTCACAGAGCTGTTGCTG</td>
<td></td>
</tr>
</tbody>
</table>

† Stemler et al. and Woodling et al.
♦ Primers were designed by Dr Aven Lee, personal communication.

### 5.4.6 Quantitative PCR Primer Validation

#### 5.4.6.1 In Silico Primer Evaluation

The qPCR primers (Table 5.1) were evaluated in silico using the National Institute of Standards and Technology: Primer Tools server to determine the potential for autodimerization and hairpin formation.

#### 5.4.6.2 Electrophoretic Evaluation of GLT-1d Quantitative PCR Primers

The specificity of the GLT-d specific qPCR primer pairs (Table 5.1) was evaluated using BioTaq™ DNA Polymerase according to the manufacturer’s instructions. Briefly, two
cDNA templates—designated cDNA\textsubscript{1} and cDNA\textsubscript{2}—were amplified using the primer pairs. Each 50 \( \mu \)L final volume PCR reaction contained:

1. 5 \( \mu \)L 10x \( \text{NH}_4^+ \) reaction buffer
2. 1.5 \( \mu \)L 50 mM MgCl\textsubscript{2} solution
3. 1 \( \mu \)L 100 mM dNTP mix
4. 1 \( \mu \)L cDNA template
5. 1 \( \mu \)L 10 \( \mu \)M forward primer
6. 1 \( \mu \)L 10 \( \mu \)M reverse primer
7. 39.5 \( \mu \)L ultrapure RNase-free H\textsubscript{2}O

Thermal cycling was performed using a MultiGene\textsuperscript{TM} OptiMax Thermal Cycler with the following thermal cycling protocol:

1. Hot-Start Activation: 95\(^\circ\)C for 2 minutes
2. Amplification (35 cycles):
   (a) Denaturation: 95\(^\circ\)C for 30 seconds
   (b) Annealing: 60\(^\circ\)C for 30 seconds
   (c) Extension: 72\(^\circ\)C for 60 seconds
3. Final Extension: 72\(^\circ\)C for 5 minutes

5 \( \mu \)L ultrapure water and 2-3 \( \mu \)L 6X DNA loading dye were combined with 5 \( \mu \)L of each amplified DNA sample. The combined amplified DNA/loading dye samples were separated by electrophoresis using a 0.75% agar gel in 1x TAE (Tris Acetate EDTA) buffer\textsuperscript{364} at 70V. The separated DNA was visualized using SYBR\textsuperscript{TM} Gold Nucleic Acid Gel Stain according to the manufacturer’s instructions (Appendix P: ThermoFisher Scientific Sybr\textsuperscript{TM} Gold Nucleic Acid Gel Stain on page 281).

### 5.4.6.3 Melting Curve Analysis

The specificity of the primer pairs (Table 5.1 on page 88) was evaluated using the high resolution melting curve analysis feature of the Rotor-Gene Q real-time PCR cycler operating software, 2.3.1 (Build 49).\textsuperscript{442}
5.4.7 Quantitative PCR

Quantitative PCR (qPCR) was performed using GoTaq™ qPCR master mix according to the manufacturer’s instructions (Appendix Q: GoTaq™ qPCR Master Mix on page 287). qPCR is considered one of the most sensitive methods available to measure mRNA expression levels. Loading samples were prepared by diluting cDNA samples 1:8, i.e., 0.5 µL cDNA/3.5 µL RNase-free water.

Each 20 µL final volume qPCR reaction contained:

1. 10 µL GoTaq™ qPCR master mix
2. 4 µL RNase-free water
3. 2 µL of primer stock (10 µM forward and 10 µM reverse primers)
4. 4 µL cDNA loading sample or 4 µL ultrapure/RNase-free H₂O (No Template Control)

Thermal cycling was performed using a Rotor-Gene Q real-time PCR cycler according to the GoTaq™ qPCR master mix manufacturer’s instructions.

The thermal cycling parameters were:

1. Hot-Start Activation: 95°C for 2 minutes
2. Amplification (40 cycles):
   (a) Denaturation: 95°C for 15 seconds
   (b) Annealing/Extension: 60°C for 60 seconds
3. Dissociation: 60–95°C (1°C/step, holding for 5 seconds at each step)

Auto-gain optimization to optimize the fluorescence signal detection was selected before the first acquisition for all qPCR experiments. The excitation wavelength was set to 470 nm with the detection wavelength at 510 nm.

Relative change in RNA expression was normalized against 18S using the $2^{\Delta\Delta C_T}$ method.
5.4.8 Protein Extraction

Frozen brains (n = 5 animals/age cohort) were crushed in a mortar and pestle under liquid nitrogen and then homogenized at 4°C in 10x (m/v) ice cold 2x Laemmli buffer (125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 10% β-mercaptoethanol, 20% glycerol) excluding bromophenol blue using a T10 basic Ultra Turrax™ homogenizer on setting 1 (2 x 10 seconds followed by 2 minutes incubation on ice; repeated 2-3 times). As mentioned previously, bromophenol blue was omitted because it is not compatible with the protein assay (Section 4.3.5.3 - Protein Assay on page 55) used for the research of this thesis (Appendix G: ThermoScientific Protein Assay Compatibility Table on page 237). The lysis buffer contained a protease inhibitor cocktail according to the manufacturer’s instructions (Appendix I: Roche cOmplete™ Ultra Protease Inhibitor Cocktail on page 250) and was cooled to 4°C to reduce proteolysis.

Homogenates were incubated on a laboratory roller for 3 hours at 4°C. After incubation on the roller, the homogenates were centrifuged at 14,000 x g for 30 minutes at 4°C. The supernatant fractions were collected, aliquoted out, and stored at -80°C until required.

5.4.9 Protein Assay

Protein assays for the relevant experiments of this chapter were performed as set out under Section 4.3.5.3 - Protein Assay on page 55.

5.4.10 Protein Sample Preparation

Protein samples for western blotting experiments of this chapter were prepared as set out previously in Section 4.3.5.4 - Protein Sample Preparation on page 55.

5.4.11 Western Blotting

Western blotting was performed as set out previously in Section 4.3.5.6 - Optimized Western Blotting Method on page 60. Prior to immunodetection, Ponceau S staining was performed to assess consistency of the amount of protein loaded per lane and to confirm the efficiency of transfer (see page 58). The GLT-1a-c, and GLAST specific antisera used in this study and the conditions used were previously described in Lee et al. The glutamine synthetase specific antiserum had been previously characterised by members of the Pow Lab (Prof. David Pow, personal communication) and the conditions were as set out previously in Lee et al.
5.4.12 Immunohistochemistry

The embedded brain tissue prepared as set out previously in Section 5.4.1 - Animal Tissue Collection and Treatment on page 85 was treated and subjected to bright-field microscopy as set out below.

A Leica rotary microtome was used to cut 8 μm serial coronal sections of the embedded tissues. The cut sections were mounted onto silanated microscope slides. The mounted sections were dewaxed in eucalyptus oil at room temperature (2x 10 minutes). As mentioned previously, eucalyptus oil is a less toxic organic solvent alternative to xylene. Endogenous peroxidase activity was quenched by incubating the mounted sections for 10 minutes in 3% H₂O₂/methanol solution at room temperature. The sections were rehydrated through a graded series of ethanol solutions, i.e., 100% ethanol (10 minutes) and 50% ethanol (10 minutes), followed by incubation in ultrapure H₂O (10 minutes).

Sections were incubated in an antigen recovery solution for 10 minutes at 85°C.

0.1 M phosphate buffer (pH 7.2) (phosphate buffer) was prepared from 0.1 M stock solutions of NaH₂PO₄ and Na₂HPO₄. The pH of 500 mL of 0.1 M Na₂HPO₄ was adjusted to pH 7.2 by adding 0.1 M NaH₂PO₄.

Following antigen recovery, sections were placed in Coplin Jars and blocked for 30 minutes in 0.1% bovine serum albumin (BSA)/0.05% NaN₃/phosphate buffer at room temperature. The blocked sections were then treated as follows:

1. Incubated in GLT-1d antiserum (1:500) in 0.1% BSA/0.05% NaN₃/phosphate buffer overnight at room temperature.
2. Washed briefly in phosphate buffer.
3. Incubated in biotinylated secondary antibody (1:300) in 0.1% BSA/phosphate buffer for 3 hours at room temperature.
5. Incubated in streptavidin–biotin–horseradish peroxidase conjugate (1:300) in 0.1% BSA/phosphate buffer for 3 hours at room temperature.
7. Immunoreactivity was visualized by incubation in 0.05% 3,3’-diamino-benzidine (DAB)/0.015% H₂O₂/phosphate buffer as the peroxidase-sensitive chromogenic substrate. The peroxidase-sensitive chromogenic substrate was prepared as set out previously in Section 4.3.5.5 - Dot Blotting on page 55.
8. Washed briefly in ultrapure H$_2$O.

9. Sections were mounted in 1:1 glycerol:ultrapure H$_2$O and cover slipped.

10. Edges of cover slips were sealed with clear nail polish.

Bright-field microscopy was performed using a standard Pow Laboratory protocol as described previously. Briefly, images were captured using a Nikon Eclipse 80i microscope equipped with a Olympus DP70 digital camera. During the preparation of the final images for this thesis minor contrast and colour balance adjustment was performed using Adobe Photoshop.

5.4.13 Statistical Analysis

Data are expressed as the mean ± SEM. The statistical significance among different experimental groups was determined by one-way ANOVA followed by Dunnett’s multiple comparisons test. The statistical significance for Ponceau S staining as loading/transfer efficiency control was determined by one-way ANOVA followed by Tukey’s multiple comparisons test. All statistical analyses were performed using GraphPad Prism, Version 6.07, June 12, 2015. Data were regarded as statistically significant when $P < 0.05$, unless indicated otherwise. Significance levels are $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) , and $P \leq 0.0001$ (****).

5.5 Results

5.5.1 Preliminary Validation of Quantitative PCR Primers and Ponceau S Staining as Loading/Transfer Control

Preliminary studies were performed to evaluate:

1. The utility of the qPCR primers set out in Table 5.1 on page 88 for the investigation of GLT-1a–d, VGLUT1–3, and glutamine synthetase expression; and

2. Ponceau S staining as loading control for western blotting.

5.5.1.1 In Silico Evaluation of Quantitative PCR Primers

The qPCR primers set out in Table 5.1 were evaluated in silico using the National Institute of Standards and Technology: Primer Tools server. Autodimerization and hairpin formation were not predicted for any of the qPCR primers.
5.5.1.2 Electrophoresis/Melt Curve Analysis of Quantitative PCR Primers

The specificity of the GLT-1d primers set out Table 5.1 on page 88 was evaluated using electrophoresis of the PCR amplified products and melt curve analysis. GLT-1d primers pairs 1 and 2 (Table 5.1) amplified DNA products that correspond in size to the predicted amplicons of 140 bp and 250 bp, respectively, but these bands were not dominant. GLT-1d primer pair 3 amplified a dominant DNA band at ~150 base pairs (bp), which is the expected size of the predicted amplicon (Figure 5.1). This primer pair also produced a single peak under melt curve analysis conditions (Figure 5.2 on page 95). Accordingly, GLT-1d primer pair 3 was selected to investigate the expression of GLT-1d.

![Ladder cDNA1 cDNA2](image)

**Figure 5.1:** DNA amplicons produced using GLT-1d quantitative PCR primer pair 3. Two cDNA templates—designated cDNA1 and cDNA2—were amplified and produced a dominant band at ~150 base pairs, i.e., at the predicted amplicon size for GLT-1d for both cDNA templates. The size of selected bands in the ladder are indicated to the left in base pairs.

The VGLUT1–3 primers (Table 5.1) that exhibited a single melt curve analysis peak, i.e., VGLUT1: PP1, VGLUT2: PP3, and VGLUT3: PP4, were selected to investigate the expression of VGLUT1–3.

5.5.1.3 Ponceau S Staining as Loading/Transfer Control

The commonly used housekeeping proteins β-actin, α-tubulin, β-tubulin, and glyceraldehyde 3-phosphate dehydrogenase are not stably expressed during brain development. Accordingly, Ponceau S staining (Figure 5.3 on page 95) was used as a loading control and to evaluate transfer efficiency. Ponceau S staining is particularly useful for the evaluation of the transfer efficiency of both large and small proteins.
5.5.2 Differential Expression of GLT-1a–d, GLAST, Glutamine Synthetase, and VGLUT1–3 in Juvenile Mouse Brain

To study the developmental expression profiles of GLT-1a–d, GLAST, VGLUT1–3, and glutamine synthetase in juvenile mouse brain, homogenates were prepared from postnatal day one (P1) to adult (~3 months) mouse brains. The homogenates were analysed for mRNA expression using quantitative PCR (qPCR) and protein expression by western blotting.
Temporal changes in GLT-1a–d, VGLUT1–3, and glutamine synthetase mRNA expression were observed (Figure 5.4 on page 97). GLT-1a, GLT-1b, and GLT-1d mRNA expression underwent a steep increase from P1 to P14–P21. Glutamine synthetase and VGLUT1 mRNA expression, on the other hand, increased progressively from P1 to P28. Glutamine synthetase mRNA expression appeared to increase slightly after P28 to adulthood, conversely VGLUT1 mRNA expression appeared to decrease slightly from P28 to adulthood. GLT-1c mRNA expression appeared to be consistent from P1 to adulthood. VGLUT 2 and 3 mRNA expression appeared to peak at P1 and P7 respectively. Both VGLUT 2 and 3 mRNA expression appeared to diminish after the relevant peaks to adulthood.

Western blots were used to investigate GLT-1a–d protein levels for the developmental stages P1–adult (Figure 5.5 on page 98). Similar temporal patterns for each of GLT-1a, GLT-1b, and GLT-1d were detected. GLT-1c protein was not detected by western blotting. A distinct prominent broad band at ~65 kDA was detected for GLT-1a, GLT-1b, and GLT-1d in juvenile mouse brain. As mentioned previously, such a prominent band is thought to represent a monomer form of other known forms of GLT-1. Accordingly, it is plausible that this prominent band is the monomer form of GLT-1a, GLT-1b, and GLT-1d. A similar prominent band was also detected for GLAST. Less prominent higher molecular weight GLT-1a and GLAST bands were also observed, which is consistent with previous reports of glutamate transporters forming multimers. GLT-1a and GLT-1d were undetectable at P1 (Figures 5.5 A and 5.5 C). The prominent monomer bands of both GLT-1a and GLT-1d were detected from P7 and increased to adulthood. The less prominent higher molecular weight GLT-1a and GLAST bands were first detected at P7 and P14, respectively, and increased to adulthood. The GLT-1b monomer band was flanked by less prominent bands. The less prominent GLT-1b bands increased from P1 to P14 and then remained relatively constant to adulthood. A distinct ~43 kDA band was first observed for glutamine synthetase at P7 and gradually increased in intensity to adulthood.

In summary, during the first four weeks of postnatal mouse brain development, GLT-1a, GLT-1b, GLT-1d, GLAST, VGLUT1, and glutamine synthetase expression increased, GLT-1c expression increased to P14 then decreased to adulthood, and VGLUT2 and VGLUT3 expression decreased from early stages of postnatal development to adulthood. These results suggest that there may be an increasing demand for the activities of GLT-1a, GLT-1b, GLT-1d, GLAST, VGLUT1, and glutamine synthetase which is initiated in the first postnatal week of mouse brain development. In contrast, the demand for VGLUT2 and VGLUT3 activities may decrease from the early stages of mouse brain development to adulthood.
Chapter 5: GLT-1d in Juvenile Mouse Brain

Figure 5.4: Developmental expression profiles of GLT-1a–d, VGLUT1–3, and glutamine synthetase mRNA in mouse brain from postnatal day 1 (P1) to adult. mRNA expression levels were measured by quantitative PCR using transcript specific primers. Each bar represents the mean ± SEM obtained for five mice/age cohort. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, and **** P ≤ 0.0001, significantly different from adult.
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5.5.3 Differential Regional and Cellular Distribution of GLT-1d in Juvenile Mouse Brain

To investigate the differential distribution of GLT-1d immunoreactivity in the juvenile mouse brain, immunohistochemistry using the GLT-1d antiserum (Rb 1713 antiserum)
was performed on postnatal day one (P$_1$)– adult (~3 months) mouse brain tissue. Labelling was not observed when sections were treated with pre-immune- and pre-absorbed sera. The pre-absorbed antisera was prepared as set out previously in Section 4.3.5.6 Pre-Absorption of Antisera on page 58.

5.5.3.1 Neocortex

Intense GLT-1d immunoreactivity is seen in layer I of the neocortex (Figure 5.6 on page 100) at postnatal day seven (P$_7$) and P$_{14}$, with the intensity in immunoreactivity decreasing away from layer I. In effect, the GLT-1d immunoreactivity distribution profile was intense in layer I, moderate in layer II/III, and low in layers IV–VI and localised to astrocytes. Unlabelled neuronal somata (red arrows) interspersed between the labelled elements throughout layers II/III–VI. At P$_{14}$, at higher magnification, labelling of astrocytes (black arrows) was predominantly associated with the somata, with minor labelling of processes proximal to the somata. GLT-1d distribution in the adult mouse neocortex is discussed in Chapter 6: GLT-1d in Adult Mouse Brain, in particular in Section 6.4.3 - Immunohistochemistry on page 120 et seq.

5.5.3.2 Olfactory Bulb

GLT-1d immunolabelling of the astrocytes (black arrows) was seen throughout the olfactory bulb at postnatal day seven (P$_7$) (Figure 5.7 on page 101), with the neuronal somata (red arrows) unlabelled for GLT-1d.

5.5.3.3 Choroid Plexus

The distribution of GLT-1d was examined in postnatal choroid plexus isolated from the fourth ventricle of juvenile and adult mouse brains. GLT-1d immunolabelling was just detectable at the apical surface of epithelia of the postnatal day seven (P$_7$) choroid plexus (Figure 5.8 A on page 102), but was readily detectable at the same location in the adult mouse brain (Figure 5.8 B on page 102).

5.5.3.4 Cerebellum

In the cerebellum at P$_7$ (Figure 5.9 A on page 104), both the molecular layer and granule cell layer were densely labelled. At P$_{14}$ (Figure 5.9 B on page 104), in addition to the granule cell layer and molecular layer, the white matter was also immunoreactive for
Figure 5.6: Representative immunohistochemical photomicrographs illustrating the distribution of GLT-1d protein in postnatal mouse neocortex. [A] Postnatal day seven (P7) and [B] postnatal day fourteen (P14) mouse neocortex was immunolabelled for GLT-1d protein. GLT-1d immunolabelling is seen in the neuropil of both P7 and P14 neocortex, with a gradient in the label decreasing away from the layer I. Note the intense labelling in layer I of the P7 neocortex [A] and the staining of the astrocytes (black arrows) in the P14 neocortex [B]. Unlabelled neuronal somata (red arrows) are distributed throughout both P7 and P14 neocortex. GLT-1d labelling is prominent in layers I and II/III of the P7 neocortex [A] and P14 [B]. Layers of P7 neocortex are shown to the left of panel (A). Scale bars: [A] 40 µm and [B] 20 µm.
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Figure 5.7: Representative immunohistochemical photomicrograph illustrating the distribution of GLT-1d in postnatal day seven (P7) mouse olfactory bulb. The astrocytes (black arrows) are predominantly perisomally labelled, while neuronal somata (red arrows) are unlabelled. Scale bar: 20 μm.
Figure 5.8: Representative immunohistochemical photomicrographs illustrating the distribution of GLT-1d in mouse choroid plexus. Choroid plexus of the fourth ventricle was immunolabelled for GLT-1d. [A] At postnatal day seven (P7) GLT-1d immunolabelling was barely detected at the apical surface (arrow) epithelia of the choroid plexus. [B] In the adult mouse, the epithelia of the choroid plexus was densely labelled for GLT-1d. Scale bars = 20 µm.
GLT-1d. At P7, the Bergmann glia processes were distinctly labelled, which is also seen at P14. The somata of the inhibitory Purkinje neurons (also known as Purkinje cells) were unlabelled in the densely labelled Purkinje cell layer at P7 and P14. Similarly, at P7 and P14 dense labelling was seen in the granule cell layer, but the granule cell somata were unlabelled. GLT-1d distribution in the adult mouse brain cerebellum is discussed in Chapter 6: GLT-1d in Adult Mouse Brain, in particular in Section 6.4.3 - Immunohistochemistry on page 120 et seq.

5.6 Discussion

This study provided the first description of the temporal expression and distribution profile of GLT-1d in juvenile mouse brain. The study showed that GLT-1d is developmentally regulated and was coordinated with other key members of the glutamate homeostasis system in juvenile mouse brain. The coordinated expression suggested that there was an increasing demand for GLT-1d activity during mouse brain development and maturation. The study also showed that, in juvenile mouse brain, GLT-1d protein was expressed in astrocytes, and not observed in neurons.

5.6.1 Coordinated Temporal Changes in GLT-1d Expression

For the analysis of the temporal changes in the expression of GLT-1d, quantitative PCR (qPCR) was used to examine GLT-1d mRNA expression. Western blotting and immunohistochemistry using the Rb 1713 antiserum (Chapter 4) was used to examine the protein expression. In addition to GLT-1d, GLT-1a–c, GLAST, glutamine synthetase, and the VGLUTs mRNA was examined by qPCR, and GLT-1a–c, GLAST, and glutamine synthetase were examined by western blotting.

This study demonstrated that GLT-1a, GLT-1b, GLT-1d, glutamine synthetase, and VGLUT1 mRNA expression increased dramatically from low levels of expression at postnatal day one (P1) and reached adult levels between P14 to P28. On the other hand, GLT-1a, GLT-1b, GLT-1d, glutamine synthetase, and GLAST protein expression increased from P7 to adult, GLT-1c protein, which is expressed at very low levels in brain, was not detected by western blotting. The delay in the detection of western blotting. The delay in the detection of GLT-1a, GLT-1b, GLT-1d, and GLAST protein was not unexpected as glutamate transporter proteins are known to be expressed at low levels during early brain development. In fact, a similar delay in GLT-1 protein expression has been reported. The dramatic increase in GLT-1d protein expression from P7 to P14 suggested that there was
Figure 5.9: Representative immunohistochemical photomicrographs illustrating the distribution of GLT-1d in juvenile mouse cerebellum. [A] At P7 both the molecular layer and granule cell layer show a high level of labelling. [B] At P14 the white matter, granule cell layer, and molecular layer are labelled for GLT-1d, with the processes of the Bergmann glia also distinctly labelled. The somata of the inhibitory Purkinje neurons are unlabelled in the distinct densely labelled Purkinje cell layer. At both P7 and P14 the Purkinje cell layer is densely labelled, presumably due to the Bergmann glia enveloping the somata of the Purkinje neurons. GLT-1d labelling is also dense around the excitatory granule cells, but the granule cell somata appear unlabelled. Abbreviations: molecular layer (ML), granule cell layer (GCL), Purkinje cell layer (PCL), Bergmann glia processes (BGP), and white matter (WM). Scale bars: 20 µm.
a considerable increase in demand for GLT-1d activity during this stage of brain development and maturation in the juvenile mouse. Furthermore, the increase in expression observed for GLT-1a, GLT-1b, GLT-1d, GLAST, glutamine synthetase, and VGLUT1 corresponded to when synaptogenesis occurs in juvenile mouse brain.\textsuperscript{429–431} Such correspondence suggested a putative interrelationship between astrocytes and neurons that mediates the onset of GLT-1a, GLT-1b, GLT-1d, GLAST, glutamine synthetase, and VGLUT1 expression in juvenile mouse brain in a coordinated manner. Support for such an interrelationship has been reported with respect to GLT-1 expression \textit{in vitro}. Cortical astrocytes in culture in isolation express GLT-1 protein at very low levels, whereas co-cultured astrocytes and neurons show a marked increase in astrocytic GLT-1 protein expression.\textsuperscript{453–455} Interestingly, when neurons were removed from astrocyte-neuron co-culture, GLT-1 expression was reduced.\textsuperscript{455} This reduction in astrocytic GLT-1 expression when neurons were removed further suggests such a putative interrelationship. In effect, at least \textit{in vitro}, an interrelationship between astrocytes and neurons may be required for the onset and ongoing maintenance of GLT-1 expression.\textsuperscript{456} It is plausible, therefore, that an astrocyte-neuron interrelationship may similarly influence the onset and ongoing maintenance of GLT-1d expression in mouse brain. A future astrocyte-neuron co-culture study will investigate whether an interrelationship exists between astrocytes and neurons with respect to GLT-1d expression.

GLT-1c, VGLUT2, and VGLUT3 mRNA expression exhibited peaks in expression at P\textsubscript{14}, P\textsubscript{1}, and P\textsubscript{7}, respectively. Such peaks in expression suggested a potential transient role for GLT-1c, VGLUT2, and VGLUT3 at the relevant peak of expression in the juvenile mouse brain. VGLUT1 and VGLUT2 expression undergoes a switch from the predominant VGLUT2 in the postnatal developing brain, to the higher levels of VGLUT1 in the adult brain.\textsuperscript{457} Such a switch has been suggested to be important for development of presynaptic terminals in the cerebral cortex.\textsuperscript{457}

It is plausible, in light of the coordinated expression of GLT-1d with GLT-1a, GLT-1b, GLAST, glutamine synthetase, and VGLUT1, that GLT-1d may play a role in the termination of glutamate signalling and in protecting against neurotoxicity in juvenile mouse brain. Given the increase in GLT-1d mRNA and protein expression observed in the juvenile mouse brain, the role that GLT-1d plays may be increasingly important as the brain matures.
5.6.2 Regional Distribution of GLT-1d

Immunohistochemistry confirmed the temporal increase in GLT-1d expression determined by qPCR and western blotting. Furthermore, immunohistochemistry demonstrated that the GLT-1d expression pattern increased in intensity from $P_1$ to adulthood. This increase was most noticeable from $P_7$ to $P_{14}$, which was consistent with previous reports for GLT-1.\textsuperscript{103,142,389,391} By $P_7$, intense GLT-1d immunoreactivity in layer I of the neocortex was accompanied by less intense immunoreactivity in layers II/III–VI (Figure 5.6 on page 100). Such distribution of GLT-1d was consistent with previous reports for GLT-1 in mouse neocortex.\textsuperscript{103,389} The dense GLT-1d immunolabelling in layer I of $P_7$ and $P_{14}$ neocortex is likely due to the presence of the glutamatergic Cajal-Retzius cells in this layer. The glutamatergic Cajal-Retzius cells have been proposed to fulfil important roles in corticogenesis, in particular with respect to neuronal migration and synaptogenesis.\textsuperscript{458} In support of this important role, ablation of the glutamatergic Cajal-Retzius cells in newborn mice resulted in the arrested migration of the late-generated neocortical neurons.\textsuperscript{459} It would appear, however, that GLAST, rather than GLT-1, mediates the extracellular glutamate concentration in the extracellular environment adjacent the glutamatergic Cajal-Retzius cells.\textsuperscript{460} The role GLT-1d protein plays in the Cajal-Retzius cells–and layer I–in postnatal developing mouse neocortex requires investigation. Also by $P_7$, the molecular layer, granule cell layer, and white matter tracts of the cerebellum and the olfactory bulb were GLT-1d immunoreactive. Interestingly, the GLT-1d immunoreactivity in juvenile mouse brain preceded the detection of GLT-1 in corresponding regions of rat brain.\textsuperscript{142,391}

5.6.3 Astrocytic, but Not Neuronal Expression of GLT-1d Protein

An important finding of this study was that GLT-1d protein was transiently expressed in astrocyte somata and processes in juvenile mouse brain, but not observed in neurons. The transient expression of GLT-1d in astrocytes observed in juvenile mouse brain was in contrast to the transient expression of GLT-1a protein in neurons observed in developing mammalian brains. During early postnatal mammalian brain development, GLT-1a protein undergoes a switch from expression in specific subsets of neurons,\textsuperscript{142,223,416} to astrocytes.\textsuperscript{141,142,213,214,223,312,391,416} The importance of astrocytic expression of GLT-1d protein in juvenile mouse brain became apparent during the investigation the GLT-1d expression in adult mouse brain (Chapter 6: GLT-1d in Adult Mouse Brain).

At the cellular level, GLT-1d immunoreactive astrocytes were distributed throughout the juvenile neocortex, i.e., defined here as $P_1$–$P_{21}$,\textsuperscript{409} with unlabelled neuronal elements interspersed. Such labelling in the neocortex corresponds to previously reported astrocytic
labelling for GLT-1 through all neocortical layers,\textsuperscript{141,144,145,213,218,313} although GLT-1 mRNA had also been detected in some neurons.\textsuperscript{214,221,313,426} Consistent with previous GLT-1 studies,\textsuperscript{103,144,213} GLT-1d immunoreactivity was observed in astrocytic somata, with immunoreactivity also observed in the proximal regions of astrocytic processes. The localisation of GLT-1d protein in astrocyte processes suggests that GLT-1d protein may participate in the rapid response to changes in glutamate levels in the extracellular space adjacent nascent synapses during synaptogenesis in the developing mouse brain.

GLT-1d immunoreactivity was not detected in somata of the inhibitory Purkinje neurons (also known as Purkinje cells). Labelling of the Purkinje cell layer was presumably due to the Bergmann glia enveloping the somata of the Purkinje neurons, as has been previously reported for GLT-1.\textsuperscript{141,213,217,313} The granule cell layer was GLT-1d immunoreactive from early in development, i.e., P\textsubscript{7}, which was similar to that observed for GLT-1 where labelling was seen between P\textsubscript{5} and P\textsubscript{10}.\textsuperscript{391} The distribution profile of GLT-1d in postnatal developing cerebellum was consistent with a potential increased role in glutamate transport as the cerebellum matures into adult glutamatergic neurocircuitry, i.e., particularly associated with the climbing fibres that project to the Purkinje neurons.\textsuperscript{461} The Purkinje neurons that align in a monolayer between the granule cell layer and the molecular layer\textsuperscript{462,463} were surrounded by dense GLT-1d immunolabelling (Figure 5.9 on page 104). Such dense immunolabelling suggests that GLT-1d immunolabelling was associated with Purkinje neuron dendrites that extend into the molecular layer where they are innervated directly by the excitatory climbing fibres that arise in the inferior olive\textsuperscript{462} early in development, i.e., by P\textsubscript{7}. Likewise, the GLT-1d immunolabelling appeared to be associated early in development with the parallel fibres of the excitatory granule cells.\textsuperscript{462,463} It is likely this GLT-1d immunolabelling was located in the Bergmann glia processes that ramify throughout the molecular layer amongst the Purkinje neuron dendrites, the climbing fibres, and the parallel fibres.\textsuperscript{462,463} The GLT-1d immunolabelling of the granule cells, located in the granule cell layer (Figure 5.9 on page 104), suggested that GLT-1d expression in this part of the mouse cerebellum was also established early in development, i.e., by P\textsubscript{7}. It is plausible that the GLT-1d immunolabelling observed in the granule cell layer was located in or adjacent the synapses between the granule cells and the excitatory mossy fibres that arise in nuclei of the pons.\textsuperscript{462}

5.7 Significance Statement

This study demonstrated that the expression of GLT-1d is developmentally regulated in juvenile mouse brain. Such developmental regulation suggests that GLT-1d may plausibly be associated with the inception of and ensuing glutamatergic neurotransmission
in developing mouse brain. This study also demonstrated that GLT-1d protein was expressed in astrocytes, but not observed in neurons, in juvenile mouse brain.

5.8 Further Observations

Observations regarding this study, and its integration into achieving the aim of the research for this thesis, are set out in Chapter 8.

5.9 Future Studies

Importantly, extracellular glutamate is known to modulate cell proliferation, migration, differentiation, and synapse formation.\textsuperscript{103,410–415} Accordingly, future studies to investigate whether GLT-1d is associated with these processes will expand our understanding of the role GLT-1d may play in mouse brain development. GLT-1 expression has been observed during embryogenesis.\textsuperscript{103,312,389} Embryonic mouse brain was not examined in this study and requires investigation.

In retrospect, the glutamine transporters, i.e., the SNATs, should have been included in this study to provide a more comprehensive perspective of the glutamate homeostasis system and how GLT-1d integrates into this system in the juvenile mouse brain. Future GLT-1d studies will include the SNATs, GLT-1a–d, the other EAATs, the VGLUTs, and glutamine synthetase in juvenile mammalian brains.

5.10 Key Points

This study was undertaken to examine the expression and regional and cellular distribution profile of GLT-1d in juvenile mouse brain. In the course of this study, the following key points were revealed:

\begin{itemize}
  \item GLT-1d exhibited an increase in temporal expression profile in juvenile mouse brain, which was consistent with postnatal changes in GLT-1 expression for various mammalian species.
  \item GLT-1d mRNA increased from low expression at postnatal day one (P\textsubscript{1}) to peak at P\textsubscript{28} and then declined to adult.
\end{itemize}
The increase in GLT-1d expression from P7–P14 suggested that there may be an increasing demand for GLT-1d activity in juvenile mouse brain development and maturation.

GLT-1d protein was expressed in astrocyte somata and processes in juvenile mouse brain, but not observed in neurons.

The increase in expression of GLT-1d was largely coincident with gliogenesis, synaptogenesis, a transition to mature forms and expression levels of glutamate receptors, and myelination.

GLT-1d may be associated with the onset of and ensuing glutamatergic neurotransmission in juvenile mouse brain.
6.1 Synopsis

Aim: The aim of this study was to examine the expression, and regional and cellular distribution profile of GLT-1d in adult mouse brain.

Research Question: What is the spatial expression and distribution profile of GLT-1d in adult mouse brain?

Methods: The expression of GLT-1d mRNA was examined by quantitative PCR (qPCR) primers using GLT-1d specific qPCR primers (Chapter 5), whereas the GLT-1d antiserum (Rb 1713 antiserum) (Chapter 4) was used to investigate the expression of GLT-1d protein by western blotting and immunohistochemistry.

Results: GLT-1d mRNA expression was highest in the neocortex and caudate putamen > hippocampus and dentate gyrus, hypothalamus and thalamus, inferior colliculus, and olfactory bulb > optic and auditory vestibular nerves > cerebellum. Western blotting detected a broad ~65 kDa band and two higher molecular weight bands for GLT-1d in forebrain, midbrain, hindbrain, and cerebellum, with the highest signal for each of these bands detected in the forebrain, with a decrease in the signal detected for each band in rostrocaudal order, i.e., forebrain ≫ midbrain ≫ hindbrain > cerebellum.
Immunohistochemistry revealed GLT-1d immunoreactivity throughout the adult mouse brain, with enriched GLT-1d immunoreactivity detected in neuronal and/or astrocyte somata in layers IV–VI of the neocortex, CA1 and CA3 regions of the hippocampus, in the dentate gyrus, and the cerebellum. Interestingly, dense GLT-1d immunoreactivity was detected in the white matter of the cerebellum and in discontinuous regions adjacent blood vessels in the neocortex and hippocampus.

**Inferences:** GLT-1d mRNA and protein expression was highest in adult mouse forebrain, with enriched GLT-1d immunoreactivity in neuronal and/or astrocytic somata in the neocortex, CA1 and CA3 regions of the hippocampus, in the dentate gyrus, and the cerebellum. The GLT-1d expression decreased in a rostrocaudal manner with the highest level in the forebrain and lowest level in the cerebellum. The high level of GLT-1d expression in the white matter of the cerebellum, despite this region having the lowest level in general, suggested a possible role for GLT-1d protein in the white matter tracts.

**Significance:** Importantly, GLT-1d protein was detected in neurons and astrocytes in adult mouse brain, in contrast to juvenile mouse brain where GLT-1d protein was detected in astrocytes. The elevated GLT-1d expression detected in white matter tracts pointed to GLT-1d as a potential therapeutic target for neurodegenerative diseases associated with white matter tracts, e.g., spinal injury and Alzheimer’s disease.

**6.2 Introduction**

Chapter 5 of this thesis investigated the developmental expression profile of GLT-1d in the developing mouse brain. The brains of C57BL/6 mice ranging in age from the day of birth to adult (~3 months) were analyzed with respect to the excitatory amino acid transporters GLT-1a–d and GLAST, the vesicular glutamate transporters VGLUT1–3, and glutamine synthetase. Since GLT-1d expression increased to adult levels by postnatal day fourteen and then remained stable, it appeared that the demand for GLT-1d activity was established early in postnatal brain development and continued in adult mouse brain.

As mentioned previously in Chapter 2, GLT-1 is the primary glutamate transporter in the brain. GLT-1 comprises a number of splice variants. Previous studies have investigated the expression and distribution of these variants in adult rodent brain. GLT-1a, the original and dominant GLT-1 splice variant, and GLT-1b protein are widely distributed throughout the adult rodent brain, with a predominance in the forebrain. The protein of the less abundant GLT-1c splice
variant is confined to the endfeet of astrocytes adjacent blood vessels and periventricular astrocytes.\textsuperscript{287} The exon-skipping variants of GLT-1, i.e., exon 4-skip\textsuperscript{282} and exon 9-skip,\textsuperscript{281} are also limited in their distribution. Exon 4-skipping GLT-1 protein is distributed in a punctate manner through the grey matter of adult rat brain.\textsuperscript{282} In contrast, exon 9-skipping GLT-1 protein is predominantly detected in white matter, i.e., corpus callosum and white matter tracts of the cerebellum, in adult rat brain.\textsuperscript{281} The variability of expression and distribution of the known GLT-1 splice variants in adult rodent brain prompted the following question: What is the spatial expression and distribution profile of GLT-1d in adult mouse brain?

6.3 Experimental Methods

All chemicals used in the experiments of this chapter were of analytical grade and purchased from commercial suppliers. Specific details of the suppliers of the animals, chemicals, and equipment cited in this chapter are set out in Table F.1 in Appendix F: Suppliers of Animals, Chemicals, Equipment, and Services on page 232.

6.3.1 Animal Tissues

The experiments of this chapter were performed on tissues of \(\sim\)3-month-old adult male mice of a common inbred strain of laboratory mouse, the C57BL/6 mouse strain.

Mice were housed under standard temperature- and light-controlled conditions, i.e., \(\sim\)22\( ^\circ\)C and 12 hours light/12 hours dark, with free access to food and water prior to the collection of tissues.

The mice were terminally anaesthetised by intraperitoneal injection of sodium pentobarbitral (150 mg/kg). Once the foot withdrawal reflex was absent, the deeply anaesthetised animals were decapitated using a pair of surgical scissors.

Each brain was rapidly dissected from the cranial vault according to a previously described method.\textsuperscript{363} Specific brain regions were identified using stereotaxic mapping as set out, for example, in Figures 6.1–6.3 on pages 113–115.

Depending on the proposed use, the isolated brains were treated as follows:

1. RNA and protein extraction:
   (a) The caudate putamen, cerebellum, cortex, hippocampus, hypothalamus and thalamus, inferior colliculus, olfactory bulb, and optic and auditory vestibular
Figure 6.1: Stereotaxic map showing a coronal section of an adult mouse brain (bregma -0.34 mm). The highlighted region is the caudate putamen (CP) (light green). Modified from Figure 34 of Paxinos and Franklin with permission from the copyright holder Elsevier Limited.
Figure 6.2: Stereotaxic map showing a coronal section of an adult mouse brain (bregma -2.34 mm). The highlighted regions are the neocortex (salmon pink), Cornu Ammonis areas CA1 (lilac) and CA3 (turquoise), the dentate gyrus (DG) (purple), and the caudate putamen (CP) (light green). The hippocampus, which comprises Cornu Ammonis areas CA1, CA2, and CA3, is highlighted in red. Modified from Figure 50 of Paxinos and Franklin with permission from the copyright holder Elsevier Limited.
Figure 6.3: Stereotaxic map showing a sagittal section of an adult mouse brain (lateral 0.96 mm). The highlighted regions are the olfactory bulb (yellow), the caudate putamen (light green), neocortex (salmon pink), Cornu Ammonis areas CA1 (lilac) and CA3 (turquoise), the inferior colliculus (pink), and the cerebellum (light blue). Modified from Figure 109 of Paxinos and Franklin with permission from the copyright holder Elsevier Limited.
nerves were isolated by microdissection, immediately frozen in liquid nitrogen, and stored at -80°C until required.

(b) Whole brains were dissected into forebrain, midbrain, hindbrain, and cerebellum, which were immediately frozen in liquid nitrogen, and stored at -80°C until required. These regions of the brain have been defined previously (Section 4.3.3 - Animal Tissue Collection on page 53), but for ease of reference are described here again:

i. Forebrain: cerebral cortex, basal ganglia, thalamus, hypothalamus, subthalamus, epithalamus, pretectum, and associated white matter;

ii. Midbrain: superior and inferior colliculi, midbrain tegmentum, crus cerebri, and substantia nigra; and

iii. Hindbrain: medulla and pons.

iv. The hindbrain is generally understood to include the cerebellum, but for the purposes of this study the cerebellum was treated as an independent structure.

2. Immunohistochemistry: Brain tissue was treated as set out previously in Section 5.4.1 - Animal Tissue Collection and Treatment on page 85.

6.3.2 Total RNA Extraction

Total RNA extraction was performed as set out previously in Section 5.4.2 - Total RNA Extraction on page 86.

6.3.3 RNA Concentration and Integrity

RNA concentration and integrity was determined using a NanoDrop™ 2000 spectrophotometer according to the manufacturer’s instructions (see Appendix M: ThermoFisher Scientific NanoDrop™ 2000 Spectrophotometer Operating Instructions on page 266).

6.3.4 Reverse Transcription

Reverse transcription was performed as set out previously in Section 5.4.4 - Reverse Transcription on page 87.
6.3.5 Quantitative PCR

Quantitative PCR was performed as set out previously in Section 5.4.7 - Quantitative PCR on page 90.

6.3.6 Protein Extraction

Protein extraction was performed as set out previously in Section 5.4.8 - Protein Extraction on page 91.

6.3.7 Protein Assay

Protein assays were performed as set out in Section 4.3.5.3 - Protein Assay on page 55.

6.3.8 Protein Sample Preparation

Protein samples were prepared as set out previously in Section 4.3.5.4 - Protein Sample Preparation on page 55.

6.3.9 Western Blotting

Western blotting was performed as set out previously in Section 4.3.5.6 - Optimized Western Blotting Method on page 60 using lysates prepared from four broad regions of the adult mouse brain, i.e, the forebrain, midbrain, hindbrain, and cerebellum. Prior to immunodetection, Ponceau S staining was performed to assess consistency of the protein loaded per lane and to confirm the efficiency of transfer (see page 58).

6.3.10 Immunohistochemistry

Immunohistochemistry was performed on adult mouse brain as set out previously in Section 5.4.12 - Immunohistochemistry on page 92.

6.3.11 Statistical Analysis

Statistical analysis was performed as set out in Section 5.4.13 - Statistical Analysis on page 93.
6.4 Results

This study investigated the expression and distribution of GLT-1d in adult mouse brain using quantitative PCR, western blotting, and immunohistochemistry.

6.4.1 Quantitative PCR

Quantitative PCR was performed using cDNA prepared from the total RNA extracted from various representative regions and nerves in adult mouse brain, i.e., the caudate putamen, cerebellum, neocortex, hippocampus and dentate gyrus, hypothalamus and thalamus, inferior colliculus, olfactory bulb, and optic and auditory vestibular nerves. GLT-1d mRNA expression was highest in the neocortex and caudate putamen > hippocampus and dentate gyrus, hypothalamus and thalamus, inferior colliculus, and olfactory bulb > optic and auditory vestibular nerves > cerebellum (Figure 6.4 D).

![Figure 6.4: Regional expression of the GLT-1 C-terminal splice variants GLT-1a–d mRNA in the C57BL/6 adult mouse brain (n = 3). Abbreviations: Cp, caudate putamen; Cb, cerebellum; Cx, neocortex; Hc, hippocampus and dentate gyrus; HT, hypothalamus and thalamus; Ic, inferior colliculus; Ob, olfactory bulb; and OAVn, optic and auditory vestibular nerves. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, and **** P ≤ 0.0001, significantly different from neocortex.](image-url)
Chapter 6: *GLT-1d in Adult Mouse Brain*

The expression profile of GLT-1a differed slightly from that of GLT-1d in that the profile was neocortex > caudate putamen, hippocampus and dentate gyrus, hypothalamus and thalamus > inferior colliculus and olfactory bulb > cerebellum >> optic and auditory vestibular nerves (Figure 6.4 A). Interestingly, similar to GLT-1d mRNA expression, GLT-1b mRNA expression was highest in the neocortex and caudate putamen > hippocampus and dentate gyrus, hypothalamus and thalamus, inferior colliculus, and olfactory bulb > cerebellum and optic and auditory vestibular nerves (Figure 6.4 B). GLT-1c, which is expressed at low levels in the brain, exhibited an expression profile with the highest mRNA expression in neocortex > hippocampus and dentate gyrus, and hypothalamus and thalamus > olfactory bulb > caudate putamen, cerebellum, inferior colliculus, and optic and auditory vestibular nerves (Figure 6.4 C).

The fold changes of the expression of each GLT-1 splice variant is set out in Table X.

**Table 6.1:** The fold change in expression of the GLT-1 splice variants in adult mouse brain relative to the neocortex.

<table>
<thead>
<tr>
<th></th>
<th>GLT-1a</th>
<th>GLT-1b</th>
<th>GLT-1c</th>
<th>GLT-1d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudate putamen</td>
<td>0.67</td>
<td>1.13</td>
<td>0.17</td>
<td>0.91</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.32</td>
<td>0.32</td>
<td>0.05</td>
<td>0.32</td>
</tr>
<tr>
<td>Neocortex</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.79</td>
<td>0.80</td>
<td>0.68</td>
<td>0.70</td>
</tr>
<tr>
<td>Hypothalamus and Thalamus</td>
<td>0.82</td>
<td>0.75</td>
<td>0.75</td>
<td>0.61</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>0.52</td>
<td>0.73</td>
<td>0.07</td>
<td>0.75</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>0.38</td>
<td>0.90</td>
<td>0.48</td>
<td>0.85</td>
</tr>
<tr>
<td>Optic and Auditory Vestibular Nerves</td>
<td>0.01</td>
<td>0.42</td>
<td>0.01</td>
<td>0.50</td>
</tr>
</tbody>
</table>

6.4.2 Western Blotting

Western blotting was performed using lysates prepared from four broad regions of adult mouse brain, i.e., the forebrain, midbrain, hindbrain, and cerebellum (see page 116 for the relevant component structures of each region). GLT-1d antiserum (Rb 1713 antiserum) detected a broad ~65 kDa band and two higher molecular weight bands for each of the four broad regions (Figure 6.5). The higher molecular weight bands were presumed to be oligomeric forms, i.e., dimer (**) and trimer (***) forms, of GLT-1d. As mentioned previously, similar bands are normally thought to represent monomer and oligomeric forms of GLT-1, at least for other known forms of GLT-1.146,212 The highest signal detected for each of these bands was detected in the forebrain, with a decrease in the signal detected for each band in rostrocaudal order, i.e., forebrain >> midbrain >> hindbrain >> cerebellum. The trimer band (***) was barely detectable in the cerebellum lysate (Figure 6.5).
Chapter 6: *GLT-1d in Adult Mouse Brain*

**Figure 6.5:** Representative western blot of GLT-1d expression in the adult mouse forebrain, midbrain, hindbrain, and cerebellum. Molecular weights are indicated to the left of the blot (kDa). The bands marked ** and *** were presumed to be GLT-1d dimer and trimer forms, respectively. Sample lanes = 5 µg protein/lane.

### 6.4.3 Immunohistochemistry

Immunohistochemistry revealed GLT-1d immunoreactivity throughout the adult mouse brain. Notably, GLT-1d labelling was detected throughout the neuropil of the adult mouse neocortex, with enriched immunoreactivity observed in neuronal somata in layers IV–VI, particularly the pyramidal cells and the apical dendrites in layer IV (Figure 6.6 on page 121). Similarly, labelling was seen throughout the CA1 and CA3 regions of the hippocampus (Figures 6.7 and 6.8 on pages 122 and 123, respectively), with dense labelling of the pyramidal cell somata and processes (Figure 6.7 B). Likewise, in the dentate gyrus, dense labelling of the somata in the granule cell layer and a number of the somata in the hilus or polymorph layer was observed (Figure 6.9 on page 124).

Both the molecular and granular layers of the cerebellum were densely labelled for GLT-1d (Figure 6.10 on page 125). The somata of the inhibitory Purkinje neurons (also known as Purkinje cells), as in the developing mouse brain (Figure 5.9 on page 104), were unlabelled in the densely labelled Purkinje cell layer at the intersection of the molecular and granular layers. Similarly, high levels of immunoreactivity was detected in the white matter. At high magnification, the astrocytic somata in the granular layer of the cerebellum presented as a clearly labelled network, with label extending into the processes (Figure 6.10 B).

Interestingly, dense GLT-1d immunoreactivity was detected in discontinuous regions adjacent blood vessels in the neocortex (Figure 6.6) and hippocampus (Figure 6.7).
A Adult mouse neocortex (low magnification)

B Pyramidal cell, adult mouse neocortex (high magnification)

Figure 6.6: Representative immunohistochemical photomicrographs illustrating the distribution of GLT-1d in the adult mouse neocortex and dense immunolabelling of pyramidal cells. [A] GLT-1d immunoreactivity was seen throughout the grey matter, with dense immunoreactivity present in the somata of neurons. Dense labelling was seen in layer IV–VI relative to layers I–II/III. GLT-1d immunolabelling was particularly dense in the somata of the pyramidal cells and their apical dendrites in layer IV. Labelling was also seen in the areas immediately adjacent blood vessels throughout the neuropil. The cortical layers are shown to the right of the image. [B] Somata of pyramidal cells and their apical dendrites in layer IV exhibited dense GLT-1d immunolabelling. Scale bars: [A] 25 µm and [B] 5 µm.
Figure 6.7: Representative immunohistochemical photomicrographs illustrating the distribution of GLT-1d in adult mouse CA1 region of the hippocampus. At low magnification [A], labelling was seen throughout the CA1, with distinct labelling of pyramidal cell somata in the pyramidal layer (P) and adjacent blood vessels. At high magnification [B], in addition to labelling of the somata in the pyramidal layer (P), immunolabelling of the proximal processes (arrows) of the pyramidal cells was also observed. Abbreviations: stratum oriens (O), pyramidal layer (P), and stratum radiatum (R). Scale bar: [A] 20 µm, and [B] 10 µm.
Figure 6.8: Representative immunohistochemical photomicrograph illustrating the distribution of GLT-1d in the adult mouse CA3 region of the hippocampus. Immunolabelling was seen throughout the CA3, with distinct labelling of the pyramidal cell somata (arrows) in the pyramidal cell layer (P). * was reaction product. Abbreviations: stratum oriens (O), pyramidal layer (P), and stratum lucidum (L). Scale bar = 10 μm.
Figure 6.9: Representative immunohistochemical photomicrographs illustrating the distribution of GLT-1d in the adult mouse dentate gyrus. At low magnification [A], immunolabelling was seen throughout the dentate gyrus, with dense labelling of the somata in the granule cell layer (G) and the hilus or polymorph layer (H). At high magnification [B], immunolabelling of the granule cell somata in the granule cell layer (G) was observed. * was reaction product. Abbreviations: molecular layer (M), granular layer (G), and hilus or polymorph layer (H). Scale bar: [A] 25 µm and [B] 10 µm.
Figure 6.10: Representative immunohistochemical photomicrographs illustrating the distribution of GLT-1d in the adult mouse cerebellum. At low magnification [A], labelling was seen throughout the molecular and granular layers of the cerebellum. Particularly dense labelling was associated with the Bergmann glia in the molecular layer and the white matter. The somata of the inhibitory Purkinje cells were unlabelled in the densely labelled Purkinje cell layer at the intersection of the molecular and granular layers. The labelling in the granular layer at high magnification [B] showed a network of dense labelling of astrocyte somata and processes. Abbreviations: molecular layer (ML), granular layer (GL), Purkinje cell layer (PCL), and white matter (WM). Scale bars: [A] 50 µm and [B] 10 µm.
6.5 Discussion

This study provided the first description of the spatial expression and distribution profile of GLT-1d in adult mouse brain. The study examined the spatial expression and distribution profiles of GLT-1a–d mRNA in adult mouse brain by quantitative PCR using specific primers designed for this thesis or based on published primer sequences (Chapter 5). At the protein level, GLT-1d protein was examined by western blotting and immunohistochemistry using the preferred GLT-1d-specific antiserum (Rb 1713 antiserum) produced for this thesis (Chapter 4).

6.5.1 Spatial Expression Profile of GLT-1d in Adult Mouse Brain

The study demonstrated that GLT-1d mRNA and protein were broadly coincident with respect to their regional distribution in adult mouse brain (Table 6.2 on page 127). GLT-1d mRNA was highly abundant in the neocortex, caudate putamen, hippocampus, hypothalamus and thalamus, and olfactory bulb, moderately abundant in the inferior colliculus and the optic and auditory vestibular nerves, and present at a low level in the cerebellum (Table 6.2 on page 127). Corresponding to GLT-1d mRNA distribution, GLT-1d protein distribution, as determined by western blotting, was highest in the forebrain, moderately expressed in the midbrain and hindbrain, with the lowest level detected in the cerebellum. Such distribution of GLT-1d, at least at the mRNA level, was markedly similar to that of GLT-1a and GLT-1b mRNA observed in this study, and that previously reported for GLT-1 in adult rat brain.150,215,228,313,318,426

6.5.2 An Unexpected Switch to Neuronal and Astrocytic GLT-1d Protein Expression

In adult mouse, consistent with GLT-1a protein distribution in rat316 and human465 neocortex, GLT-1d protein was detected in the neocortex. Enriched GLT-1d immunoreactivity was present in the neuronal somata and neuronal processes in layers IV–VI, particularly the pyramidal cells and their apical dendrites in layer IV (Figure 6.6 B on page 121). Interestingly, GLT-1d immunoreactivity in the adult mouse neocortex contrasted with that observed in juvenile mouse neocortex. GLT-1d immunoreactivity detected in P7 and P14 mouse brain (Figure 5.6 on page 100) was intense in layer I, moderate in layer II/III, and low in layers IV–VI and localised to astrocytes. One possible explanation for the dense GLT-1d immunolabelling in layer I of P7 and P14 neocortex relative to adult mouse neocortex may be the presence of glutamatergic Cajal-Retzius cells in this layer in juvenile mouse brain. The glutamatergic Cajal-Retzius cells decline

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Table 6.2: Distribution of GLT-1d mRNA and protein in adult mouse brain.

<table>
<thead>
<tr>
<th>GLT-1d mRNA</th>
<th>GLT-1d Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR</td>
<td>Western Blotting</td>
</tr>
<tr>
<td>Neocortex</td>
<td>+++</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>+++</td>
</tr>
<tr>
<td>Forebrain</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>+++</td>
</tr>
<tr>
<td>Hypothalamus &amp; Thalamus</td>
<td>+++</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>+++</td>
</tr>
<tr>
<td>Midbrain</td>
<td>++</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>++</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>ND</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>OAVNs</td>
<td>++</td>
</tr>
</tbody>
</table>

OAVNs: Optic and Auditory Vestibular Nerves.
Signal intensities: +++ high, ++ moderate, and + low, and ND not determined.
Chapter 6: GLT-1d in Adult Mouse Brain

in number after P7.\textsuperscript{466} Such a decline in the number of the glutamatergic Cajal-Retzius cells would plausibly lead to a reduction in glutamatergic neurotransmission in layer I, and possibly a reduction in GLT-1d immunoreactivity. Furthermore, as the glutamatergic neurons in layers II/III–VI assume mature functions, i.e., consolidate into the glutamatergic circuitry of the adult mouse brain, a change in GLT-1d immunoreactivity is conceivable. Support for such a change was seen in the increasing GLT-1d protein expression in the early postnatal mouse brain, i.e., from P1–P14, (Figure 5.5 C on page 98).

Dense GLT-1d immunoreactivity was detected in neuronal somata in the granule cell layer and in the hilus or polymorph layer of the adult mouse dentate gyrus (Figure 6.9 on page 124), in contrast to that previously observed for GLT-1a where the immunolabelling surrounded the somata.\textsuperscript{270} Dense GLT-1d immunoreactivity was also observed in the pyramidal cell somata and processes of the CA3 of the adult mouse hippocampus (Figure 6.8 on page 123) The CA3 is innervated by the glutamatergic mossy fibres, which are unmyelinated axons of the granule cells of the dentate gyrus.\textsuperscript{467} Similarly, dense GLT-1d immunoreactivity was also observed in the pyramidal cell somata and processes of the CA1 of the adult mouse hippocampus (Figure 6.7 on page 122). The glutamatergic Schaffer collaterals that arise from pyramidal cells in the CA3 extend to the CA1 pyramidal cells.\textsuperscript{468} The abundance of GLT-1d detected in the dentate gyrus, CA1, and CA3 suggest an active role for GLT-1d in the glutamatergic neurocircuitry of these regions. If, as in adult rat CA1,\textsuperscript{469} not all CA1 synapses in adult mouse brain are apposed to astrocytes and/or astrocytic processes, it is plausible that GLT-1d protein in this region may fulfil an active role in limiting the inter-synaptic glutamate diffusion.

In adult mouse cerebellum, the Purkinje cell layer and molecular layer were densely GLT-1d immunoreactive (Figure 6.10 A on page 125). The Purkinje neuron (also known as Purkinje cell) dendrites extend into the molecular layer (Figure 6.11 A) where they are innervated directly by the excitatory climbing fibres, which arise in the inferior olive.\textsuperscript{462} The Purkinje neuron dendrites are also innervated by the parallel fibres of the excitatory granule cells.\textsuperscript{462,463} The parallel fibres are bifurcated extensions of the granule cell axons (Figure 6.11 B).\textsuperscript{463} It is plausible that the dense GLT-1d immunoreactivity seen in the molecular cell layer is GLT-1d localised to excitatory climbing fibres and parallel fibres. The localisation of GLT-1d in the molecular layer requires further investigation. Interestingly, in adult mouse cerebellum granular layer, the astrocytic somata presented as a clearly labelled GLT-1d immunoreactive network, with GLT-1d immunoreactivity extending into the processes (Figure 6.10 B on page 125).

Also interestingly, the white matter tracts of the adult mouse cerebellum were densely GLT-1d immunoreactive (Figure 6.10 A). The location and potential roles of GLT-1d
6.5.3 GLT-1d in Adult Mouse Brain White Matter Tracts

An intriguing finding of the present study was that the GLT-1d mRNA expression in the optic and auditory vestibular nerves was strikingly higher than that observed for GLT-1a mRNA (Figures 6.4 D and 6.4 A on page 118). Consistent with the GLT-1d mRNA level, dense GLT-1d immunoreactivity was also observed in the white matter tracts of the adult mouse brain, particularly in the cerebellum (Figure 6.10 A on page 125), which was consistent with a previous study relating to GLT-1. Reduced GLT-1 activity and levels in white matter tracts was associated with excitotoxic damage of the oligodendrocytes in the optic nerve. Accordingly, it is plausible that GLT-1d may be expressed in one or more of the cells types found in white matter tracts, i.e., neurons, astrocytes, and oligodendrocytes. If GLT-1d is expressed in different cells types, then it is plausible that it fulfils different functions in different cell types. Furthermore, it is conceivable that the function GLT-1d fulfils in white matter is distinct with respect to the function it fulfils in grey matter. The cellular localisation of GLT-1d in white matter tracts and its potential role(s) in such tracts requires investigation.
It is possible that GLT-1 may play a role in the control of the levels of ambient extracellular glutamate and the restriction of glutamate diffusion in white matter tracts.\textsuperscript{171} The elevated GLT-1d expression in white matter tracts suggested GLT-1d be investigated as a potential therapeutic target for neurodegenerative diseases associated with white matter tracts, e.g., spinal injury and Alzheimer’s disease.\textsuperscript{473–475}

### 6.5.4 GLT-1d and Blood Vessels in the Adult Mouse Brain

The GLT-1d immunoreactivity observed adjacent a number of blood vessels in adult mouse neocortex (Figure 6.6 on page 121) and hippocampus (Figure 6.7 A on page 122) was discontinuous. Such discontinuous distribution of GLT-1d immunoreactivity was consistent with that previously observed for GLT-1a\textsuperscript{270} and GLT-1c.\textsuperscript{287} One possible explanation for such distribution of GLT-1d immunoreactivity is that astrocytes apposed to blood vessels differentially express and selectively localize GLT-1d protein, as has been suggested for GLT-1c.\textsuperscript{287} If GLT-1d protein is similarly located in astrocyte endfeet, it is possible that GLT-1d protein may be associated with the function of the brain vascular network.\textsuperscript{476} More particularly, GLT-1d protein may be associated with the function of the blood-brain-barrier\textsuperscript{477,478} and/or mediation of neurovascular coupling.\textsuperscript{479}

### 6.6 Significance Statement

This study demonstrated that the expression of GLT-1d protein underwent a developmental transition from expression in astrocytes in juvenile mouse brain to expression in neurons and astrocytes in adult mouse brain. This study also demonstrated that in adult mouse brain GLT-1d expression was elevated in white matter tracts, particularly in the cerebellum. Such elevated levels of expression of GLT-1d protein in white matter tracts suggests that GLT-1d be investigated as a potential therapeutic target for neurodegenerative diseases associated with white matter tracts, e.g., spinal injury and Alzheimer’s disease.

### 6.7 Further Observations

Observations regarding this study, and its integration into achieving the aim of the research for this thesis, are set out in Chapter 8.
6.8 Future Studies

Future confocal and electronmicroscopy GLT-1d immunolabelling studies are required to investigate the subcellular distribution of GLT-1d in neurons and astrocytes, in particular whether GLT-1d is localised to synaptic terminals in adult mouse brain. Future studies should also investigate the electrophysiological and pharmacological properties of GLT-1d in adult mouse brain.

In retrospect, the glutamine transporters, i.e., the SNATs, should have been included in this study to provide a more comprehensive perspective of the glutamate homeostasis system and how GLT-1d integrates into this system in the adult mouse brain. Future GLT-1d studies will include the SNATs, GLT-1a–d, the other EAATs, the VGLUTs, and glutamine synthetase in adult mammalian brains.

6.9 Key Points

This study was undertaken to examine the expression, and regional and cellular distribution profiles of GLT-1d in adult mouse brain. In the course of this study, the following key points were revealed:

* GLT-1d protein was detected in neurons and astrocytes in adult mouse brain, in contrast to juvenile mouse brain where GLT-1d protein was detected in astrocytes.

* GLT-1d mRNA expression was highest in the neocortex and caudate putamen > hippocampus and dentate gyrus, hypothalamus and thalamus, inferior colliculus, and olfactory bulb > optic and auditory vestibular nerves > cerebellum.

* The highest GLT-1d protein level was detected in forebrain and decreased in rostrocaudal order, i.e., forebrain ≫ midbrain ≫ hindbrain > cerebellum.

* GLT-1d expression was high in optic and auditory vestibular nerves and the white matter of the cerebellum, and in discontinuous regions adjacent blood vessels in the neocortex and hippocampus.

* Elevated GLT-1d expression in white matter tracts pointed to GLT-1d as a potential therapeutic target for neurodegenerative diseases associated with white matter tracts, e.g., spinal injury and Alzheimer’s disease.
GLT-1d in Pathological Mouse Brains, a Preliminary Study

7.1 Synopsis

Aim: The aim of this study was to examine the expression profile of GLT-1d in an acute pathological condition, i.e., transient focal cerebral ischaemic insult, and a chronic pathological condition, i.e., amyotrophic lateral sclerosis, in mouse models of the respective pathological conditions.

Research Questions: (i) What is the pathological expression profile of GLT-1d in an acute pathological condition in mouse brain? (ii) What is the pathological expression profile of GLT-1d in a chronic pathological condition in mouse brain?

Methods: Two animal models were used in this study to investigate the expression of GLT-1d in pathological mouse brain: a transient focal cerebral ischaemia and reperfusion mouse model (acute pathological condition) and an hSOD1G93A amyotrophic lateral sclerosis mouse model (chronic pathological condition). GLT-1d expression was examined using quantitative PCR (mRNA) and western blotting (protein).
Results: In the acute pathological condition, i.e., the transient focal cerebral ischaemia and reperfusion mouse model, GLT-1d mRNA was downregulated in both the ipsilateral and contralateral hemispheres, albeit to a lesser extent in the contralateral hemisphere. Intriguingly, no significant change was observed for GLT-1a mRNA in both the ipsilateral and contralateral hemispheres. In the chronic pathological condition, i.e., the hSOD1<sup>G93A</sup> amyotrophic lateral sclerosis mouse model, both GLT-1a and GLT-1d mRNA expression was downregulated in motor cortex at the onset of symptoms stage relative to the pre-symptomatic stage, at mid-stage and end-stage both GLT-1a and GLT-1d mRNA expression was upregulated relative to the immediately preceding stage, with the highest level of expression detected in the end-stage motor cortex.

Inferences: In the acute pathological condition, downregulation of the GLT-1d expression in the contralateral hemisphere suggested that transient ischaemia in the ipsilateral hemisphere influenced GLT-1d expression in the contralateral hemisphere within 24 hours post-reperfusion. The modest downregulation of GLT-1d expression in the contralateral hemisphere may have been due to degeneration of intercortical glutamatergic neurons projecting from the injured area of the ipsilateral hemisphere into the contralateral hemisphere. The degeneration of these projection neurons may in turn have led to a reduction in the secretion of neurotrophic factor(s) known to modulate GLT-1 expression by these projection neurons in the target regions. Alternatively, or in addition, the modest reduction in the GLT-1d expression in the contralateral hemisphere may have been due to a reduction in the GLT-1d mRNA and/or protein in the intercortical projection neurons projecting from the injured ipsilateral hemisphere into the contralateral hemisphere. With respect to GLT-1a mRNA expression, one possible explanation for no change in the expression of GLT1a mRNA being detected in the present study may be that the neuronal and astrocytic GLT-1d may exhibit different kinetics in response to cerebral ischaemic insult relative to the predominantly astrocytic GLT-1a. The findings of the present study relating to GLT-1a mRNA expression may be due to the mild cerebral ischaemic insult induced during this study. In the chronic pathological condition, GLT-1a and GLT-1d mRNA expression may be subject to biphasic regulation in the hSOD1<sup>G93A</sup> mouse motor cortex over the course of disease progression. The elevated GLT-1a and GLT-1d mRNA expression observed in the pre-symptomatic hSOD1<sup>G93A</sup> mouse motor cortex relative to the age-matched wildtype may be a compensatory phase during which neurons were more active in a hostile environment. The marked decline in GLT-1a and GLT-1d mRNA expression as the hSOD1<sup>G93A</sup> mouse motor cortex neurons approached the symptomatic stage, i.e., onset stage, may have been due to the neurons dying, which was followed by further compensatory phase, i.e. from disease onset to end-stage, where the remaining neurons endeavoured to restore glutamate homeostasis in their immediate environment.
Significance: The sensitivity of GLT-1d to a mild cerebral ischaemic insult is an important finding. Furthermore, the differential regulation of GLT-1d expression in both acute and chronic pathological conditions strongly suggested future studies investigate GLT-1d as a potential therapeutic target.

### 7.2 Introduction

A loss of the glutamate transporter GLT-1 has been associated with both acute and chronic neurodegenerative diseases such as cerebral ischaemia and amyotrophic lateral sclerosis, respectively. Our understanding of cerebral ischaemia and amyotrophic lateral sclerosis has been aided by the development of animal models of these diseases. Accordingly, the aim of this study was to examine the expression of GLT-1d in an acute pathological condition, i.e., transient focal cerebral ischaemic insult, and a chronic pathological condition, i.e., amyotrophic lateral sclerosis, in brains of mouse models of the respective pathological conditions.

### 7.3 Cerebral Ischaemia

Cerebral ischaemia is associated with a number of pathological conditions including cardiac arrest and resuscitation, stroke, and traumatic brain injury. Ischaemic stroke is a leading cause of mortality and permanent disability of adult humans in industrialised countries worldwide. Cerebral ischaemia is also associated with surgical procedures in the brain, when blood flow has to be reduced to at least part of the brain.

The interruption of blood flow during cerebral ischaemia deprives at least part of the brain of oxygen and glucose. The reduction of oxygen and glucose supply in turn leads to an energy deficiency in the affected area. As a consequence of the energy deficiency, cells in the affected area are unable to maintain the ionic gradient across the plasma membrane and cellular function and homeostasis become dysregulated. The Na$^+$-dependent excitatory amino acid transporters located in the plasma membrane of cells, including GLT-1, are functionally dependent on the ionic gradient. As the ionic gradient collapses, glutamate uptake by the Na$^+$-dependent excitatory amino acid transporters located in the plasma membrane of cells, typically neuronal cells, is slowed and eventually reversed, leading to elevated levels of glutamate in the synaptic cleft and extracellular space. The elevated levels of glutamate in the synaptic cleft and extracellular space result in enhanced activation of glutamate receptors, which facilitates an increased influx of Na$^+$ and Ca$^{2+}$ into cells.
intracellular Na\(^+\) further exacerbates reversed glutamate transport into the synaptic cleft and extracellular space.\(^{515-517}\) Increased intracellular Ca\(^{2+}\), on the other hand, can stimulate a deleterious cascade of cellular and molecular events that either cause harm or cell death.\(^{515,518,519}\)

There are several categories of animal models of cerebral ischaemia, i.e., occlusion of the middle cerebral artery,\(^{520-522}\) photothrombotic,\(^{523}\) and thromboembolic models.\(^{524,525}\) The occlusion of the middle cerebral artery animal model most closely approximates the conditions that occur in human ischaemic stroke patients and, for this reason, is the most popular model for cerebral ischaemia research.\(^{526}\) Within the occlusion of middle cerebral artery category there are four specific models: intraluminal filament model, electrocoagulation model, middle cerebral artery ligation model, and surgical clip model.\(^{526}\) Transient occlusion of the middle cerebral artery using an intraluminal filament, as used in this study, represents a well-established cerebral ischaemia model.\(^{527,528}\) This model is particularly favoured as the injuries caused in rodent brain correspond closely with those seen in human cerebral ischaemia patients, i.e., injuries to the frontoparietal cortex and lateral caudate putamen, without requiring major invasive cranial surgery.\(^{522,526,529}\)

Downregulation of GLT-1 expression, without considering variability in splice variant expression, has been associated with cerebral ischaemia in rodents.\(^{224,482-489}\) The study of Chapter 6 had established that GLT-1d was expressed in both neurons and astrocytes in adult mouse brain. On the other hand, the dominant GLT-1 splice variant, GLT-1a, is predominantly expressed in astrocytes.\(^{141,144,145,213,329}\) Neurons are known to be substantially more sensitive to glutamate than astrocytes.\(^{530}\) Accordingly, it was considered possible that the predominantly astrocytic GLT-1a and neuronal and astrocytic GLT-1d would be differentially regulated in mouse brain that had been subjected to cerebral ischaemic insult.

### 7.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig’s disease and motor neurone disease (MND),\(^{531}\) is typically an adult-onset neurodegenerative disease that begins focally within the motor neurons of the spinal cord, brain stem, and motor cortex.\(^{532}\) As the disease develops, degeneration of the motor neurons results in progressive paralysis and muscular atrophy.\(^{532,533}\) ALS is ultimately fatal.\(^{532}\) A majority of ALS cases are sporadic (SALS), whereas \(\sim5-10\%\) are known as familial ALS (FALS).\(^{534}\) SALS is largely idiopathic, with no clearly defined single cause yet identified.\(^{532}\) FALS cases, on the other hand, arise due to mutations within a number of genes.\(^{532}\) Some of the most
common mutations associated with FALS, i.e., \( \sim 20\% \), are found in the ubiquitously expressed Cu/Zn superoxide dismutase gene (\( SOD1 \)).\textsuperscript{532,535,536} Superoxide dismutase is a cytoplasmic and mitochondrial enzyme that catalyses the breakdown of reactive oxygen species and thus ameliorates oxidative stress.\textsuperscript{537} Notwithstanding the recognised association of mutations in Cu/Zn superoxide dismutase with at least some cases of FALS, the aetiology of ALS is still largely uncertain.\textsuperscript{196}

Elevated extracellular glutamate levels in the central nervous system, i.e., excitotoxicity, is one of the key pathological mechanisms associated with ALS.\textsuperscript{538,539} Interestingly, decreased GLT-1 expression, without considering variability in splice variant expression, has been reported both in human ALS patients,\textsuperscript{17,490,540–542} as well as SOD1\textsuperscript{G93A} rodent models.\textsuperscript{492,543,544} Mutations in the \( SOD1 \) gene, i.e., gain of function, leads to the production of hydrogen peroxide, which impairs GLT-1 function.\textsuperscript{24} GLT-1 is known to be susceptible to oxidation,\textsuperscript{545} which has been suggested may result in the loss of GLT-1 protein as observed in both human patients as well as SOD1 ALS animal models.\textsuperscript{196}

The SOD1\textsuperscript{G93A} (glycine 93 to alanine) mutation is the most widely studied mutation within the transgenic amyotrophic lateral sclerosis animal models.\textsuperscript{531,546,547} The transgenic hSOD1\textsuperscript{G93A} mouse, which was used in this study, carries a high copy number of the mutated allele of the human \( SOD1 \) gene\textsuperscript{548} and accurately reproduces amyotrophic lateral sclerosis symptoms\textsuperscript{534,547} and disease progression in humans.\textsuperscript{491,546,549–553}

7.5 Experimental Methods

All chemicals used in the experiments of this chapter were of analytical grade and purchased from commercial suppliers. Specific details of the suppliers of the animals, chemicals, and equipment cited in this chapter are set out in Table F.1 in Appendix F: Suppliers of Animals, Chemicals, Equipment, and Services on page 232.

7.5.1 Cerebral Ischaemia

7.5.1.1 Animals and Housing Conditions

Eight–twelve week old male mice of a common inbred strain of laboratory mouse, the C57BL/6 mouse strain, were used in the focal cerebral ischaemia and reperfusion experiments.
Mice were housed under standard temperature- and light-controlled conditions, i.e., ∼22°C and 12 hours light/12 hours dark, with free access to food and water prior to the induction of transient cerebral ischaemia and reperfusion.

7.5.1.2 Transient Focal Cerebral Ischaemia and Reperfusion

To minimize post-operative pain, the mice were administered a long-acting analgesic (buprenorphine: 0.05mg/kg) by subcutaneous injection two hours before surgery. Shortly before the surgical procedure, the mice were anaesthetised with a combination of anaesthetic agents (ketamine: 150 mg/kg and xylazine: 10 mg/kg) administered by intraperitoneal injection.

Since this mouse model is associated with hypothermia, the body temperature of each anaesthetised mouse undergoing the procedure was carefully monitored using a rectal temperature probe. The rectal temperature probe measured the mouse’s body temperature, which was used by a temperature regulator to control a heat lamp. The heat lamp maintained the mouse’s body temperature at ∼37°C throughout the surgical procedure and until the mouse regained consciousness.

During each surgical procedure, the regional cerebral blood flow in the area of the cortex irrigated by the middle cerebral artery was measured using laser Doppler flowmetry. While in a prone position, the skin covering the calvaria of the anaesthetised mouse was reflected laterally and a laser Doppler flowmetry flexible probe was securely attached perpendicular to the right parietal skull, i.e., ∼1–2 mm posterior and 5 mm lateral to the bregma. The regional cerebral blood flow was monitored throughout the surgical procedure, and for the initial 10 minutes of reperfusion.

The anaesthetised mouse, with the laser Doppler flowmetry flexible probe securely attached, was carefully placed in a supine position under a dissecting microscope. Care was taken to avoid detaching the laser Doppler flowmetry flexible probe while placing the mouse under the dissecting microscope. A 5 mm incision was made in the skin on the right side of the neck and the skin reflected to expose the right external carotid artery. Using blunt dissection, the right external carotid artery was freed from surrounding connective tissue. A branch of the right external carotid artery was selected and cauterised to provide a right external carotid artery stump.

Using blunt dissection, the right common carotid and the right internal carotid arteries were freed from the surrounding connective tissue and separated from the right vagus nerve. The right common carotid artery was temporarily clamped and tension applied to the right internal carotid artery using a suture bridge. An arteriotomy was made in
the right external carotid artery stump. A 6-0 nylon monofilament with a silicone-coated tip was introduced via the arteriotomy into the right internal carotid. The 6-0 nylon monofilament was advanced \(\sim 11-12\) mm until the silicone-coated tip was adjacent, and occluding, the junction with the middle cerebral artery. A >70% reduction in the middle cerebral artery irrigation as measured by laser Doppler flowmetry confirmed occlusion of the middle cerebral artery junction. The filament remained \textit{in situ} for 50 minutes, following which the filament was carefully retracted to permit reperfusion (>80%) and the right external carotid artery stump was ligated. Reperfusion was also confirmed by laser Doppler flowmetry. The regional cerebral blood flow during and after the surgical procedure was measured relative to the pre-surgical procedure regional cerebral blood flow.

The laser Doppler flowmetry flexible probe was carefully removed, both wounds sutured, and a local anaesthetic (0.5% bupivacaine: 6mg/kg) was applied topically to the wounds. The mice were then returned to their cages and allowed to recover.

The sham surgeries were identical to as set out above insofar as the right external carotid artery and right common carotid were visualized, but the arteriotomy and introduction of the 6-0 nylon monofilament was not performed.

\subsection{7.5.1.3 Neurological Assessment}

The mice were monitored for neurological abnormalities according to the following scale:

1. No abnormalities;
2. A tendency to circle, i.e., turn toward the left as they walk, due to the stroke damage on the right side of the brain;
3. Crouched on all fours and unresponsive to external stimuli; and
4. Spinning.

The mice were closely monitored for 8 hours post surgery. Mice that were observed to crouch on all fours and became unresponsive to external stimuli or spin were humanely killed. Mice that showed no neurological abnormalities consistent with 50 minutes of transient focal cerebral ischaemia were excluded from the study.
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7.5.1.4 Animal Tissue Collection

Twenty four hours after the surgery was initiated, the mice were terminally anaesthetised by intraperitoneal injection of sodium pentobarbital (150 mg/kg). Once the foot withdrawal reflex was absent, the deeply anaesthetised mice were decapitated using a pair of surgical scissors. Each brain was rapidly dissected from the cranial vault according to a previously described method, sagitally separated into ipsilateral (right) and contralateral (left) hemispheres, and immediately frozen in liquid nitrogen. The ipsilateral hemisphere was the brain hemisphere that was subjected to transient focal ischaemia. The separated brain hemispheres were stored at -80°C until required.

7.5.1.5 Total RNA Extraction

Total RNA extraction was performed as set out previously in Section 5.4.2 - Total RNA Extraction on page 86.

7.5.2 Amyotrophic Lateral Sclerosis

7.5.2.1 Animals and Housing Conditions

Wild type (control) and hSOD1\textsuperscript{G93A} transgenic B6.Cg-Tg(SOD1-G93A)1Gur/J\textsuperscript{550} male mice on the same background were used in this study. The mice were housed under standard temperature- and light-controlled conditions, i.e., ∼22°C and 12 hours light/12 hours dark, with free access to food and water prior to the collection of tissues.

7.5.2.2 Neurological Assessment

The hSOD1\textsuperscript{G93A} mice were monitored for the neurological symptoms associated with disease progression. The stages of development of the neurological symptoms were defined as set out in Table 7.1 on the next page.

Wild type (control) mice were age-matched for each symptomatic stage. Symptomatic hSOD1\textsuperscript{G93A} mice were provided with readily accessible wet food (standard chow) and water, i.e., located on the floor of their cage. hSOD1\textsuperscript{G93A} mice that had progressed to loss of righting reflex were humanely killed.
Table 7.1: Stages of development of clinical symptoms of hSOD1<sup>G93A</sup> mice.\textsuperscript{548}

<table>
<thead>
<tr>
<th>Stage</th>
<th>Age</th>
<th>Clinical Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-symptomatic</td>
<td>30 days</td>
<td>No detectable clinical symptoms</td>
</tr>
<tr>
<td>Onset</td>
<td>70 days</td>
<td>Signs of hindlimb weakness, slight hindlimb tremor, and decreasing hindlimb grip strength</td>
</tr>
<tr>
<td>Mid-stage</td>
<td>130 days</td>
<td>Moderate hindlimb weakness and moderate hindlimb tremor</td>
</tr>
<tr>
<td>End-stage</td>
<td>150—175 days</td>
<td>Significant hindlimb weakness, hindlimb paralysis, and loss of the righting reflex, i.e., inability to right itself within thirty seconds after being placed in supine position</td>
</tr>
</tbody>
</table>

7.5.2.3 Animal Tissue Collection

The mice were deeply anaesthetised by intraperitoneal injection of sodium pentobarbital (150 mg/kg). Once the foot withdrawal reflex was absent, the deeply anaesthetised mice were decapitated using a pair of surgical scissors. Each brain was rapidly dissected from the cranial vault according to a previously described method.\textsuperscript{363} The motor cortex was collected, immediately frozen in liquid nitrogen, and stored at -80\textdegree C until required.

7.5.2.4 Total RNA Extraction

Total RNA was isolated from mouse motor cortex using the TRIzol\textsuperscript{TM} reagent\textsuperscript{437} according to the manufacturer’s instructions (Appendix R: Invitrogen\textsuperscript{TM} TRIzol\textsuperscript{TM} Reagent Protocol on page 297). Briefly, the following protocol was used to isolate the total RNA:

1. Frozen motor cortex was crushed in a mortar and pestle under liquid nitrogen.
2. 1 mL TRIzol\textsuperscript{TM} reagent was added per 50–100 mg of motor cortex and then homogenized using a T10 basic Ultra Turrax\textsuperscript{TM} homogenizer on setting 1 (2 x 10 seconds followed by 2 minutes incubation on ice; repeated 2-3 times).
3. Since brain has a high lipid content, the lysate was centrifuged for 5 minutes at 12,000 x g at 4\textdegree C. Centrifugation facilitates removal of the lipid component of the sample, which improves the total RNA yield.
4. The clear supernatant was transferred to a new tube.
5. The supernatant was incubated at room temperature for 5 minutes to permit complete dissociation of the nucleoproteins.

6. 0.2 mL chloroform was added per mL TRIzol\textsuperscript{TM} reagent used for lysis, the tube was securely sealed.

7. The chloroform/supernatant solution was vigorously shaken for 15 seconds and then incubated at room temperature for 3 minutes.

8. The chloroform/supernatant solution was centrifuged for 15 minutes at 12,000 x \( g \) at 4\(^\circ\)C.

9. The colourless upper aqueous phase was transferred to a new tube, taking care not to disturb the interphase.

10. 0.5 mL isopropanol was added per mL TRIzol\textsuperscript{TM} reagent used for lysis and gently mixed with the aqueous phase.

11. The isopropanol/aqueous phase solution was incubated at room temperature for 10 minutes and then centrifuged for 15 minutes at 12,000 x \( g \) at 4\(^\circ\)C.

12. The supernatant was carefully removed from the total RNA pellet, i.e., a white gel-like pellet.

13. The total RNA pellet was resuspended in 1 mL 75\% ethanol per mL TRIzol\textsuperscript{TM} reagent used for lysis by gently pipetting up and down.

14. The total RNA/75\% ethanol solution was gently mixed in a vortex mixer and then centrifuged for 5 minutes at 7,500 x \( g \) at 4\(^\circ\)C.

15. The supernatant was carefully removed from the total RNA pellet.

16. The total RNA pellet was allowed to air dry for 10 minutes.

17. The total RNA pellet was resuspended in 20 \( \mu \)L ultrapure-RNase-free water by gently pipetting up and down.

18. The resuspended total RNA was incubated in a heat block set at 60\(^\circ\)C for 10 minutes.

19. Total RNA was aliquoted out and stored at -80\(^\circ\)C until required.

### 7.5.3 RNA Concentration and Integrity

RNA concentration and integrity was determined using a NanoDrop\textsuperscript{TM} 2000 spectrophotometer according to the manufacturer’s instructions (Appendix M: ThermoFisher Scientific NanoDrop\textsuperscript{TM} 2000 Spectrophotometer Operating Instructions on page 266).
7.5.4 Reverse Transcription

Reverse transcription was performed as set out previously in Section 5.4.4 - Reverse Transcription on page 87.

7.5.5 Quantitative PCR

Quantitative PCR was performed as set out previously in Section 5.4.7 - Quantitative PCR on page 90.

7.5.6 Protein Extraction

Protein extraction was performed as set out previously in Section 5.4.8 - Protein Extraction on page 91.

7.5.7 Protein Assay

Protein assay was performed as set out previously in Section 4.3.5.3 - Protein Assay on page 55.

7.5.8 Protein Sample Preparation

Protein samples were prepared as set out previously in Section 4.3.5.4 - Protein Sample Preparation on page 55.

7.5.9 Western Blotting

Western blotting was performed as set out previously in Section 4.3.5.6 - Optimized Western Blotting Method on page 60. Prior to immunodetection, Ponceau S staining was performed to assess consistency of the amount of protein loaded per lane and to confirm the efficiency of transfer (see page 58).

7.5.10 Statistical Analysis

Statistical analysis for this chapter was performed as set out previously in Section 5.4.13 Statistical Analysis on page 93.
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7.6 Results

7.6.1 Cerebral Ischaemia

Quantitative PCR was used to investigate the change in expression of GLT-1a and GLT-1d mRNA following transient focal cerebral ischaemic insult and reperfusion. Brains that had been subjected to transient focal cerebral ischaemic insult and reperfusion were compared with sham-surgery control brains. After 50 minutes of middle cerebral artery occlusion and \( \sim 23 \) hours and 10 minutes reperfusion, downregulation of GLT-1d mRNA was observed in the ipsilateral hemisphere when compared to either the contralateral hemisphere or the sham-surgery control (Figure 7.1 A on page 144). Similarly, the GLT-1d mRNA of the contralateral hemisphere was downregulated relative to the sham-surgery control, but to a lesser extent than observed for the ipsilateral hemisphere (Figure 7.1 A).

Intriguingly, no change was observed for GLT-1a mRNA in both the ipsilateral and contralateral hemispheres after 50 minutes of middle cerebral artery occlusion and \( \sim 23 \) hours and 10 minutes reperfusion (Figure 7.2).

Western blotting was used to investigate the change in expression of GLT-1d protein following transient focal cerebral ischaemia and reperfusion. Brains that had been subjected to transient focal cerebral ischaemia were compared to sham-surgery control brains. After 50 minutes of middle cerebral artery occlusion and \( \sim 23 \) hours and 10 minutes reperfusion, GLT-1d protein was downregulated in the ipsilateral hemisphere compared to the sham-surgery control (Figures 7.1 B and 7.1 C on page 144). A modest downregulation of GLT-1d protein was also observed in the contralateral hemisphere when compared to the sham-surgery control, but again to a lesser extent than observed for the ipsilateral hemisphere.

No significant difference in GLT-1d mRNA and protein was observed when the two hemispheres of the sham-surgery control brains were compared (data not shown).

7.6.2 Amyotrophic Lateral Sclerosis

Quantitative PCR (qPCR) was used to investigate the change in expression of GLT-1d mRNA in hSOD1<sup>G93A</sup> mouse motor cortex over various stages of development of amyotrophic lateral sclerosis-like symptoms (age in brackets): pre-symptomatic (30 days), onset (70 days), mid-stage (130 days), and end-stage (150—175 days). Symptomatic motor cortices were compared to appropriate age-matched wild type (control) motor cortices for each stage. The neurological assessment of the progression of the disease
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A GLT-1d mRNA expression

B GLT-1d protein expression

C Composite GLT-1d western blot

Figure 7.1: Effect of transient middle cerebral artery occlusion on the expression of GLT-1d mRNA and protein in mouse brain. GLT-1d mRNA [A] and protein [B] expression was determined in ipsilateral [tMCAO (i)] and contralateral [tMCAO (c)] mouse brain hemispheres by quantitative PCR (qPCR) and western blotting as set out in the Experimental Methods. Each bar represents the mean ± SEM obtained for four mice. [C] Representative composite western blots probed with GLT-1d antiserum (1:100,000) for ipsilateral (Ipsi) and contralateral (Contra) mouse brain hemispheres as set out in the Experimental Methods. Molecular weights (kDa) are indicated to the left of the western blots [C]. Sample lanes = 2.5 µg whole brain protein/lane. ** P ≤ 0.01, significantly different from sham. The statistical significance among different experimental groups was determined by one-way ANOVA followed by Dunnett’s multiple comparisons test. Abbreviations: MW, molecular weight markers; tMCAO (i), transient middle cerebral artery occlusion (ipsilateral hemisphere); and tMCAO (c), transient middle cerebral artery occlusion (contralateral hemisphere).
in the hSOD1^{G93A} mice and the clinical symptoms observed were assessed as set out previously (Table 7.1 on page 140). The hSOD1^{G93A} mice exhibited clinical symptoms that correlated with the onset and progression of disease, as well as decreased weight gain relative to age-matched wild type (control) mice (data not shown).

Interestingly, the expression of both GLT-1a and GLT-1d mRNA was downregulated in hSOD1^{G93A} motor cortex at the onset of symptoms stage relative to the pre-symptomatic stage in the hSOD1^{G93A} motor cortex (Figure 7.3). At mid-stage and end-stage, GLT-1a and GLT-1d mRNA expression was upregulated in the hSOD1^{G93A} motor cortex relative to the immediately preceding stage, with the highest level of expression detected in the end-stage hSOD1^{G93A} motor cortex. Both GLT-1a and GLT-1d mRNA expression was relatively stable for all age-matched wild type (control) motor cortices. Interestingly, with the exception of the onset of symptoms stage, the GLT-1a and GLT-1d mRNA expression in hSOD1^{G93A} motor cortex was higher than that observed in wild type (control) motor cortex for all other stages.

### 7.7 Discussion

Loss of GLT-1 been reported with respect to both acute and chronic neurodegenerative diseases. Furthermore, the relative expression levels of the known C-terminal GLT-1 splice variants, i.e., GLT-1a, GLT-1b, and GLT-1c, are known to differ in pathological brains relative to healthy brains. Accordingly, two animal models were used in this preliminary study to investigate the expression of GLT-1d in pathological brain: a transient focal cerebral ischaemia and reperfusion mouse model (an...
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Figure 7.3: Expression of [A] GLT-1a and [B] GLT-1d mRNA in hSOD1<sup>G93A</sup> (SOD1) and wildtype (Control) mouse motor cortex. Each bar represents the mean ± SEM obtained for three mice. ** P ≤ 0.01 and *** P ≤ 0.001, significantly different from PSC. The statistical significance among different experimental groups was determined by one-way ANOVA followed by Dunnett’s multiple comparisons test. Abbreviations: pre-symptomatic SOD1, PSS; pre-symptomatic control, PSC; onset SOD1, OSS; onset control, OSC; mid-stage SOD1, MSS; mid-stage control, MSC; end-stage SOD1, ESS; and end-stage control, ESC.

Acute pathological condition) and the hSOD1<sup>G93A</sup> amyotrophic lateral sclerosis mouse model (a chronic pathological condition).

7.7.1 Cerebral Ischaemia

Cerebral ischaemic insult leads to excitotoxic levels of glutamate in the synaptic cleft and extracellular space and consequential enhanced activation of glutamate receptors. The enhanced activation of glutamate receptors in turn leads to neuronal damage or cell death. Several studies have underscored the importance of GLT-1 dysfunction in the neuronal damage or cell death that are consequences of cerebral ischaemic insult. In fact, irrespective of animal species, hypoxic-ischaemia,
global ischaemia,\textsuperscript{486,487,559–563} and transient focal ischaemia\textsuperscript{484,485,489,564} have all been associated with the downregulation of GLT-1 expression.

The present study demonstrated that transient focal cerebral ischaemia (50 minutes) followed by reperfusion (\(\sim 23\) hours and 10 minutes) was associated with the downregulation of GLT-1d mRNA and protein expression in the ipsilateral hemisphere relative to the sham-surgery control (Figure 7.1 on page 144). Consistent with the GLT-1d downregulation in the ipsilateral hemisphere, a number of studies using global ischaemia models have shown that GLT-1 mRNA and protein expression was downregulated in the hippocampus, particularly CA1, by 24 hours post-reperfusion.\textsuperscript{482,484,486,487,559} Interestingly, however, the present study showed that GLT-1d mRNA and protein expression was also downregulated in the contralateral hemisphere, albeit to a more modest extent than observed in the ipsilateral hemisphere (Figure 7.1 A). Downregulation of the GLT-1d expression in the contralateral hemisphere suggests that transient ischaemia in the ipsilateral hemisphere influenced GLT-1d expression in the contralateral hemisphere within 24 hours post-reperfusion. Previous studies have demonstrated that following ischaemia GLT-1 expression is downregulated in the hippocampal CA1 region in the early stages of reperfusion following global ischemia, i.e., within 12 hours of reperfusion.\textsuperscript{486,561} The present study demonstrated that GLT-1d downregulation was detectable in mouse brain at \(\sim 23\) hours and 10 minutes post-reperfusion. It is plausible, therefore, that downregulation of GLT-1d expression was associated with neuronal dysfunction that occurs shortly after ischaemic insult due to oxidative stress and excitotoxicity,\textsuperscript{234,565–568} i.e., prior to neuronal death.\textsuperscript{484} It is plausible that the downregulation of GLT-1d, at least in the ipsilateral hemisphere, was associated with neuronal damage that occurs shortly after ischaemic insult due to oxidative stress and excitotoxicity.

Downregulation of GLT-1 expression in target regions has been associated \textit{in vivo} with both deafferentation and lesioning of projection neurons.\textsuperscript{569–572} It is plausible that the ischaemic injury may have led to degeneration of intercortical glutamatergic neurons projecting from an injured area of the ipsilateral hemisphere into the contralateral hemisphere (Figure 7.4 on page 149). The degeneration of these projection neurons may in turn have led to a reduction in the secretion of neurotrophic factor(s) by these projection neurons in the target regions.\textsuperscript{238–249} Neurotrophic factors including growth factors and peptides such as endothelins, BDNF, EGF, FGF-2, PACAP, TGF\(\alpha\), and TNF\(\alpha\) are known to modulate GLT-1 expression.\textsuperscript{238–249,573} Interestingly, decreased levels of BDNF and its receptor TrkB mRNA were observed in the contralateral hemisphere in a focal cerebral ischaemia animal model.\textsuperscript{574} It is possible, therefore, that changes in expression of any one or more of these growth factors and peptides in the contralateral hemisphere, triggered by the degeneration of intercortical glutamatergic neurons projecting from the injured area of the ipsilateral hemisphere into the contralateral hemisphere, led to the
reduction in GLT-1d expression in the contralateral hemisphere. Such a reduction in GLT-1d expression was consistent with previous reports of glutamate transporter expression being modulated by the secretion of such neurotrophic factors. The reduction in GLT-1d expression in the contralateral hemisphere observed here was consistent with the reduction of GLT-1 expression in the contralateral hemisphere observed in rats under similar experimental conditions. In that study, the reduction in GLT-1 expression in the contralateral hemisphere was suggested to be due to a reduction in PACAP secretion by the intercortical glutamatergic neurons projecting from the injured area of the ipsilateral hemisphere into the contralateral hemisphere. It is also possible that the decline in GLT-1a mRNA and protein expression observed in the contralateral hemisphere may be related to a reduction in the GLT-1d mRNA and/or protein in the intercortical projection neurons projecting from the injured ipsilateral hemisphere into the contralateral hemisphere. In this paradigm, in a healthy brain, GLT-1d mRNA produced in the intercortical projection neurons projecting from the ipsilateral hemisphere into the contralateral hemisphere would be transported into the terminals in the contralateral hemisphere for local translation, whereas the protein would be transported into/or produced locally in the terminals to fulfill its function, i.e., glutamate transport. Following the ischaemic insult, this transport would be impaired or terminated. Such impairment or termination would lead to a reduction, potentially modest, in the GLT-1d mRNA and protein in the target region in the contralateral hemisphere. On the other hand, the reduction in the expression of GLT-1d in the ipsilateral hemisphere would be due to a combination of the dysfunction of the local glutamatergic neurocircuitry as well as dysfunction within/associated the terminals of the intercortical glutamatergic neurons projecting into the ipsilateral hemisphere. Consequently, the reduction in GLT-1d expression in the ipsilateral hemisphere would be more substantial than observed in the contralateral hemisphere, as was observed here (Figure 7.1 on page 144).

It will be appreciated that a decline in protein expression typically exhibits a lag phase relative to the decline in mRNA expression. It is plausible, therefore, that a longer post-reperfusion period will be associated with a more substantial decline in GLT-1d protein expression in the contralateral hemisphere than was observed here (Figure 7.1 on page 144).

Interestingly, no change in the GLT-1a mRNA expression was detected in both the ipsilateral and the contralateral hemispheres. One possible explanation for this finding may be related to the cellular distribution of GLT-1a and GLT-1d. As mentioned previously, GLT-1a, is predominately expressed in astrocytes, whereas GLT-1d was shown to be expressed in both neurons and astrocytes in adult mouse brain (Chapter 6 of this thesis). It is plausible, therefore, given the substantial differences in sensitivity of
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• Ipsilateral projection neuron
• Columnar neuron
• Callosal projection neuron: ipsilateral-contralateral
• Callosal projection neuron: contralateral-ipsilateral

**Figure 7.4:** Schematic illustration of selected neurons of the mouse neocortex. The illustrated neurons include columnar, ipsilateral projection, callosal projection: ipsilateral-contralateral, and callosal projection: contralateral-ipsilateral neurons. The columnar and ipsilateral projection neurons project locally within the same hemisphere, whereas the callosal projection neurons project across the midline into the opposite hemisphere. Modified from Figure 1 of Franco and Müller with permission under copyright held by Elsevier.

neurons and astrocytes to glutamate that the neuronal and astrocytic GLT-1d may exhibit different kinetics in response to cerebral ischaemic insult relative to the predominantly astrocytic GLT-1a. In contrast to the present study, previous studies have shown downregulation of GLT-1a expression. The findings of the present study relating to no change being observed for GLT-1a mRNA expression may be due to the mild cerebral ischaemic insult induced during this study, i.e., no seizures were observed in the mice of the present study, which is in stark contrast to the more severe insults of previous studies. In effect, the sensitivity of GLT-1d to such a mild cerebral ischaemic insult is an important finding.

### 7.7.2 Amyotrophic Lateral Sclerosis

Decreased GLT-1 protein is a key feature of disease progression in human amyotrophic lateral sclerosis (ALS) patients, as well as SOD1 rodents. Previous studies in human patients have shown a significant decrease in GLT-1 (EAAT2) protein in the motor cortex and spinal cord in a majority of the patients. Similarly,
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SOD1 animal models of ALS exhibit decreased GLT-1 protein levels.\textsuperscript{491,492,543} Interestingly, differences in the expression profiles of GLT-1 splice variants were observed in \textit{in vivo} and \textit{in vitro} ALS animal model studies.\textsuperscript{297,556,580,581} In human ALS patients, the motor cortex also exhibits differential regulation of EAAT2,\textsuperscript{556} the human homolog of GLT-1. Interestingly, changes in mRNA expression were also reported for 5’ GLT-1 splice variants in an hSOD1\textsuperscript{G93A} mouse model.\textsuperscript{295} Such changes in mRNA expression suggest that GLT-1a and GLT-1b,\textsuperscript{581} and 5’ GLT-1 splice variants\textsuperscript{295} may be subject to differential regulation during the early stages of disease progression in the hSOD1\textsuperscript{G93A} mouse model.\textsuperscript{295,581} Thought-provokingly, the studies were typically end-stage focussed and, as such, would not detect changes in the GLT-1 expression profile over the course of disease progression. Accordingly, the present preliminary study investigated the effect of disease progression, i.e., from pre-symptomatic (30 days) to end-stage (150–175 days), on the expression profile of GLT-1d mRNA in hSOD1\textsuperscript{G93A} mouse motor cortex.

The present study demonstrated that GLT-1a and GLT-1d mRNA expression was subject to biphasic regulation over the course of disease progression in hSOD1\textsuperscript{G93A} mouse motor cortex (Figure 7.3 B on page 146). From pre-symptomatic (30 days) to onset (70 days), GLT-1a and GLT-1d mRNA expression was observed to decrease, but from disease onset (70 days) to end-stage (150–175 days) GLT-1a and GLT-1d mRNA increased in expression. It is possible that the elevated GLT-1a and GLT-1d mRNA expression observed in the pre-symptomatic hSOD1\textsuperscript{G93A} mouse motor cortex relative to the age-matched wildtype may be a compensatory phase during which neurons were more active in a hostile environment. It is plausible that the neurons in the hSOD1\textsuperscript{G93A} mouse motor cortex were already more active in the pre-symptomatic stage than in the age-matched wildtype control. Support for the possibility of such a compensatory phase of GLT-1a and GLT-1d mRNA expression was observed in a study relating to an exon-9 skipping splice variant of the excitatory amino acid transporter GLAST, GLAST1b.\textsuperscript{582} This study demonstrated that neurons that were stressed in a hostile environment up-regulated GLAST1b expression in response to glutamate dyshomeostasis. The marked decline in GLT-1a and GLT-1d mRNA expression as the hSOD1\textsuperscript{G93A} mouse motor cortex neurons approached the symptomatic stage, i.e., onset stage, may have been due to the neurons dying. The increase in GLT-1a and GLT-1d mRNA expression from disease onset (70 days) to end-stage (150–175 days) may potentially be a further compensatory phase during which the remaining neurons endeavoured to restore glutamate homeostasis in their immediate environment. In hSOD1\textsuperscript{G93A} rat fronto-temporal cortex, GLT-1a mRNA decreased in expression and GLT-1b mRNA increased in expression from pre-symptomatic (60 days) to adult (120 days).\textsuperscript{581} In that study, GLT-1a and GLT-1b mRNA expression remained relatively stable from adult (120 days) to end-stage (195 days).\textsuperscript{581} It is regrettable that Dumont \textit{et al.}\textsuperscript{581} did not report the expression levels of
GLT-1a and GLT-1b mRNA at an earlier stage, i.e., 30 days, as set out in the present preliminary study.

The results of the present preliminary study suggest that both GLT-1a and GLT-1d mRNA expression may be differentially regulated in the hSOD1\textsuperscript{G93A} mouse motor cortex over the course of disease progression. It is plausible that differential regulation of GLT-1a and GLT-1d mRNA expression during the early stage of disease progression may be associated with the development of disease in hSOD1\textsuperscript{G93A} mouse motor cortex. A deeper understanding of the regulation of GLT-1d throughout the course of disease progression, particularly the early stages, would clarify what role, if any, GLT-1d may play in the development of disease to end-stage in hSOD1\textsuperscript{G93A} mouse motor cortex. Future studies on the effect of disease progression on GLT-1d expression in hSOD1\textsuperscript{G93A} mouse motor cortex, brain stem, and spinal cord should hopefully expand our understanding of GLT-1d and its association with ALS.

Mutations in the \textit{SOD1} gene, i.e., gain of function, leads to the production of hydrogen peroxide, which impairs GLT-1 function.\textsuperscript{24} Impaired GLT-1 function may be due to the susceptibility of GLT-1 protein to oxidation,\textsuperscript{545} which may result in the loss of GLT-1 protein as observed in both human patients,\textsuperscript{490} as well as SOD1 ALS animal models.\textsuperscript{319,492,543} Patently, the effect of disease progression on the GLT-1d protein expression profile in hSOD1\textsuperscript{G93A} mouse motor cortex requires investigation.

### 7.8 Significance Statement

This study indicated that GLT-1d mRNA expression was more sensitive to a mild cerebral ischaemic insult that GLT-1a mRNA expression in mouse brain. This study also indicated both acute and pathological conditions in mouse brain was associated with differential regulation of GLT-1d expression, which strongly suggests that future studies investigate GLT-1d as a potential therapeutic target.

### 7.9 Further Observations

Observations regarding this study, and its integration into achieving the aim of the research for this thesis, are set out in Chapter 8.
7.10 Future Studies

Future studies will examine the expression and distribution of GLT-1d mRNA and protein within the 24 hours post-reperfusion and for at least 72 hours post-reperfusion to provide a more comprehensive understanding of the effect of cerebral ischaemic insult on GLT-1d expression and distribution. Future studies will also examine GLT-1d protein expression and distribution in hSOD1\textsuperscript{G93A} mouse motor cortex and spinal cord over the course of disease progression.

7.11 Key Points

This study was undertaken to examine the expression of GLT-1d in an acute pathological condition, i.e., transient focal cerebral ischaemic insult and reperfusion, and a chronic pathological condition, i.e., amyotrophic lateral sclerosis, in mouse models of the respective pathological conditions. In the course of this study, the following key points were revealed:

✴ Downregulation of GLT-1d mRNA expression was detectable in brain at 24 hours post-reperfusion and may be associated with neuronal damage due to oxidative stress and excitotoxicity that occurs shortly after ischaemic insult.

✴ Expression of GLT-1a mRNA did not appear to be affected at 24 hours post-reperfusion under the present experimental conditions and requires further investigation.

✴ Downregulation of GLT-1d expression in the contralateral hemisphere suggests that transient ischaemia in the ipsilateral hemisphere influenced GLT-1d expression in the contralateral hemisphere within 24 hours post-reperfusion.

✴ Importantly, GLT-1d mRNA expression was more sensitive to a mild cerebral ischaemic insult than GLT-1a mRNA expression.

✴ GLT-1a and GLT-1d mRNA expression may be subject to biphasic regulation over the course of disease progression in hSOD1\textsuperscript{G93A} mouse motor cortex.

✴ Downregulation of GLT-1a and GLT-1d mRNA expression during the early stage of disease progression may be associated with the development of disease in hSOD1\textsuperscript{G93A} mouse motor cortex.
Future studies on GLT-1d expression in pathological conditions in the central nervous system, i.e., brain and spinal cord, may expand our understanding of GLT-1d as a potential therapeutic target.
Part IV

Summation of the Present Studies
Final Observations and Principal Findings

The research carried out for this thesis focused on examining the expression and distribution of a novel GLT-1 C-terminal splice variant, GLT-1d, in juvenile, adult, and pathological mouse brains. Final observations and the principal findings of the studies for this thesis are set out below.

The *raison d’être* for the first study of this thesis was simple: GLT-1d was a newly discovered excitatory amino acid transporter (EAAT). Understandably, given the novelty of GLT-1d, GLT-1d specific antibodies/antisera were not available. Accordingly, to investigate the expression and distribution of GLT-1d protein in mouse brain, GLT-1d specific antibodies/antisera had to be produced.

Typically, pure antigens are preferred as immunogens. In this case, however, pure antigen was not a suitable option as immunogen as this would most likely produce antisera that would recognise all of the GLT-1 splice variants. Furthermore, given the homology of the amino acid sequence of the other excitatory amino acid transporters (EAATs) and alanine serine cysteine threonine transporters (ASCTs), antisera produced using pure antigen as immunogen would also recognise the other EAATS and the ASCTs. In effect, antibodies/antisera produced using pure GLT-1d protein as immunogen would have been non-specific and hence not a useful research tool to investigate GLT-1d in mouse
Chapter 8: Final Observations and Principal Findings

brain. The alternative was to use synthetic peptides that corresponded to the unique C-terminal amino acid sequences of rat and human GLT-1d identified during preliminary investigations (Chapter 3: Preliminary Study - Discovering GLT-1d). Accordingly, the first study of this thesis (Chapter 4: Production and Validation of Antisera to Detect GLT-1d in Mouse Brain) investigated whether synthetic peptides that corresponded to unique C-terminal amino acid sequences of rat and human GLT-1d could be used to produce antisera and, if so, whether the produced antisera were useful in the investigation of GLT-1d expression and distribution in mouse brain. This study established that the antisera produced—designated Rb 1713, Rb 1714, Rb 1715, and Rb 1716 antisera—could be used to detect the relevant synthetic peptides used to generate the relevant antisera. Based on the results of dot blotting and preliminary western blotting experiments, one of the antisera, Rb 1713 antiserum, was preferred for use in western blotting and immunohistochemistry to investigate the expression and distribution of GLT-1d in mouse brain.

Interestingly, the first study disclosed that heating samples prior to western blotting led to a ~50 kDa downward shift in the apparent molecular weight of a higher molecular weight oligomer form of GLT-1d detected by the Rb 1713 antiserum. One possible explanation for this downward shift was the heat-induced dissociation of a potential protein partner that was associated with a higher molecular weight oligomer form of GLT-1d. The potential protein partner that dissociated from the higher molecular

The first study therefore established that synthetic peptides that corresponded to the unique C-terminal amino acid sequences of rat and human GLT-1d could be used to produce antisera that would be useful in the investigation of GLT-1d expression and distribution in mouse brain.
weight oligomer form of GLT-1d was initially thought to be PICK1 based on PICK1’s known interaction with GLT-1b via a PDZ motif and its known molecular weight of ∼50 kDa. GLT-1b contains a class I PDZ motif, whereas GLT-1d (both rat and human forms) contain a putative class II PDZ motif. Nonetheless, PICK1 is known to interact promiscuously with all three classes of PDZ motifs, which meant that PICK1 could not simply be discounted. At least in vitro support for PICK1 being a potential protein partner was recently provided in a pull-down experiment performed by Dr Aven Lee. In order to understand GLT-1d and its potential interaction with other proteins, future studies are required to identify protein partners that bind to GLT-1d via the PDZ motif.

The limited animal facilities of RMIT University represented a challenge to this study. With access to more extensive animal facilities, the production of the antisera would have been carried out in more rabbits than the four in the present case, i.e., more sets of antisera would have been produced. Furthermore, production would have been more extensive, i.e., the rabbits would not have been culled in general, rather those producing the most effective antisera would have been maintained.

The second study of this thesis (Chapter 5: GLT-1d in Juvenile Mouse Brain) investigated the temporal expression and distribution profile of GLT-1d in juvenile mouse brain. Interestingly, this study established that GLT-1a, GLT-1b, GLT-1d, GLAST, glutamine synthetase, and VGLUT1 exhibited expression profiles that were broadly consistent during postnatal mouse brain development. The consistency in the patterns of these expression profiles suggested a potential interrelationship between these members of the glutamate homeostasis system in juvenile mouse brain.

An important finding of this study was that GLT-1d protein was expressed in astrocytes in juvenile mouse brain. The importance of this finding became apparent during the investigation of the cellular distribution of GLT-1d protein in adult mouse brain (Chapter 6: GLT-1d in Adult Mouse Brain). The increase in GLT-1d expression during early postnatal mouse brain development and maturation suggested that there was an increasing demand for GLT-1d activity. Another important finding of this study was that the increase in GLT-1d expression—and GLT-1a, GLT-1b, GLAST, glutamine synthetase, and VGLUT1—was largely coincident with important events that occur in the first weeks of postnatal mouse brain development, namely gliogenesis, synaptogenesis, an increase in glutamate uptake, an increase in AMPA receptor-mediated activity and a transition to mature forms and expression levels of glutamate receptors, and myelination. The consistency of these patterns of expression and the coincidence of the above-mentioned important events with the increase in GLT-1d expression emphasised the possibility that GLT-1d may be associated with the onset of and ensuing glutamatergic neurotransmission in juvenile mouse brain.
The second study therefore established that GLT-1d exhibited a temporal expression and distribution profile in juvenile mouse brain that was coordinated with the expression profiles of GLT-1a, GLT-1b, GLAST, glutamine synthetase, and VGLUT1. Importantly, this study also established that the GLT-1d cellular distribution was astrocytic in juvenile mouse brain. Another important finding of this study was that the increase in GLT-1d expression coincided with important events associated with brain development in mouse brain.

A comprehensive immunohistochemical investigation of the distribution of the GLT-1 splice variants, GLT-1a–d, would have benefited this study, especially with regard to understanding the role of GLT-1d in proliferation, migration, differentiation, and synapse formation. Given that GLT-1 expression has been observed during embryogenesis, this study would have benefited had the distribution and expression of GLT-1d also been investigated during embryogenesis.

The third study of this thesis (Chapter 6: GLT-1d in Adult Mouse Brain) investigated the spatial expression and distribution profile of GLT-1d in adult mouse brain. The study demonstrated that GLT-1d mRNA and protein were broadly coincident with respect to their regional distribution in adult mouse brain. The study also demonstrated that GLT-1d protein expression was highest in the forebrain, moderately expressed in the midbrain and hindbrain, with the lowest level detected in the cerebellum. Regionally within the adult mouse brain, GLT-1d mRNA was highly abundant in the neocortex, caudate putamen, hippocampus, hypothalamus and thalamus, and olfactory bulb, moderately abundant in the inferior colliculus and the optic and auditory vestibular nerves, and present at a low level in the cerebellum, which was broadly consistent with the distribution of the mRNA of GLT-1a and GLT-1b. Importantly, this study established that GLT-1d protein expression underwent a developmental transition from astrocytic expression in the juvenile mouse brain to neuronal and astrocytic expression in the adult mouse brain. Interestingly, the optic and auditory vestibular nerves and white matter of the cerebellum exhibited high levels of GLT-1d expression. The high level of GLT-1d expression in the white matter of the cerebellum, despite the cerebellum having the lowest GLT-1d expression level in general, pointed to a possible role for GLT-1d protein in white matter tracts, which warrants further investigation. Such investigation may consider GLT-1d as a potential therapeutic target for neurodegenerative disease associated with white matter tracts, e.g., spinal injury and Alzheimer’s disease.

The unexpected switch to neuronal and astrocytic GLT-1d protein expression in adult mouse brain is intriguing in light of the ongoing debate surrounding neuronal GLT-1 expression in adult and normal brain. The detection of GLT-1d protein expression
in normal adult mouse neurons may add a further element to the efforts to resolving the so-called "conundrum" alluded to earlier in this thesis. As mentioned previously (Section 2.7.4 - Neuronal Expression of GLT-1 on page 27), despite a concerted effort on the part of several laboratories around the world, until now evidence of GLT-1 protein in the neurons of normal adult brain has been considered elusive. The reasons for the lack of success in identifying a neuronal GLT-1 include the existence of splice variants not recognised by then existing antibodies, although given the vast array of antibodies specific to various epitopes within the known GLT-1 proteins, this would appear to be unlikely. An important question that arises when considering the expression and distribution of GLT-1 protein in adult neurons, in particular presynaptic neurons, is what physiological role GLT-1 protein may play in this location. Expression of GLT-1 in presynaptic neurons appears to be in conflict with the canonical view that the glutamate-glutamine cycle is responsible for the transport of glutamate (in the form of glutamine) into presynaptic neurons. Considering the elusiveness of the identity of a neuronal GLT-1 to date and what role such a neuronal GLT-1 may play in presynaptic neurons, it is critical that the expression of GLT-1d protein in adult mouse brain neurons be further investigated. Such investigation should include knockout animals to confirm expression and distribution. The global GLT-1 homozygote knockout mouse represents a problem to studying GLT-1d protein expression in adult mouse brain as juveniles of this knockout mouse are subject to high levels of mortality and typically do not survive into adulthood due to intractable epileptic seizures. Alternative conditional knockout mice have also been produced. Of these conditional knockout mice, only the Petr et al. mice have a normal lifespan into adulthood and, consequently, would be useful in further investigation relating to the expression and distribution of GLT-1d in adult mouse brain. Such mice would also be beneficial with respect to the investigation of the expression and distribution of GLT-1d in juvenile mouse brain, i.e., the second study of this thesis (Chapter 5: GLT-1d in Juvenile Mouse Brain).

The third study therefore established that the spatial expression and distribution profiles of GLT-1d mRNA and protein were broadly coincident in terms of regional distribution, and that GLT-1d protein expression was highest in the forebrain, and decreased along the neuraxis to the lowest level in the cerebellum. The most important finding of this study was that GLT-1d protein expression underwent a developmental transition from astrocytic expression in the juvenile mouse brain to neuronal and astrocytic expression in the adult mouse brain.

A comprehensive immunohistochemical investigation comprising confocal and electron-microscopy GLT-1d immunolabelling studies including markers for neurons, astrocytes,
Chapter 8: Final Observations and Principal Findings

and oligodendrocytes in cell-type-specific immunolabelling experiments would have benefited this study. Similarly, counterstaining experiments that would elucidate the localisation of GLT-1d protein expression would have expanded the understanding of GLT-1d distribution in the adult mouse brain. Such confocal and electronmicroscopy studies would also have assisted in clarifying the sub-cellular localisation of GLT-1d protein in synaptic terminals in adult mouse brain.

The final study of this thesis (Chapter 7: GLT-1d in Pathological Mouse Brains, a Preliminary Study) investigated the pathological expression profile of GLT-1d in an acute pathological condition and a chronic pathological condition of mouse brain. Two animal models were used in the study to investigate the expression of GLT-1d in pathological mouse brains: a transient focal cerebral ischaemia and reperfusion mouse model (an acute pathological condition) and an hSOD1G93A amyotrophic lateral sclerosis mouse model (a chronic pathological condition).

In the transient focal cerebral ischaemia and reperfusion mouse model, at \(\sim 24\) hours post-reperfusion, GLT-1d expression was downregulated in both the ipsilateral and contralateral hemispheres. Plausibly, the downregulation of GLT-1d expression, at least in the ipsilateral hemisphere, was associated with neuronal damage that occurs shortly after ischaemic insult due to oxidative stress and excitotoxicity. GLT-1d downregulation in the contralateral hemisphere suggested that transient ischaemia in the ipsilateral hemisphere influenced GLT-1d expression in the contralateral hemisphere within 24 hours post-reperfusion. The downregulation of GLT-1d mRNA and protein expression observed in the ipsilateral hemisphere was understandable. The downregulation of GLT-1d mRNA and protein expression, albeit modest, observed in the contralateral hemisphere was, however, intriguing.

Deafferentation and lesioning of projection neurons has been associated with the downregulation of GLT-1 expression in target regions. It is plausible that the injury in the ipsilateral hemisphere resulted in an interruption in afferent connections of intercortical projection neurons projecting into the contralateral hemisphere. It will be appreciated that the cerebral ischaemic insult in the ipsilateral hemisphere would likely injure somata of intercortical glutamatergic neurons projecting into the contralateral hemisphere. Such injury may result in dysfunction within the terminals of the intercortical projection neurons in the contralateral hemisphere. This dysfunction could potentially result in the reduction of GLT-1d mRNA and protein levels in the terminals and/or cells associated with the terminals, and/or changes in the secretion of neurotrophic factors that modulate GLT-1d mRNA and protein expression in cells adjacent to the terminals.

Importantly, this study indicated that GLT1-d mRNA expression was more sensitive to a mild cerebral ischaemic insult than GLT-1a mRNA expression. One possible explanation
for the difference in sensitivity between GLT-1a and GLT-1d mRNA expression to a mild cerebral ischaemic insult may lie in the cellular distribution of these two splice variants. GLT-1a is predominantly expressed in astrocytes, whereas GLT-1d was shown to be expressed in both neurons and astrocytes in adult mouse brain (Chapter 6: GLT-1d in Adult Mouse Brain). It is plausible, given the substantial differences in sensitivity of neurons and astrocytes to glutamate, that the neuronal and astrocytic GLT-1d may exhibit different kinetics, i.e. be more sensitive, in response to a mild cerebral ischaemic insult than the predominantly astrocytic GLT-1a. Such sensitivity of GLT-1d mRNA expression to a mild cerebral ischaemic insult requires further investigation.

In adult mouse brain (Chapter 6: GLT-1d in Adult Mouse Brain), GLT-1d mRNA was highly abundant in the neocortex and caudate putamen, which are areas that are susceptible to cerebral ischaemic injury in human cerebral ischaemia patients. A focused study to investigate the effect of ischaemic insult on GLT-1d protein expression in specific areas of adult mouse brain that are susceptible to cerebral ischaemic injury is required.

In the hSOD1$^{G93A}$ amyotrophic lateral sclerosis mouse model, GLT-1a and GLT-1d mRNA expression was subject to biphasic regulation over the course of disease progression. In effect, the elevated level of GLT-1a and GLT-1d mRNA expression observed in the pre-symptomatic hSOD1$^{G93A}$ mouse motor cortex may have been a compensatory phase during which neurons were more active in a hostile environment. Initially, high levels of GLT-1a and GLT-1d mRNA expression was observed, which was potentially a consequence of the neurons responding to a hostile environment. The decline in GLT-1a and GLT-1d mRNA expression may have been a consequence of neurons dying during the initial stages of symptom development, whereas the subsequent increase in expression could represent a further compensatory phase during which the remaining neurons endeavoured to restore glutamate homeostasis. Mutations in the SOD1 gene are known to result in the production of hydrogen peroxide. These conditions may be associated with GLT-1 dysfunction in both humans and SOD1 animal models. Importantly, GLT-1d protein expression in hSOD1$^{G93A}$ mouse motor cortex requires investigation.
Chapter 8: Final Observations and Principal Findings

The final study therefore demonstrated that both the acute and chronic pathological conditions that were examined were associated with changes in the GLT-1d expression in mouse brain. The important findings of this study were: In the acute pathological condition, i.e., the transient focal cerebral ischaemia and reperfusion mouse model, the reduction in GLT-1d expression observed in the contralateral hemisphere, and the increased sensitivity of GLT-1d mRNA expression to a mild cerebral ischaemic insult relative to GLT-1a mRNA expression. In the chronic pathological condition, i.e., the hSOD1<sup>G93A</sup> amyotrophic lateral sclerosis mouse model, biphasic regulation of GLT-1a and GLT-1d mRNA expression was observed in the progression of disease in the hSOD1<sup>G93A</sup> mouse motor cortex. Furthermore, the differential regulation of GLT-1d expression in both acute and chronic pathological conditions strongly suggested future studies investigate GLT-1d as a potential therapeutic target.

The present study was a preliminary study that was constrained by low animal numbers for both the acute and the chronic disease states. The acute disease state, i.e., cerebral ischaemia followed by reperfusion, would have benefited from the inclusion of time points earlier than 24 hours after reperfusion and, additionally, at least until 72 hours after reperfusion. Likewise, while the material for the chronic study, i.e., total RNA, was greatly appreciated, the lack of both immunohistochemical and western blotting data meant that protein distribution and expression could not be resolved. Furthermore, underpowering of these studies constrained the outcomes of both the acute and chronic pathological brain studies.

Ideally, the glutamine transporters, i.e., the SNATs, should have been included in the studies relating to the juvenile (Chapter 5: GLT-1d in Juvenile Mouse Brain) and adult (Chapter 6: GLT-1d in Adult Mouse Brain) mouse brain. Inclusion of the SNATs would have provided a more comprehensive perspective of the glutamate homeostasis system and how GLT-1d integrates into this system in mouse brain. Future GLT-1d studies in the brains of other mammalian species will include the SNATs as well as GLT-1a–c, the other EAATs, the VGLUTs, and glutamine synthetase.

In summary, this thesis describes, for the first time, the expression, distribution, and potential relevance of GLT-1d, a newly discovered splice variant of the glutamate transporter GLT-1, in juvenile, adult, and pathological mouse brains. The characterisation of GLT-1d as set out in this thesis expanded the knowledge regarding GLT-1 and its association with the development, normal function, and pathological conditions of mouse brains. It is plausible that the knowledge derived here with respect to mouse brain
may be useful in understanding the expression, distribution, and potential relevance of GLT-1d, and perhaps GLT-1 in general, in the brains of other mammalian species.
Part V

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Part VI

Appendices
GenBank Submission KP966086.1 EAAT2d

(Homo sapiens)
Appendix A: GenBank Submission KP966086.1 EAAT2d Homo sapiens

Homo sapiens excitatory amino acid transporter 2 splice variant d mRNA, complete cds, alternatively spliced

GenBank: KP966086

FASTA Graphics

Go to:
LOCUS KP966086.1
ORGANISM Homo sapiens (human)
SOURCE Homo sapiens (human)
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REFERENCE 2 (bases 1 to 1701)
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/organism="Homo sapiens"
/translation="MASTEGANNMPKQVEVRMHDSHLGSEEPKHRHLGLRLCDKGLNLWLTLTVFGVILGAVCGGLLRLASPIHPDVVMLIAFPGDILMRMLKMLILPLIISSLSTGLDDRASGRLTMVYMTTIIIAMVLAVLIDMPWYKLQXLQSGKRNDOVEUSMMLYLQNPILQXFOQXTYLVKRVPMEDANATAVSNVNETVPEEPVYXIKXLEFOSNMGDLYPIAPFINDN1INEVMVMIVNLSPLISILGEXHLSVEARQGKYGMPTVYGILHMGSGPLFLPBPFTVFDFGSWGMLALTACTAGACGTPFRCLEKEDKWVTPGFATINMPKPORTMTTAVMILVNDLLMTHMTYIVSVMGSGVSGVLVAYSVTLDSTIIALIWHNCEDTVSNKIIISGKVIQVAMKVVTVECKMVCQGMHNASL"
GenBank Submission KP966087.1 GLT-1d

(Rattus norvegicus)
Appendix B: GenBank Submission KP966087.1 GLT-1d Rattus norvegicus
Appendix B: GenBank Submission KP966087.1 GLT-1d *Rattus norvegicus*

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1621 ccacaactctg tcgtaataga tgagtgcaag ggcacctggt tctctgttcc cagcatgctg
1681 catgttcttg atgcatgcat ttccttccca cttgggtta
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Animal Ethics Committee Approval Letter - AEC
1421
18 September 2014

Professor David Pow
School of Medical Sciences
RMIT University

Dear David,

AEC 1421: Understanding the role of the glutamate transporter GLT1D in the developing brain.

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from 18 September 2014 until 17 September 2017. An approved version of the application is attached.

Animals
Your application has been approved to use up to \( n=42 \) mice (C57BL/6JArc dams + litters, as outlined in your application) over the duration of the project.

The use of animals in scientific procedures is strictly regulated by the Australian code of practice for the care and use of animals for scientific purposes. The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare.

Responsibilities of investigators
1. Professor David Pow
2. Mr Seán Klinkradt
3. Dr Daria Camera

Responsibilities of investigators are described in the Australian code of practice for the care and use of animals for scientific purposes (section 3). Investigators have a ‘personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project’ (s.3.1.1).

Amendments and extensions
If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with ‘minor’ amendment requests. Major amendments to projects normally require a new project application.

Adverse events or unexpected outcomes
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.

Unwell animals must be immediately reported via the care forms available at the RMIT Animal Facility. In the case of any emergency, the Animal Welfare Officer, Dr Rebbecca Wilcox, may be contacted on 0409...
In case of any unexpected animal death, the researcher has a responsibility to organise an autopsy so as to determine the cause of death.

**Investigator guidelines for record keeping**
Investigators are required to adhere to the strict guidelines regarding record keeping for their project. Note that records associated with a project ‘should be available for audit by the institution and authorised external reviewers’. Failure to maintain proper records may result in a compliance breach of the Code and place at risk the researcher’s capacity to carry out research with animals.

**Conditions of approval**
The AEC may apply conditions of approval beyond the submission of annual/final reports. There are no specific conditions attached to this project, except that described elsewhere in this letter.

**Reports**
Approval to continue a project is conditional on the submission of annual and final reports. Annual reports are requested in December each year, and must be submitted whether or not the project has commenced or is inactive. Report forms are available at http://www.rmit.edu.au/governance/committees/aec.

Failure to submit reports will mean that a project is no longer approved, and/or that approval will be withheld from future projects.

All reports or communication regarding this project are to be forwarded to the secretary.

On behalf of the AEC I wish you well with your research.

Dr Brad Hayward
Coordinator, Animal Ethics and Gene Modification
*On behalf of*
RMIT Animal Ethics Committee

cc:   Ms Tricia Murphy, RAF manager
Animal Ethics Committee Approval Letter - AEC
1513
Dear Alyson,

AEC 1513: *Understanding the role of the glutamate transporter GLT1d in the ischaemic brain using a mouse model of stroke.*

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from 26 June 2015 until 25 June 2018. An approved version of the application is attached.

**Animals**

Your application has been approved to use up to n=140 mice (Male, C57BL/6) over the duration of the project.

The use of animals in scientific procedures is strictly regulated by the *Australian code of practice for the care and use of animals for scientific purposes.* The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare.

**Responsibilities of investigators**

1. Dr Alyson Miller
2. Professor David Pow
3. Mr Sean Klinkradt

Responsibilities of investigators are described in the *Australian code of practice for the care and use of animals for scientific purposes* (section 3). Investigators have a ‘personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project’ (s.3.1.1).

**Amendments and extensions**

If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with ‘minor’ amendment requests. Major amendments to projects normally require a new project application.

**Adverse events or unexpected outcomes**

As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.
Unwell animals must be immediately reported via the care forms available at the RMIT Animal Facility. In the case of any emergency, the Animal Welfare Officer, Dr Rebbecca Wilcox, may be contacted on 0409 521 234 at any time. In case of any unexpected animal death, the researcher has a responsibility to organise an autopsy so as to determine the cause of death.

**Investigator guidelines for record keeping**
Investigators are required to adhere to the strict guidelines regarding record keeping for their project. Note that records associated with a project 'should be available for audit by the institution and authorised external reviewers'. Failure to maintain proper records may result in a compliance breach of the Code and place at risk the researcher’s capacity to carry out research with animals.

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The AEC may apply conditions of approval beyond the submission of annual/final reports. There are no specific conditions attached to this project, except that described elsewhere in this letter.

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Approval to continue a project is conditional on the submission of annual and final reports. Annual reports are requested in December each year, and must be submitted whether or not the project has commenced or is inactive. Report forms are available at http://www.rmit.edu.au/governance/committees/aec.

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All reports or communication regarding this project are to be forwarded to the secretary.

On behalf of the AEC I wish you well with your research.

[Signature]

Dr Brad Hayward
Research Ethics Coordinator

*On behalf of*
RMIT Animal Ethics Committee

cc:   Ms Tricia Murphy, RAF manager
Synthetic Peptides - Quality Control Information
Appendix E: Synthetic Peptides - Quality Control Information

Certificate of Analysis

| Sequence: Ac-KGTWFSVPSMLHVLDACISFPLG-OH |
| Peptide Name: | Date: 11/26/2013 |
| Name: David Pow | Lot #: A7435 | Milligrams: 5.0 |

1.0 Product Name and Formulation: Research Grade Peptide. Final product is supplied as a lyophilized powder containing traces of Trifluoroacetate (TFA) salts.

2.0 Quality Control Specifications:

<table>
<thead>
<tr>
<th>QC Test</th>
<th>QC Specifications</th>
<th>Results</th>
<th>Approval/Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purity</td>
<td>Performed by HPLC, must be 85% by percent area on standard HPLC gradient (see column QC).</td>
<td>&gt;85%</td>
<td>MB</td>
</tr>
<tr>
<td>Mass Identification</td>
<td>Performed mass spectral analysis, mass to be within 0.1% of calculated average molecular weight: 2548</td>
<td>2546</td>
<td>MB</td>
</tr>
<tr>
<td>Concentration (if required)</td>
<td>Spectrophotometric analysis (A280) or Amino Acid Analysis confirming net peptide content.</td>
<td>N/A</td>
<td>MB</td>
</tr>
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</table>

3.0 Notes:

4.0 Indirect Materials

<table>
<thead>
<tr>
<th>Description</th>
<th>Source</th>
<th>Part Number</th>
<th>Lot #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile / Isopropanol / Methanol</td>
<td>Mallinckrodt</td>
<td>2856/3032/9093</td>
<td>418</td>
</tr>
<tr>
<td>Water</td>
<td>MilliQ</td>
<td>ZMQK6V0T1</td>
<td>106</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>American Bioanalytical</td>
<td>AB2010</td>
<td>14341400</td>
</tr>
<tr>
<td>HPLC column</td>
<td>YMC</td>
<td>C18</td>
<td>NEP289</td>
</tr>
<tr>
<td>DMF / NMP</td>
<td>J.T. Baker</td>
<td>9344-13/9261-13</td>
<td>41057</td>
</tr>
<tr>
<td>Alpha cyano cinnamic acid</td>
<td>SIGMA</td>
<td>C2020</td>
<td>03120BC</td>
</tr>
</tbody>
</table>

For Science... From Science.

New England Peptide Inc., 65 Zub Lane, Gardner, MA 01440 • Phone 888-343-5974 • Fax 978-630-0021

www.NewEnglandPeptide.com
Appendix E: Synthetic Peptides - Quality Control Information

5.0 Quality Control Equipment

HPLC – Gilson HPLC
Spectrophotometer – Milton Roy Spectronic 21DU

6.0 Reagent Preparation:

HPLC Buffers: A: HPLC grade water with 0.1% TFA; B: Acetonitrile with 0.08% TFA

Mass Spectral Matrix: 10mg 4 hydroxy-alpha cyano cinnamic acid in 500uL A, 500uL B
Dissolve in eppendorf, spin down pellet, decant and use supernatant

7.0 Quality Control Protocol:

A. HPLC Analysis
Gradient: as shown
Injection: approximately 1mg/mL peptide in HPLC grade water, 100uL (100ug) per injection
* Other solubility techniques may be required for hydrophobic sequences*
Percent purity based on peak area

B. Mass Spec
Spot 1uL matrix with 1uL/mL peptide solution
Let air dry, run sample
Different voltages, ion charges, and setting shown on Mass Spectral Analysis
Mass to be within 0.1% of exact molecular weight. (Note: if MW is less than 2000 daltons, MW to be within 2 daltons.)

C. Spectrophotometric Analysis (If required)
After final lyophilization weigh out 2 vials of peptide (from 1-5mg/s)
Dissolve in 1-5mL HPLC water at approximately 1.0mg/mL
Read abs of each sample at 280nm
Calculate concentration and net peptide content

D. AAA Analysis (If required)
Sample must be within 20% for each amino acid
Sequence ratios must be confirmed by analysis
Note: Cysteine and tryptophan residues are destroyed during analysis, number will not be accurate

8.0 Reassay Interval of Stored Samples:

Every two years or each time a lot is aliquotted from bulk storage.

9.0 Stability Information:

Lyophilized peptides generally have excellent stabilities, often showing little or no degradation after a few years at -20 deg C. Long term storage (>1 year) should be at -80 deg C desiccated, medium term storage (1-12 months) should be at -20 deg C desiccated, short term storage (<1 month) may be at 4 deg C.

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www.NewEnglandPeptide.com

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Certificate of Analysis

Sequence: Ac-KHVVCGHGDACSLPLG-OH

Peptide Name:  
Date: 12/9/2013

Name: David Pow  
Lot #: V1125-3  
Milligrams: 5.0

1.0 Product Name and Formulation: Research Grade Peptide. Final product is supplied as a lyophilized powder containing traces of Trifluoroacetate (TFA) salts.

2.0 Quality Control Specifications:

<table>
<thead>
<tr>
<th>QC Test</th>
<th>QC Specifications</th>
<th>Results</th>
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<tbody>
<tr>
<td>Purity</td>
<td>Performed by HPLC, must be 85% by percent area on standard HPLC gradient (see column QC).</td>
<td>85%</td>
<td>MB</td>
</tr>
<tr>
<td>Mass Identification</td>
<td>Performed mass spectral analysis, mass to be within 0.1% of calculated average molecular weight: 1691</td>
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<td>MB</td>
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<tr>
<td>Concentration (if required)</td>
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<td>MB</td>
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4.0 Indirect Materials

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<td>418</td>
</tr>
<tr>
<td>Water</td>
<td>MilliQ</td>
<td>ZMQ56V011</td>
<td>106</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>American Bioanalytical</td>
<td>AB2010</td>
<td>14380600</td>
</tr>
<tr>
<td>HPLC column</td>
<td>YMC</td>
<td>C18</td>
<td>NEP292</td>
</tr>
<tr>
<td>DMF / NMP</td>
<td>J.T. Baker</td>
<td>9344-13/9261-13</td>
<td>59295</td>
</tr>
<tr>
<td>Alpha cyano cinnamic acid</td>
<td>SIGMA</td>
<td>C2020</td>
<td>031208C</td>
</tr>
</tbody>
</table>

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Appendix E: Synthetic Peptides - Quality Control Information

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Dissolve in appendorf, spin down pellet, decant and use supernatant

7.0 Quality Control Protocol:

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Injection: approximately 1mg/mL peptide in HPLC grade water, 100uL (100ug) per injection
* Other solubility techniques may be required for hydrophobic sequences*
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B. Mass Spec
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Mass to be within 0.1% of exact molecular weight. (Note: if MW is less than 2000 daltons, MW to be within 2 daltons.)

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www.NewEnglandPeptide.com
## Appendix E: Synthetic Peptides - Quality Control Information

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>Ret. Time</th>
<th>Area</th>
<th>Area%</th>
<th>RP-HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1</td>
<td>12.28</td>
<td>52244</td>
<td>56.0</td>
<td>7/15.5-27.4</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>13.70</td>
<td>37375</td>
<td>40.0</td>
<td>7/15.5-27.4</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>14.32</td>
<td>8015</td>
<td>8.7</td>
<td>7/15.5-27.4</td>
</tr>
</tbody>
</table>

![Graph of % Mobile Phase vs. mVolts](image-url)

- X-axis: % Mobile Phase
- Y-axis: mVolts

### Graph Details:
- Peaks 1, 2, and 3 are clearly visible.
- The graph shows the separation of different peptide samples.

---

Page 1

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Suppliers of Animals, Chemicals, Equipment, and Services
### Table F.1: List of suppliers of animals, chemicals, equipment, and services.

<table>
<thead>
<tr>
<th>Animals, Chemicals, Equipment, and Services</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>All reagents not specifically mentioned</td>
<td>Sigma, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>3,3’,5,5’-tetramethylbenzidine</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>3,3’-diaminobenzidine (DAB)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>6-0 nylon monofilament</td>
<td>Doccol Co., Redlands, CA, USA</td>
</tr>
<tr>
<td>6X DNA Loading Dye</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>Adobe Photoshop</td>
<td>Adobe Systems, San Jose, CA, USA</td>
</tr>
<tr>
<td>Antigen recovery solution</td>
<td>Revealit-Ag™, ImmunoSolution, Newcastle, NSW, Australia</td>
</tr>
<tr>
<td>Anti-rabbit secondary antibody horseradish peroxidase complex</td>
<td>Cell Signaling Technology, Danvers, MA, USA</td>
</tr>
<tr>
<td>BCA Protein Assay Kit (Pierce)</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>BioTaq™ DNA Polymerase</td>
<td>Bioline (Aust) Pty Ltd, Alexandria, NSW, Australia</td>
</tr>
<tr>
<td>Biotinylated anti-rabbit secondary antibody</td>
<td>GE Healthcare, Castle Hill, NSW, Australia</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Branson Ultrasonics Sonifier™ S-450 Digital Ultrasonic Cell Disruptor/Homogenizer</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Bioline (Aust) Pty Ltd, Alexandria, NSW, Australia</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>ChemiDoc XRS+ system</td>
<td>Animal Resources Centre, Perth, WA, Australia</td>
</tr>
<tr>
<td>CLARIOstar™ microplate reader</td>
<td>Bio-Rad, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>cOmplete™ Ultra protease inhibitor cocktail</td>
<td>BMG LABTECH, Mornington, VIC, Australia</td>
</tr>
<tr>
<td>Super Signal West Femto enhanced chemiluminescence substrate</td>
<td>Roche Diagnostics Australia Pty. Ltd, North Ryde, NSW, Australia</td>
</tr>
</tbody>
</table>

Continued on next page
### Table F.1 – continued from previous page

<table>
<thead>
<tr>
<th>Animals, Chemicals, Equipment, and Services</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucalyptus oil</td>
<td>Bosisto, Oakleigh South, VIC, Australia</td>
</tr>
<tr>
<td>Glycine</td>
<td>VWR, Tingalpa, QLD, Australia</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG horseradish peroxidase</td>
<td>Rockland, Limerick, PA, USA</td>
</tr>
<tr>
<td>GoTaq&lt;sup&gt;™&lt;/sup&gt; qPCR Master Mix</td>
<td>Promega Australia, Alexandria, NSW, Australia</td>
</tr>
<tr>
<td>GraphPad Prism</td>
<td>GraphPad Software, Inc., La Jolla, CA, USA</td>
</tr>
<tr>
<td>HCl</td>
<td>Sigma, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>Heat lamp</td>
<td>Exo Terra, Rolf C. Hagen (U.S.A.) Corp., Mansfield, MA, USA</td>
</tr>
<tr>
<td>High-Capacity cDNA Reverse Transcription Kit</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>hSOD1&lt;sup&gt;G93A&lt;/sup&gt; transgenic mice</td>
<td>University of Queensland Animal Facility, St Lucia, QLD, Australia</td>
</tr>
<tr>
<td>Hyperladder 1 kb DNA markers</td>
<td>Bioline (Aust) Pty Ltd, Alexandria, NSW, Australia</td>
</tr>
<tr>
<td>Image Lab software, version 5.2.1, Build 11</td>
<td>Bio-Rad, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Sigma, Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Laser Doppler Perfusion Monitor (Perimed)</td>
<td>Hyperbaric Health Pty Ltd, Keaysborough, VIC, Australia</td>
</tr>
<tr>
<td>MARS Data Analysis software</td>
<td>BMG LABTECH, Mornington, VIC, Australia</td>
</tr>
<tr>
<td>Methanol</td>
<td>VWR, Tingalpa, QLD, Australia</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Mini BlotBoy (laboratory rocker)</td>
<td>Benchmark Scientific, Edison, NJ, USA</td>
</tr>
<tr>
<td>Mini-PROTEAN&lt;sup&gt;™&lt;/sup&gt; Tetra Cell 1-D Vertical Gel Electrophoresis System</td>
<td>Bio-Rad, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>Mini-Trans-Blot&lt;sup&gt;™&lt;/sup&gt; Electrophoretic Transfer Cell System</td>
<td>Bio-Rad, Sydney, NSW, Australia</td>
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Continued on next page
## Table F.1 – continued from previous page

<table>
<thead>
<tr>
<th>Animals, Chemicals, Equipment, and Services</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight Markers (Precision Plus Protein™ Dual Color Standards)</td>
<td>Bio-Rad, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>MultiGene™ OptiMax Thermal Cycler</td>
<td>Edison, New Jersey, USA</td>
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<td>Na$_2$HPO$_4$</td>
<td>Sigma, St. Louis, MO, USA</td>
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<tr>
<td>NaCl</td>
<td>UNIVAR, Redmond, CA, USA</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>NanoDrop™ 2000 spectrophotometer</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>Nikon Eclipse 80i microscope</td>
<td>Nikon Australia, Rhodes, NSW, Australia</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Bio-Rad, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>NP-40 (Nonyl Phenoxypolyethoxylethanol)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Oligo Explorer 1.2</td>
<td>Gene Link, Inc., Hawthorne, NY, USA</td>
</tr>
<tr>
<td>Olympus DP70 digital camera</td>
<td>Olympus Australia Pty Ltd, Notting Hill, NSW, Australia</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>Lomb Scientific, Sydney, NSW, Australia</td>
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<tr>
<td>Paraformaldehyde</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Ponceau S stain</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>qPCR primers</td>
<td>Sigma, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>Rb 1713–1716 antisera</td>
<td>New England Peptide, Inc., Gardner, MA, USA</td>
</tr>
<tr>
<td>Rectal temperature probe</td>
<td>Testronics, Kinglake, VIC, Australia</td>
</tr>
<tr>
<td>RNase inhibitor (RNAsin)</td>
<td>Promega, Alexandria, NSW, Australia</td>
</tr>
<tr>
<td>RNAse-free water</td>
<td>Promega Australia, Alexandria, NSW, Australia</td>
</tr>
<tr>
<td>RNaseOUT™ recombinant RNase inhibitor</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
</tbody>
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<td>RNeasy Mini Kits</td>
<td>QIAgen Pty Ltd – Australia, Chadston, VIC, Australia</td>
</tr>
<tr>
<td>Rotor-Gene Q</td>
<td>QIAgen Pty Ltd – Australia, Chadston, VIC, Australia</td>
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<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Sodium pentobarbital</td>
<td>Virbac (Australia) Pty Ltd, Milperra, NSW, Australia</td>
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<tr>
<td>SpectraMax Plus 384 Microplate Reader</td>
<td>Molecular Devices, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Streptavidin horseradish peroxidase complex</td>
<td>GE Healthcare, Castle Hill, NSW, Australia</td>
</tr>
<tr>
<td>SureBlue TMB 1-Component</td>
<td>KPL, Inc. Gaithersburg, MD, USA</td>
</tr>
<tr>
<td>Microwell Peroxidase Substrate</td>
<td>Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>(3,3’,5,5’-tetramethylbenzidine)</td>
<td>New England Peptide, Inc., Gardner, MA, USA</td>
</tr>
<tr>
<td>SYBR™ Gold Nucleic Acid Gel Stain</td>
<td>New England Peptide, Inc., Gardner, MA, USA</td>
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<tr>
<td>Synthetic peptides</td>
<td>IKA, Staufen, Germany</td>
</tr>
<tr>
<td>Synthetic peptide-keyhole limpet hemocyanin conjugates</td>
<td>Testronics, Kinglake, VIC, Australia</td>
</tr>
<tr>
<td>T10 basic Ultra Turrax™ homogenizer</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Temperature regulator</td>
<td>Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>( N,N,N',N' - ) Tetramethylethylenediamine</td>
<td>Thermo Fisher Scientific, Scoresby, VIC, Australia</td>
</tr>
<tr>
<td>Tris Base</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>TRIzol™ reagent</td>
<td>University of Queensland Animal Facility, St Lucia, QLD, Australia</td>
</tr>
<tr>
<td>Tween-20</td>
<td></td>
</tr>
<tr>
<td>Wild-type mice</td>
<td></td>
</tr>
</tbody>
</table>
ThermoScientific Protein Assay Compatibility Table
**TECH TIP #68**

Protein assay compatibility table

---

**Introduction**

No single protein assay method is compatible with all sample components that might be encountered in typical laboratory research. To alleviate the frustration of discovering these incompatibilities by trial and error, our technical support scientists have characterized the effects of commonly used buffers and sample components on the behavior of seven different Thermo Scientific Pierce* Protein Assays.

Results of these compatibility experiments are summarized in tabular form on the following two pages. The table serves as a guide for assessing which protein assay(s) might be most effective with a given sample type. Use this information as a guide only. It is the researcher’s responsibility to validate the compatibility of specific sample buffers. Even minor changes in buffers and components affect each assay method to some extent. Researchers must decide if these effects are acceptable for their specific purposes.

**About the Compatibility Table**

The table indicates the maximum compatible concentrations for substances tested with the Test Tube procedure on the midpoint concentration of BSA standard (i.e., 1000µg/mL for most assays; 10µg/mL for the Micro BCA Assay). Substances were considered compatible at the indicated concentration if the error in protein concentration estimation caused by the presence of the substance in the sample was less than or equal to 10%.

**Notes and Symbols:**

- Compounds are listed alphabetically using common names or abbreviations, except sodium compounds which are alphabetized under “Na”.
- Dilutions are expressed as “neet” (= undiluted) or in the form of a ratio, where “1:2” means 2-fold dilution.
- n/a Denotes that the compound was not tested in this assay.
- Ø Denotes compounds that were not compatible at the lowest concentration tested.
- * Thermo Scientific product trademark; “PER Reagents” are Thermo Scientific Pierce Cell Lysis Reagents.
- ** Trademarks that are the property of other companies; see end of document.
- † Value when the 660nm Assay is run using the ionic detergent compatibility reagent (IDCR, Part No. 22663).
- †† Selected values for the regular BCA-RAC Kit are given in parentheses in the column for the Microplate BCA-RAC.
- ‡ Compound (buffer) whose formulation is described more fully in the following table:

<table>
<thead>
<tr>
<th>Part No.</th>
<th>Buffer</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>2-D sample buffer</td>
<td>(6M urea, 4% CHAPS) or (7M urea, 2M thiourea, 4% CHAPS)</td>
</tr>
<tr>
<td>28390</td>
<td>MES-buffered saline pH 4.7</td>
<td>0.1M MES, 150mM NaCl pH 4.7</td>
</tr>
<tr>
<td>28374</td>
<td>Modified Dulbecco’s PBS</td>
<td>8mM sodium phosphate, 2mM potassium phosphate, 0.14M NaCl, 10mM KCl, pH 7.4</td>
</tr>
<tr>
<td>28382</td>
<td>Na carbi-bicarb pH 9</td>
<td>0.2M sodium carbonate-bicarbonate pH 9.4</td>
</tr>
<tr>
<td>28388</td>
<td>Na citrate-carbonate pH 9</td>
<td>0.6M sodium citrate, 0.1M sodium-carbonate pH 9</td>
</tr>
<tr>
<td>28386</td>
<td>Na citrate-MOPS pH 7.5</td>
<td>0.6M sodium citrate 0.1M MOPS pH 7.5</td>
</tr>
<tr>
<td>28372</td>
<td>Phosphate-buffered saline (PBS)</td>
<td>100mM sodium phosphate, 150mM NaCl pH 7.2</td>
</tr>
<tr>
<td>89900</td>
<td>RIPA Buffer</td>
<td>50mM Tris, 150mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS pH 8.0</td>
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<tr>
<td>28379</td>
<td>Tris-buffered saline (TBS)</td>
<td>25mM Tris, 150mM NaCl pH 7.6</td>
</tr>
<tr>
<td>28380</td>
<td>Tris-glycine pH 8.0</td>
<td>25mM Tris, 192mM glycine pH 8.0</td>
</tr>
<tr>
<td>28378</td>
<td>Tris-glycine-SDS pH 8.3</td>
<td>25mM Tris, 192mM glycine, 0.1% SDS pH 8.3</td>
</tr>
<tr>
<td>28388</td>
<td>Tris-HEPES-SDS</td>
<td>100mM Tris, 100mM HEPES, 3mM SDS</td>
</tr>
</tbody>
</table>

---

*Pierce Biotechnology PO Box 117 (815) 968-0747 www.thermoscientific.com/pierce
3747 N. Meridian Road Rockford, IL 61105 USA (815) 966-7316 fax
## Thermo Scientific Pierce Protein Assay

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>660 nm</th>
<th>BCA</th>
<th>Micro BCA</th>
<th>1:1Microplate BCA/RAC</th>
<th>Coomassie Plus</th>
<th>Coomassie</th>
<th>Modified Lowry</th>
</tr>
</thead>
<tbody>
<tr>
<td>J-O sample buffer</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>2-Mercaptoethanol</td>
<td>1M</td>
<td>0.01%</td>
<td>1mM</td>
<td>25mM (35)</td>
<td>1M</td>
<td>1M</td>
<td>1mM</td>
</tr>
<tr>
<td>ACES, pH 7.8</td>
<td>50mM</td>
<td>25mM</td>
<td>100mM</td>
<td>Ø</td>
<td>100mM</td>
<td>100mM</td>
<td>n/a</td>
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<tr>
<td>Acetone</td>
<td>50%</td>
<td>10%</td>
<td>1%</td>
<td>10%</td>
<td>10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetotriite</td>
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<td>1%</td>
<td>30%</td>
<td>10%</td>
<td>10%</td>
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<tr>
<td>Ammonium sulfate</td>
<td>125mM</td>
<td>1.5M</td>
<td>Ø</td>
<td>Ø</td>
<td>1M</td>
<td>1M</td>
<td>Ø</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>2mM</td>
<td>10mg/mL</td>
<td>10mg/L</td>
<td>Ø</td>
<td>10mg/L</td>
<td>10mg/L</td>
<td>10mg/L</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>500mM</td>
<td>Ø</td>
<td>Ø</td>
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<td>50mM</td>
<td>50mM</td>
<td>1mM</td>
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<tr>
<td>Asparagin</td>
<td>40mM</td>
<td>1mM</td>
<td>n/a</td>
<td>Ø</td>
<td>10mM</td>
<td>10mM</td>
<td>5mM</td>
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<tr>
<td>Bicine</td>
<td>&gt;1M</td>
<td>2mM</td>
<td>2mM</td>
<td>1mM</td>
<td>100mM</td>
<td>100mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Bis-Tris pH 6.5</td>
<td>50mM</td>
<td>33mM</td>
<td>0.2mM</td>
<td>16.5mM</td>
<td>100mM</td>
<td>100mM</td>
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<td>Borate (50mM), pH 8.5</td>
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<td>neat</td>
<td>1.4</td>
<td>Ø</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
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<tr>
<td>B-PER® Reagent</td>
<td>1:2</td>
<td>neat</td>
<td>n/a</td>
<td>1.3</td>
<td>1.2</td>
<td>1.2</td>
<td>n/a</td>
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<tr>
<td>B-PER Reagent II</td>
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<td>1.4</td>
<td>1.4</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>B-PER Reagent PBS</td>
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<td>n/a</td>
<td>1.4</td>
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<td>n/a</td>
<td>n/a</td>
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<td>Bis**-35</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>0.63%</td>
<td>0.062%</td>
<td>0.125%</td>
<td>0.031%</td>
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<tr>
<td>Biq-D6</td>
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<td>1%</td>
<td>1%</td>
<td>n/a</td>
<td>0.031%</td>
<td>0.031%</td>
<td>0.092%</td>
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<tr>
<td>Biq-D8</td>
<td>5%</td>
<td>1%</td>
<td>1%</td>
<td>0.50%</td>
<td>0.015%</td>
<td>0.031%</td>
<td>0.092%</td>
</tr>
<tr>
<td>Bromophenol blue (in 50mM NaOH)</td>
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<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride (in TBS pH 7.2)</td>
<td>40mM</td>
<td>10mM</td>
<td>10mM</td>
<td>1mM</td>
<td>10mM</td>
<td>10mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Cesium bicarbonate</td>
<td>100mM</td>
<td>100mM</td>
<td>100mM</td>
<td>Ø</td>
<td>100mM</td>
<td>100mM</td>
<td>100mM</td>
</tr>
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<td>Cetylpyridinium chloride</td>
<td>2.5%</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<td>CHAPS</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>10% (10)</td>
<td>5%</td>
<td>5%</td>
<td>0.082%</td>
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<td>CHAPSO</td>
<td>4%</td>
<td>5%</td>
<td>5%</td>
<td>Ø</td>
<td>9%</td>
<td>5%</td>
<td>0.031%</td>
</tr>
<tr>
<td>CHES</td>
<td>&gt;500mM</td>
<td>100mM</td>
<td>100mM</td>
<td>50mM</td>
<td>100mM</td>
<td>100mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Cobalt chloride (in TBS pH 7.2)</td>
<td>20mM</td>
<td>0.6mM</td>
<td>Ø</td>
<td>0.4mM</td>
<td>10mM</td>
<td>10mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>250mM</td>
<td>1mM</td>
<td>Ø</td>
<td>Ø</td>
<td>2.5mM</td>
<td>1mM</td>
<td>1mM</td>
</tr>
<tr>
<td>Dithioerythritol (DTE)</td>
<td>50mM</td>
<td>1mM</td>
<td>Ø</td>
<td>5mM (5)</td>
<td>5mM</td>
<td>5mM</td>
<td>Ø</td>
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<td>DTPA</td>
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<td>1%</td>
<td>5%</td>
<td>10%</td>
<td>10%</td>
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<td>DTAB</td>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<td>EDTA</td>
<td>20mM</td>
<td>10mM</td>
<td>0.5mM</td>
<td>5mM (2)</td>
<td>100mM</td>
<td>100mM</td>
<td>1mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>20mM</td>
<td>Ø</td>
<td>Ø</td>
<td>5mM (1)</td>
<td>2mM</td>
<td>2mM</td>
<td>1mM</td>
</tr>
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<td>EPPS pH 8.0</td>
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<td>100mM</td>
<td>100mM</td>
<td>Ø</td>
<td>100mM</td>
<td>100mM</td>
<td>n/a</td>
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<td>Ethanol</td>
<td>50%</td>
<td>10%</td>
<td>1%</td>
<td>Ø</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>Ferric chloride (in TBS pH 7.2)</td>
<td>5mM</td>
<td>10mM</td>
<td>0.5mM</td>
<td>5mM</td>
<td>10mM</td>
<td>10mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Glucose</td>
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<td>10mM</td>
<td>1mM</td>
<td>Ø</td>
<td>1M</td>
<td>1M</td>
<td>100mM</td>
</tr>
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<td>Glutathione (reduced)</td>
<td>100mM</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>10mM</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>Glyceraldehyde</td>
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<td>10%</td>
<td>1%</td>
<td>5%</td>
<td>10%</td>
<td>10%</td>
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<td>Glycine-HCl pH 2.8</td>
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<td>100mM</td>
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<td>50mM</td>
<td>100mM</td>
<td>100mM</td>
<td>100mM</td>
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<td>4M</td>
<td>4M</td>
<td>1.5M (2)</td>
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<td>3.5M</td>
<td>n/a</td>
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<td>HEPES pH 7.6</td>
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<td>100mM</td>
<td>100mM</td>
<td>200mM (200)</td>
<td>100mM</td>
<td>100mM</td>
<td>10mM</td>
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<tr>
<td>Hydrolysis (Na2BH3 or NaCNBH3)</td>
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<td>Ø</td>
<td>Ø</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>Hydroxylamine acid (HCl)</td>
<td>125mM</td>
<td>100mM</td>
<td>100mM</td>
<td>Ø</td>
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<td>100mM</td>
<td>100mM</td>
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<tr>
<td>Imidazole pH 7.0</td>
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<td>50mM</td>
<td>12.5mM</td>
<td>30mM (50)</td>
<td>200mM</td>
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<td>25mg</td>
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<tr>
<td>I-PER® Reagent</td>
<td>1:4</td>
<td>neat</td>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
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<td>Leupeptin</td>
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<td>10mg/L</td>
<td>10mg/L</td>
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<td>n/a</td>
<td>n/a</td>
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<td>n/a</td>
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<tr>
<td>Malachite</td>
<td>500µM</td>
<td>Ø</td>
<td>Ø</td>
<td>n/a</td>
<td>100mM</td>
<td>100mM</td>
<td>25mM</td>
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<tr>
<td>Mem-PER® Reagent</td>
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<td>neat</td>
<td>neat</td>
<td>1.2</td>
<td>neat</td>
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<td>n/a</td>
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<td>M-Buffer buffered saline pH 4.7</td>
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<td>neat</td>
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<td>Ø</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
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<td>MES pH 4.1</td>
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<td>100mM</td>
<td>100mM (100)</td>
<td>100mM</td>
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<td>125mM</td>
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<td>Methanol</td>
<td>50%</td>
<td>10%</td>
<td>1%</td>
<td>0.5%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>&gt;1M</td>
<td>n/a</td>
<td>n/a</td>
<td>100mM</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Modified Dulbecco’s PBS</td>
<td>neat</td>
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<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
</tr>
<tr>
<td>MOPS pH 7.2</td>
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<td>100mM</td>
<td>100mM</td>
<td>200mM</td>
<td>100mM</td>
<td>100mM</td>
<td>n/a</td>
</tr>
<tr>
<td>M-PER® Reagent</td>
<td>1:2</td>
<td>neat</td>
<td>n/a</td>
<td>1:2</td>
<td>neat</td>
<td>n/a</td>
<td>n/a</td>
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</table>
## Appendix G: ThermoScientific Protein Assay Compatibility Table

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>660 nm</th>
<th>BCA</th>
<th>Micro BCA</th>
<th>T-Microplate BCA-RAC</th>
<th>Coomassie Plus</th>
<th>Coomassie Lowry</th>
<th>Modified Lowry</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Acetylglucosamine</td>
<td>100 mM</td>
<td>10 mM</td>
<td>Ø</td>
<td>Ø</td>
<td>100 mM</td>
<td>100 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Na acetate pH 4.8</td>
<td>100 mM</td>
<td>200 mM</td>
<td>200 mM</td>
<td>Ø</td>
<td>180 mM</td>
<td>180 mM</td>
<td>200 mM</td>
</tr>
<tr>
<td>Na azide</td>
<td>0.125%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>0.01%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.2%</td>
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<td>Na bicarbonate</td>
<td>100 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>Ø</td>
<td>100 mM</td>
<td>100 mM</td>
<td>100 mM</td>
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<tr>
<td>Na carbonate</td>
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<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
</tr>
<tr>
<td>Na chloride</td>
<td>1.25M</td>
<td>1M</td>
<td>1M</td>
<td>150mM</td>
<td>5M</td>
<td>5M</td>
<td>1M</td>
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<tr>
<td>Na citrate pH 4.8</td>
<td>12.5 mM</td>
<td>200 mM</td>
<td>5 mM</td>
<td>50 mM</td>
<td>200 mM</td>
<td>200 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Na citrate-carbonate pH 9.1</td>
<td>Ø</td>
<td>1.8</td>
<td>1.60</td>
<td>Ø</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
</tr>
<tr>
<td>Na citrate-MOPS pH 7.5</td>
<td>1:16</td>
<td>1:8</td>
<td>1:60</td>
<td>neat</td>
<td>n/a</td>
<td>neat</td>
<td>n/a</td>
</tr>
<tr>
<td>Na deoxycholate (SDC)</td>
<td>0.25%</td>
<td>5%</td>
<td>n/a</td>
<td>5%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>n/a</td>
</tr>
<tr>
<td>Na hydroxide (NaOH)</td>
<td>125 mM</td>
<td>100 mM</td>
<td>50 mM</td>
<td>Ø</td>
<td>100 mM</td>
<td>100 mM</td>
<td>100 mM</td>
</tr>
<tr>
<td>Na phosphate</td>
<td>500 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>100 mM</td>
</tr>
<tr>
<td>NE-PER Reagent (CER)</td>
<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>1:2</td>
<td>1:1</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>NE-PER Reagent (NER)</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
<td>1.4</td>
<td>neat</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Nickel chloride (in TBS pH 7.2)</td>
<td>10 mM</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>Ø</td>
<td>10 mM</td>
<td>10 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>NP-40</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>Ø</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.016%</td>
</tr>
<tr>
<td>Octyl beta-galactoside</td>
<td>5%</td>
<td>5%</td>
<td>0.1%</td>
<td>2.5% (10)</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.031%</td>
</tr>
<tr>
<td>Octylthioglucoside</td>
<td>10%</td>
<td>5%</td>
<td>5%</td>
<td>7%</td>
<td>3%</td>
<td>3%</td>
<td>n/a</td>
</tr>
<tr>
<td>N-acetylglucosamine (in PBS pH 7.2)</td>
<td>500 mM</td>
<td>1 mM</td>
<td>1mM</td>
<td>0.5 mM</td>
<td>1 mM</td>
<td>1 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.5 mg/mL</td>
<td>Ø</td>
<td>3.125 µg/mL</td>
<td>0.5 mg/mL</td>
<td>0.5 mg/mL</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Phosphate-buffered saline (PBS)</td>
<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>100 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>25 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>PMFS in isopropanol</td>
<td>1 mM</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.125 mM</td>
<td>1 mM</td>
<td>1 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>Potassium thioctylate</td>
<td>250 mM</td>
<td>3 M</td>
<td>n/a</td>
<td>Ø</td>
<td>3 M</td>
<td>3 M</td>
<td>100 mM</td>
</tr>
<tr>
<td>P-PER Reagent</td>
<td>1:2</td>
<td>Ø</td>
<td>n/a</td>
<td>1:2</td>
<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
</tr>
<tr>
<td>RIPA buffer II</td>
<td>neat</td>
<td>neat</td>
<td>1:10</td>
<td>1:2</td>
<td>1:40</td>
<td>1:10</td>
<td>n/a</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
<td>5%</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.016%</td>
<td>0.125%</td>
<td>1%</td>
</tr>
<tr>
<td>Sodium compounds</td>
<td>(see Na)</td>
<td>(see Na)</td>
<td>(see Na)</td>
<td>(see Na)</td>
<td>(see Na)</td>
<td>(see Na)</td>
<td>(see Na)</td>
</tr>
<tr>
<td>Spars*</td>
<td>20%</td>
<td>1%</td>
<td>1%</td>
<td>n/a</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.25%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>50%</td>
<td>40%</td>
<td>4%</td>
<td>40% (40)</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>TCEP</td>
<td>400 mM</td>
<td>Ø</td>
<td>Ø</td>
<td>10 mM (10)</td>
<td>125 mM</td>
<td>126 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Thimerosal</td>
<td>0.25%</td>
<td>0.01%</td>
<td>Ø</td>
<td>0.03%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Threonine</td>
<td>2M</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>TCLK</td>
<td>5 mg/mL</td>
<td>0.1 mg/L</td>
<td>0.1 mg/L</td>
<td>Ø</td>
<td>0.1 mg/mL</td>
<td>0.1 mg/L</td>
<td>0.01 mg/L</td>
</tr>
<tr>
<td>TPCK</td>
<td>4 mg/mL</td>
<td>0.1 mg/L</td>
<td>0.1 mg/L</td>
<td>Ø</td>
<td>0.1 mg/mL</td>
<td>0.1 mg/L</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>T-PER Reagent</td>
<td>1:2</td>
<td>1:2</td>
<td>n/a</td>
<td>n/a</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
</tr>
<tr>
<td>Tricine pH 8.0</td>
<td>500 mM</td>
<td>25 mM</td>
<td>2.5 mM</td>
<td>0.5 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Triethanolamine pH 7.8</td>
<td>100 mM</td>
<td>25 mM</td>
<td>0.5 mM</td>
<td>25 mM</td>
<td>100 mM</td>
<td>100 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Tri-buffered saline (TBS)</td>
<td>neat</td>
<td>neat</td>
<td>1:10</td>
<td>neat</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
</tr>
<tr>
<td>Tris-glycine pH 8.0</td>
<td>neat</td>
<td>1:3</td>
<td>1:10</td>
<td>Ø</td>
<td>neat</td>
<td>neat</td>
<td>n/a</td>
</tr>
<tr>
<td>Tris-glycine-PBS pH 8.3</td>
<td>neat</td>
<td>Ø</td>
<td>Ø</td>
<td>1:4</td>
<td>1:2</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Tris-HEPES-PBS</td>
<td>250 mM</td>
<td>250 mM</td>
<td>250 mM</td>
<td>250 mM</td>
<td>250 mM</td>
<td>250 mM</td>
<td>100 mM</td>
</tr>
<tr>
<td>T-PER Reagent</td>
<td>1:2</td>
<td>Ø</td>
<td>Ø</td>
<td>1:2</td>
<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1%</td>
<td>5%</td>
<td>5%</td>
<td>7% (10)</td>
<td>0.062%</td>
<td>0.125%</td>
<td>0.031%</td>
</tr>
<tr>
<td>Triton X-114</td>
<td>0.5%</td>
<td>5%</td>
<td>0.99%</td>
<td>2% (2)</td>
<td>0.062%</td>
<td>0.125%</td>
<td>0.031%</td>
</tr>
<tr>
<td>Triton X-305</td>
<td>9%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>0.125%</td>
<td>0.5%</td>
<td>0.031%</td>
</tr>
<tr>
<td>Triton X-405</td>
<td>9%</td>
<td>1%</td>
<td>1%</td>
<td>Ø</td>
<td>0.25%</td>
<td>0.5%</td>
<td>0.031%</td>
</tr>
<tr>
<td>Tween 20*</td>
<td>10%</td>
<td>5%</td>
<td>5%</td>
<td>10% (10)</td>
<td>0.016%</td>
<td>0.062%</td>
<td>0.062%</td>
</tr>
<tr>
<td>Tween 60</td>
<td>5%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.25%</td>
<td>0.1%</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Tween 80</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>2.5%</td>
<td>0.01%</td>
<td>0.062%</td>
<td>0.031%</td>
</tr>
<tr>
<td>Urea</td>
<td>12%</td>
<td>3 M</td>
<td>3 M</td>
<td>3 M</td>
<td>3 M</td>
<td>3 M</td>
<td>3 M</td>
</tr>
<tr>
<td>V-PER Reagent</td>
<td>Ø</td>
<td>neat</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>V-PER Plus Reagent</td>
<td>1:2</td>
<td>neat</td>
<td>n/a</td>
<td>n/a</td>
<td>neat</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Zinc chloride (in TBS pH 7.2)</td>
<td>10 mM</td>
<td>10 mM</td>
<td>0.5 mM</td>
<td>Ø</td>
<td>10 mM</td>
<td>10 mM</td>
<td>n/a</td>
</tr>
<tr>
<td>Zwittergent™ 3-14</td>
<td>0.05%</td>
<td>1%</td>
<td>Ø</td>
<td>2% (2)</td>
<td>0.025%</td>
<td>0.025%</td>
<td>n/a</td>
</tr>
</tbody>
</table>

* Triton™ is a registered trademark of Rohm & Haas Co.
** NP-40, Tween® and Spars* are trademarks of ICI Americas.
*** Zwittergent™ is a registered trademark of American Hoechst Corporation.

Current versions of product instructions are available at [www.thermoscientific.com/pierce](http://www.thermoscientific.com/pierce). For a faxed copy, call 800-874-3723 or your local distributor.

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Pierce™ BCA™ Protein Assay MicroTitre Plate
Reader Protocol
INTRODUCTIONS

Pierce BCA Protein Assay Kit

23225  23227

Number  Description
23225  Pierce BCA Protein Assay Kit, sufficient reagents for 500 test-tube or 5000 microplate assays
23227  Pierce BCA Protein Assay Kit, sufficient reagents for 250 test-tube or 2500 microplate assays

Kit Contents:

BCA Reagent A, 1000mL (in Product No. 23225) or 500mL (in Product No. 23227), containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide

BCA Reagent B, 25mL, containing 4% cupric sulfate

Albumin Standard Ampules, 2mg/mL, 10 × 1mL ampules, containing bovine serum albumin (BSA) at 2mg/mL in 0.9% saline and 0.05% sodium azide

Storage: Upon receipt store at room temperature. Product shipped at ambient temperature.

Note: If either Reagent A or Reagent B precipitates upon shipping in cold weather or during long-term storage, dissolve precipitates by gently warming and stirring solution. Discard any kit reagent that shows discoloration or evidence of microbial contamination.

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Introduction

The Thermo Scientific™ Pierce™ BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu²⁺ to Cu⁺ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu⁺) using a unique reagent containing bicinchoninic acid.¹ The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000µg/mL).

The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.² Studies with di-, tri- and tetrapeptides suggest that the extent of color formation caused by more than the mere sum of individual color-producing functional groups.³ Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration are prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve. If precise quantitation of an unknown protein is required, it is advisable to select a protein
standard that is similar in quality to the unknown; for example, a bovine gamma globulin (BGG) standard (see Related Thermo Scientific Products) may be used when assaying immunoglobulin samples.

Two assay procedures are presented. Of these, the Test Tube Procedure requires a larger volume (0.1 mL) of protein sample; however, because it uses a sample to working reagent ratio of 1:20 (v/v), the effect of interfering substances is minimized. The Microplate Procedure affords the sample handling ease of a microplate and requires a smaller volume (10-25 µL) of protein sample; however, because the sample to working reagent ratio is 1:8 (v/v), it offers less flexibility in overcoming interfering substance concentrations and obtaining low levels of detection.

Note: For peptide sample concentration measurements, use the Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay or the Pierce™ Quantitative Colorimetric Peptide Assay (see Related Thermo Scientific Products).

Preparation of Standards and Working Reagent (required for both assay procedures)

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1 mL ampule of 2mg/mL Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of Diluent (µL)</th>
<th>Volume and Source of BSA (µL)</th>
<th>Final BSA Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300 of Stock</td>
<td>2000</td>
</tr>
<tr>
<td>B</td>
<td>125</td>
<td>375 of Stock</td>
<td>1500</td>
</tr>
<tr>
<td>C</td>
<td>325</td>
<td>325 of Stock</td>
<td>1000</td>
</tr>
<tr>
<td>D</td>
<td>175</td>
<td>175 of vial B dilution</td>
<td>750</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325 of vial C dilution</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>325 of vial E dilution</td>
<td>250</td>
</tr>
<tr>
<td>G</td>
<td>325</td>
<td>325 of vial F dilution</td>
<td>125</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>100 of vial G dilution</td>
<td>25</td>
</tr>
<tr>
<td>I</td>
<td>400</td>
<td>0</td>
<td>0 = Blank</td>
</tr>
</tbody>
</table>

Dilution Scheme for Enhanced Test Tube Protocol (Working Range = 5–250µg/mL)

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of Diluent (µL)</th>
<th>Volume and Source of BSA (µL)</th>
<th>Final BSA Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>700</td>
<td>100 of Stock</td>
<td>250</td>
</tr>
<tr>
<td>B</td>
<td>400</td>
<td>400 of vial A dilution</td>
<td>125</td>
</tr>
<tr>
<td>C</td>
<td>450</td>
<td>300 of vial B dilution</td>
<td>50</td>
</tr>
<tr>
<td>D</td>
<td>400</td>
<td>400 of vial C dilution</td>
<td>25</td>
</tr>
<tr>
<td>E</td>
<td>400</td>
<td>100 of vial D dilution</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>400</td>
<td>0</td>
<td>0 = Blank</td>
</tr>
</tbody>
</table>

B. Preparation of the BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

\[(\# \text{ standards} + \# \text{ unknowns}) \times (\# \text{ replicates}) \times (\text{volume of WR per sample}) = \text{total volume WR required}\]

Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample:

\[(9 \text{ standards} + 3 \text{ unknowns}) \times (2 \text{ replicates}) \times (2\text{mL}) = 48\text{mL} \text{ WR required}\]

Note: 2.0mL of the WR is required for each sample in the test-tube procedure, while only 200µL of WR reagent is required for each sample in the microplate procedure.
2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50mL of Reagent A with 1mL of Reagent B.

**Note:** When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

### Procedure Summary (Test-tube Procedure, Standard Protocol)

**Test-tube Procedure (Sample to WR ratio = 1:20)**

1. Pipette 0.1mL of each standard and unknown sample replicate into an appropriately labeled test tube.
2. Add 2.0mL of the WR to each tube and mix well.
3. Cover and incubate tubes at selected temperature and time:
   - **Standard Protocol:** 37°C for 30 minutes (working range = 20-2000 µg/mL)
   - **RT Protocol:** RT for 2 hours (working range = 20-2000 µg/mL)
   - **Enhanced Protocol:** 60°C for 30 minutes (working range = 5-250 µg/mL)

**Notes:**
- Increasing the incubation time or temperature increases the net 562nm absorbance for each test and decreases both the minimum detection level of the reagent and the working range of the protocol.
- Use a water bath to heat tubes for either Standard (37°C incubation) or Enhanced (60°C incubation) Protocol. Using a forced-air incubator can introduce significant error in color development because of uneven heat transfer.

4. Cool all tubes to RT.
5. With the spectrophotometer set to 562nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

**Note:** Because the BCA assay does not reach a true end point, color development will continue even after cooling to RT. However, because the rate of color development is low at RT, no significant error will be introduced if the 562nm absorbance measurements of all tubes are made within 10 minutes of each other.

6. Subtract the average 562nm absorbance measurement of the Blank standard replicates from the 562nm absorbance measurement of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

### Microplate Procedure (Sample to WR ratio = 1:8)

1. Pipette 25µL of each standard or unknown sample replicate into a microplate well (working range = 20-2000µg/mL) (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041).

**Note:** If sample size is limited, 10µL of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2000µg/mL.

2. Add 200µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader.

Notes:
- Wavelengths from 540-590nm have been used successfully with this method.
- Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ratio to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562nm measurements are desired, increase the incubation time to 2 hours.
- Increasing the incubation time or ratio of sample volume to WR increases the net 562nm measurement for each well and lowers both the minimum detection level of the reagent and the working range of the assay. As long as all standards and unknowns are treated identically, such modifications may be useful.

5. Subtract the average 562nm absorbance measurement of the Blank standard replicates from the 562nm measurements of all other individual standard and unknown sample replicates.

6. Prepare a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No color in any tubes</td>
<td>Sample contains a copper chelating agent</td>
<td>Dialyze, desalt or dilute sample</td>
</tr>
<tr>
<td>Blank absorbance is OK, but standards and samples show less color than expected</td>
<td>Strong acid or alkaline buffer, alters working reagent pH</td>
<td>Dialyze, desalt, or dilute sample</td>
</tr>
<tr>
<td>Color of samples appears darker than expected</td>
<td>Protein concentration is too high</td>
<td>Dilute sample</td>
</tr>
<tr>
<td>Sample contains lipids or lipoproteins</td>
<td>Add 2% SDS to the sample to eliminate interference from lipids</td>
<td></td>
</tr>
<tr>
<td>Remove interfering substances from sample using Product No. 23215</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All tubes (including blank) are dark purple</td>
<td>Buffer contains a reducing agent</td>
<td>Dialyze or dilute sample</td>
</tr>
<tr>
<td>Buffer contains a thiol</td>
<td>Remove interfering substances from sample using Product No. 23215</td>
<td></td>
</tr>
<tr>
<td>Buffer contains biogenic amines (catecholamines)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Need to measure color at a different wavelength</td>
<td>Spectrophotometer or plate reader does not have 562nm filter</td>
<td>Color may be measured at any wavelength between 540nm and 590nm, although the slope of standard curve and overall assay sensitivity will be reduced</td>
</tr>
</tbody>
</table>

A. Interfering substances

Certain substances are known to interfere with the BCA assay including those with reducing potential, chelating agents, and strong acids or bases. Because they are known to interfere with protein estimation at even minute concentrations, avoid the following substances as components of the sample buffer:

- Ascorbic acid
- Catecholamines
- Creatinine
- Cysteine
- EGTA
- Impure sucrose
- Impure glycerol
- Hydrogen peroxide
- Hydrazides
- Iron
- Lipids
- Melibiose
- Impure sucrose
- Impure glycerol
- Hydrogen peroxide
- Hydrazides
- Iron
- Lipids
- Melibiose

Other substances interfere to a lesser extent with protein estimation using the BCA assay, and these have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Standard Test Tube Protocol are listed in Table 2 (see last page of Instructions). Substances were
compatible at the indicated concentration in the Standard Test Tube Protocol if the error in protein concentration estimation caused by the presence of the substance was less than or equal to 10%. The substances were tested using WR prepared immediately before each experiment. Blank-corrected 562nm absorbance measurements (for a 1000µg/mL BSA standard + substance) were compared to the net 562nm measurements of the same standard prepared in 0.9% saline. Maximum compatible concentrations will be lower in the Microplate Procedure where the sample to WR ratio is 1:8 (v/v).

Furthermore, it is possible to have a substance additive affect such that even though a single component is present at a concentration below its listed compatibility, a sample buffer containing a combination of substances could interfere with the assay.

### B. Strategies for eliminating or minimizing the effects of interfering substances

The effects of interfering substances in the Pierce BCA Protein Assay may be eliminated or overcome by one of several methods.

- Remove the interfering substance by dialysis or gel filtration.
- Dilute the sample until the substance no longer interferes. This strategy is effective only if the starting protein concentration is sufficient to remain in the working range of the assay upon dilution.
- Precipitate the proteins in the sample with acetone or trichloroacetic acid (TCA). The liquid containing the substance that interfered is discarded and the protein pellet is easily solubilized in ultrapure water or directly in the alkaline BCA WR. A protocol detailing this procedure is available from our website. Alternatively, Product No. 23215 may be used (see Related Thermo Scientific Products).
- Increase the amount of copper in the WR (prepare WR as 50:2 or 50:3, Reagent A:B), which may eliminate interference by copper-chelating agents.

**Note:** For greatest accuracy, the protein standards must be treated identically to the sample(s).

**Note:** The Thermo Scientific™ Pierce™ Detergent Compatible Bradford Assay Kit (Product No. 23246) is an alternative related product compatible with a wide range of detergents.

### Related Thermo Scientific Products

- 15041 Pierce 96-Well Plates, 100/pkg.
- 15075 Reagent Reservoirs, 200/pkg.
- 15036 Sealing Tape for 96-Well Plates, 100/pkg.
- 23209 Albumin Standard Ampules, 2mg/mL, 10 × 1mL ampules, containing bovine serum albumin (BSA)
- 23208 Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set, 7 × 3.5mL
- 23212 Bovine Gamma Globulin Standard, 2mg/mL, 10 × 1mL ampules
- 23213 Pre-Diluted Protein Assay Standards, (BGG) Set, 7 × 3.5mL aliquots
- 23246 Pierce Detergent Compatible Bradford Assay Kit
- 23235 Pierce Micro BCA Protein Assay Kit
- 23290 Pierce Quantitative Fluorometric Peptide Assay
- 23275 Pierce Quantitative Colorimetric Peptide Assay
- 23236 Coomassie Plus™ (Bradford) Assay Kit
- 23215 Compat-Able™ Protein Assay Preparation Reagent Set
- 23250 Pierce BCA Protein Assay Kit—Reducing Agent Compatible
Additional Information

A. Please visit our website for additional information including the following items:
   • Tech Tip #8: Eliminate interfering substances from samples for BCA Protein Assay

B. Alternative Total Protein Assay Reagents
   If interference by a reducing substance or metal-chelating substance contained in the sample cannot be overcome, try the
   Coomassie Plus (Bradford) Assay Kit (Product No. 23236), which is less sensitive to such substances.

C. Cleaning and Re-using Glassware
   Exercise care when re-using glassware. All glassware must be cleaned and given a thorough final rinse with ultrapure water.

D. Response characteristics for different proteins
   Each of the commonly used total protein assay methods exhibits some degree of varying response toward different proteins. These differences relate to amino acid sequence, pl, structure and the presence of certain side chains or prothetic groups that can dramatically alter the protein’s color response. Most protein assay methods use BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined (Figure 1). However, if great accuracy is required, prepare the standard curve from a pure sample of the target protein.

   Typical protein-to-protein variation in color response is listed in Table 3. All proteins were tested at 1000µg/mL using the
   30-minute/37°C Test Tube Protocol. The average net color response for BSA was normalized to 1.00 and the average net
   color response of the other proteins is expressed as a ratio to the response of BSA.

   ![Figure 1. Typical color response curves for BSA and BGG using the Standard Test Tube Protocol](image-url)

   **Table 3. Protein-to-protein variation.** Absorbance ratios (562nm) for proteins relative to BSA using the Standard Test Tube Protocol.

<table>
<thead>
<tr>
<th>Protein Tested</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin, bovine serum</td>
<td>1.00</td>
</tr>
<tr>
<td>Aldolase, rabbit muscle</td>
<td>0.85</td>
</tr>
<tr>
<td>α-Chymotrypsinogen, bovine</td>
<td>1.14</td>
</tr>
<tr>
<td>Cytochrome C, horse heart</td>
<td>0.83</td>
</tr>
<tr>
<td>Gamma globulin, bovine</td>
<td>1.11</td>
</tr>
<tr>
<td>IgG, bovine</td>
<td>1.21</td>
</tr>
<tr>
<td>IgG, human</td>
<td>1.09</td>
</tr>
<tr>
<td>IgG, mouse</td>
<td>1.18</td>
</tr>
<tr>
<td>IgG, rabbit</td>
<td>1.12</td>
</tr>
<tr>
<td>IgG, sheep</td>
<td>1.17</td>
</tr>
<tr>
<td>Insulin, bovine pancreas</td>
<td>1.08</td>
</tr>
<tr>
<td>Myoglobin, horse heart</td>
<td>0.74</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>0.93</td>
</tr>
<tr>
<td>Tranferrin, human</td>
<td>0.89</td>
</tr>
</tbody>
</table>

   **Average Ratio:** 1.02
   **Standard Deviation:** 0.15
   **Coefficient of Variation:** 14.7%
# Appendix H: Pierce™ BCA™ Protein Assay MicroTitre Plate Reader Protocol

## Table 2. Compatible substance concentrations in the Thermo Scientific Pierce BCA Protein Assay (see text for details).³

<table>
<thead>
<tr>
<th>Substance</th>
<th>Compatible Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salts/Buffers</strong></td>
<td></td>
</tr>
<tr>
<td>ACES, pH 7.8</td>
<td>25mM</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>1.5M</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1mM</td>
</tr>
<tr>
<td>Bis-Tris, pH 6.5</td>
<td>20mM</td>
</tr>
<tr>
<td>Borate (50mM), pH 8.5 (#28384)</td>
<td>undiluted</td>
</tr>
<tr>
<td>B-PER™ Reagent (#78248)</td>
<td>undiluted</td>
</tr>
<tr>
<td>Calcium chloride in TBS, pH 7.2</td>
<td>10mM</td>
</tr>
<tr>
<td>Na-Carbonate/Na-Bicarbonate (0.2M), pH 9.4 (#25382)</td>
<td>undiluted</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>100mM</td>
</tr>
<tr>
<td>CHES, pH 9.0</td>
<td>100mM</td>
</tr>
<tr>
<td>Na-Citrate (0.6M), Na-Carbonate (0.1M), pH 9.0 (#28388)</td>
<td>1.8 dilution*</td>
</tr>
<tr>
<td>Na-Citrate (0.6M), MOPS (0.1M), pH 7.5 (#28388)</td>
<td>1.8 dilution*</td>
</tr>
<tr>
<td>Cobalt chloride in TBS, pH 7.2</td>
<td>0.8mM</td>
</tr>
<tr>
<td>EPPS, pH 8.0</td>
<td>100mM</td>
</tr>
<tr>
<td>Ficin chloride in TBS, pH 7.2</td>
<td>10mM</td>
</tr>
<tr>
<td>Glycine-HCl, pH 2.8</td>
<td>100mM</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>4M</td>
</tr>
<tr>
<td>HEPES, pH 7.5</td>
<td>100mM</td>
</tr>
<tr>
<td>Imidazole, pH 7.0</td>
<td>50mM</td>
</tr>
<tr>
<td>MES, pH 6.1</td>
<td>100mM</td>
</tr>
<tr>
<td>MES (0.1M), NaCl (0.9%), pH 4.7 (#28390)</td>
<td>undiluted</td>
</tr>
<tr>
<td>MOPS, pH 7.2</td>
<td>100mM</td>
</tr>
<tr>
<td>Modified Dulbecco’s PBS, pH 7.4 (#28374)</td>
<td>undiluted</td>
</tr>
<tr>
<td>Nickel chloride in TBS, pH 7.2</td>
<td>10mM</td>
</tr>
<tr>
<td>PBS; Phosphate (0.1M), NaCl (0.15M), pH 7.2 (#28372)</td>
<td>undiluted</td>
</tr>
<tr>
<td>Pipes, pH 6.8</td>
<td>100mM</td>
</tr>
<tr>
<td>RIPA lysis buffer, 50mM Tris, 150mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0</td>
<td>undiluted</td>
</tr>
<tr>
<td>Sodium acetate, pH 4.8</td>
<td>200mM</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.2%</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>100mM</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1M</td>
</tr>
<tr>
<td>Sodium citrate, pH 4.8 or pH 6.4</td>
<td>200mM</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>100mM</td>
</tr>
<tr>
<td>Tricine, pH 8.0</td>
<td>25mM</td>
</tr>
<tr>
<td>Triethanolamine, pH 7.8</td>
<td>25mM</td>
</tr>
<tr>
<td>Tris</td>
<td>250mM</td>
</tr>
<tr>
<td>TBS; Tris (25mM), NaCl (0.15M), pH 7.6 (#28376)</td>
<td>undiluted</td>
</tr>
<tr>
<td>Tris (25mM), Glycine (192mM), pH 8.0 (#28380)</td>
<td>1:3 dilution*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance</th>
<th>Compatible Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detergents</strong>²</td>
<td></td>
</tr>
<tr>
<td>Brij™-35</td>
<td>5.0%</td>
</tr>
<tr>
<td>Brij-56, Brij-68</td>
<td>1.0%</td>
</tr>
<tr>
<td>CHAPS, CHAPSO</td>
<td>5.0%</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>5.0%</td>
</tr>
<tr>
<td>Octyl β-glucoside</td>
<td>5.0%</td>
</tr>
<tr>
<td>Nonidet P-40 (NP-40)</td>
<td>5.0%</td>
</tr>
<tr>
<td>Octyl β-D-glucopyranoside</td>
<td>5.0%</td>
</tr>
<tr>
<td>SDS</td>
<td>5.0%</td>
</tr>
<tr>
<td>Span™ 20</td>
<td>1.0%</td>
</tr>
<tr>
<td>Triton™ X-100</td>
<td>5.0%</td>
</tr>
<tr>
<td>Triton X-114, X-305, X-405</td>
<td>1.0%</td>
</tr>
<tr>
<td>Tween™-20, Tween-60, Tween-80</td>
<td>5.0%</td>
</tr>
<tr>
<td>Zwittergent™ 3-14</td>
<td>1.0%</td>
</tr>
</tbody>
</table>

## Chelating agents

- EDTA: 10mM
- EGTA: ---
- Sodium citrate: 200mM

## Reducing & Thiol-Containing Agents

- N-acetylglucosamine in PBS, pH 7.2: 10mM
- Ascorbic acid: ---
- Cysteine: ---
- Dithioerythritol (DTE): 1mM
- Dithiothreitol (DTT): 1mM
- Glucose: 10mM
- Melamine: ---
- 2-Mercaptoethanol: 0.01%
- Potassium thiocyanate: 3.0M
- Thimerosal: 0.01%

## Misc. Reagents & Solvents

- Acetone: 10%
- Acetonitrile: 10%
- Aprotinin: 10mL/G
- DMF, DMSO: 10%
- DMSO: 10%
- Ethanol: 10%
- Glucose (Fresh): 10%
- Hydrazides: ---
- Hydrides (NaBH₄ or NaCNBH₃): ---
- Hydrochloric Acid: 100mM
- Leupeptin: 10mL/G

---

³ For a more extensive list of substances, download Tech Tip # 68: Protein assay compatibility table from our website. This Tech Tip includes compatible substances for all of our protein assays and enables easy comparisons.

---

Dashed-line entry indicates that the material is incompatible with the assay.

Diluted with ultrapure water.

² Detergents were tested using high-purity Thermo Scientific™ Surface-Amps™ Products, which have low peroxide content.
Appendix H: Pierce™ BCA™ Protein Assay MicroTitre Plate Reader Protocol

Cited References


Product References


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Roche cOmplete™ Ultra Protease Inhibitor Cocktail
cOmplete ULTRA Tablets, Mini, EASYpack

Protease Inhibitor Cocktail Tablets in foil blister packs

For the inhibition of proteases during cell extraction and in lysates from mammalian, bacterial, yeast, or plant cells. Each tablet is sufficient for a volume of 10 ml extraction solution.

Cat. No. 05 892 970 001 30 tablets

Do not eat

1. What this Product Does

1.1 Properties
During cell lysis and purification, target proteins are at risk of being cleaved by proteases, which are naturally present in cells. With 8 powerful protease inhibitors, cOmplete ULTRA Tablets, Mini effectively inhibit a broad range of proteases to protect target proteins and to preserve their full length and structure.

cOmplete ULTRA Tablets, Mini have been optimized for lysates from yeast, E.coli, and several mammalian cell lines such as HEK-293 and CHO. As a result, cOmplete ULTRA Tablets, Mini protect proteins from serine, cysteine, and aspartic proteases as well as from metalloproteases in all of these lysates.

cOmplete ULTRA Tablets, Mini contain both irreversible and reversible inhibitors.

1.2 Contents
30 individually packed cOmplete ULTRA Tablets, Mini in foil blister packs. Each tablet gives a volume of 10 ml extraction solution.

1.3 Stability
• The tablets are stable at +2 to +8°C, stored dry, until the expiration date printed on the label.
• The stock solution in water is stable for 4 weeks, stored at +2 to +8°C, and for at least 4 weeks at 15 to 25°C as well.

1.4 Application
Used for the inhibition of serine, cysteine, and aspartic proteases in bacterial, yeast, plant, and mammalian cell extracts.

cOmplete ULTRA Tablets, Mini contain reversible protease inhibitors (see properties). For best protein protection we therefore recommend their addition continuously after each experiment step and not only during the initial purification steps.

cOmplete ULTRA Tablets, Mini contain EDTA (3.7 mg/tablet, yielding a 1 mM solution of EDTA in 10 ml). As a result, the extraction buffer should not contain divalent cations, such as Ca²⁺, Mg²⁺, or Mn²⁺; otherwise, the inhibition of the metalloproteases might be incomplete. If the downstream application involves purification of his-tagged proteins with IMAC (Immobilized Metal Affinity Chromatography) the cOmplete His-Tag Purification Resin and Columns can be used even with EDTA (see order information).

Unless explicitly stated otherwise, EDTA must be eliminated with all other IMAC matrices prior to the chromatography (e.g. by dialysis).

2. How to Use this Product

2.1 Handling
• Separate one individually packed unit from the rest of the blister.
• Wear gloves and carefully push the tablet through the foil packaging using the base of your thumb (not your fingernail) to prevent the breakage of tablets.

2.2 Direct Use with Lysates
One cOmplete ULTRA Tablet is sufficient for the inhibition of the proteolytic activity in 10 ml extraction solution. Tablets can be added directly to the extraction medium.

If very high proteolytic activity is present, two tablets may be used in 10 ml extraction solution.

2.3 Preparation of a Stock Solution (2 concentrated)
Alternatively to the direct use with lysates, a 2× stock solution can be prepared, with the following procedure:
• Add cOmplete ULTRA Tablet to 5 ml double-distilled water in a PE or PP tube.
• Vortex 3 min or until the tablet is completely dissolved.
• The stock solution is ready to use even if there are very fine particles still visible in solution. These particles do not interfere with the inhibition performance of the tablets.

• The stock solution is stable for 4 weeks, stored at +2 to +8°C, and for at least 4 weeks at 15 to 25°C as well.

• During storage of the 2x stock solution, the particles can sediment. Briefly vortex the stock solution prior to use.

3. Additional Information on this Product

3.1 Quality Control
The inhibitory activity of each lot is tested using a concentrated pancreas extract, as well as with Proteinase K, Trypsin and Thermolysin. The inhibitory effect is measured after one hour of incubation using Universal Protease Substrate (casein, resorufin-labeled).

3.2 References

For life science research only.
Not for use in diagnostic procedures.
4. Supplementary Information

4.1 Ordering Information

<table>
<thead>
<tr>
<th>Product</th>
<th>Pack Size</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>cOmplete ULTRA Tablets, Mini, EASYpack</td>
<td>30 tablets (for 10 ml each)</td>
<td>05 892 970 001</td>
</tr>
<tr>
<td>cOmplete ULTRA Tablets, Glass Vials</td>
<td>2 x 10 tablets (for 50 ml each)</td>
<td>05 892 988 001</td>
</tr>
<tr>
<td></td>
<td>6 x 10 tablets (for 50 ml each)</td>
<td>06 538 304 001</td>
</tr>
<tr>
<td>cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack</td>
<td>30 tablets (for 10 ml each)</td>
<td>05 892 791 001</td>
</tr>
<tr>
<td>cOmplete ULTRA Tablets, EDTA-free, Glass Vials</td>
<td>2 x 10 tablets (for 50 ml each)</td>
<td>05 892 953 001</td>
</tr>
<tr>
<td></td>
<td>6 x 10 tablets (for 50 ml each)</td>
<td>06 538 282 001</td>
</tr>
</tbody>
</table>

Related Products

<table>
<thead>
<tr>
<th>Product</th>
<th>Pack Size</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhosSTOP Tablets, EASYpack</td>
<td>10 tablets (for 10 ml each)</td>
<td>04 906 845 001</td>
</tr>
<tr>
<td>PhosSTOP Tablets, EASYpack</td>
<td>20 tablets (for 10 ml each)</td>
<td>04 906 837 001</td>
</tr>
<tr>
<td>Universal Protease Substrate (casein, resorufin-labeled)</td>
<td>15 mg</td>
<td>11 080 733 001</td>
</tr>
<tr>
<td></td>
<td>40 mg</td>
<td>11 734 334 001</td>
</tr>
<tr>
<td>cOmplete His-Tag Purification Resin</td>
<td>25 ml settled resin volume</td>
<td>05 893 682 001</td>
</tr>
<tr>
<td>cOmplete His-Tag Purification Column</td>
<td>5 x 1 ml prepacked column</td>
<td>06 781 543 001</td>
</tr>
</tbody>
</table>

The above product list includes only the most important products related to the product described. Refer to our latest catalog, visit www.roche-applied-science.com, or contact your local Roche representative to learn more about our entire product line.

4.2 Changes to Previous Version

Complete revision due to optimized formulation of the product.

Trademarks

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4.3 Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

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Proteins with Putative B-Cell Epitopes
### Appendix J: Proteins with Putative B-Cell Epitopes

<table>
<thead>
<tr>
<th>Protein</th>
<th>ID</th>
<th>Predicted MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCG148414 [Mus musculus]</td>
<td>EDL40777.1</td>
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## Appendix J: Proteins with Putative B-Cell Epitopes

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<th>Protein</th>
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</table>
Qiagen MiniKit Purification of Total RNA Protocol
Procedure

1. Excise the tissue sample from the animal or remove it from storage. Remove RNA Later stabilized tissues from the reagent using forceps. Determine the amount of tissue. Do not use more than 30 mg.

   Weighing tissue is the most accurate way to determine the amount.

   Note: If the tissues were stored in RNA Later Reagent at −20°C, be sure to remove any crystals that may have formed.

2. Follow either step 2a or 2b.

2a. For RNA Later stabilized tissues:

   If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed to step 3.

   If using only a portion of the tissue, cut it on a clean surface. Weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed to step 3.

   RNA in RNA Later stabilized tissues is protected during cutting and weighing of tissues at ambient temperature (15–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNA Later RNA Stabilization Reagent. Previously stabilized tissues can be stored at −80°C without the reagent.

2b. For unstabilized fresh or frozen tissues:

   If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed immediately to step 3.

   If using only a portion of the tissue, weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed immediately to step 3.

   RNA in harvested tissues is not protected until the tissues are treated with RNA Later RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in step 3. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

   Note: Remaining fresh tissues can be placed into RNA Later RNA Stabilization Reagent to stabilize RNA (see protocol on page 34). However, previously frozen tissues thaw too slowly in the reagent, preventing the reagent from diffusing into the tissues quickly enough to prevent RNA degradation.
3. Disrupt the tissue and homogenize the lysate in Buffer RLT (do not use more than 30 mg tissue) according to step 3a, 3b, 3c, or 3d.

See “Disrupting and homogenizing starting material”, pages 18–21, for more details on disruption and homogenization.

Note: Ensure that β-ME is added to Buffer RLT before use (see “Things to do before starting”).

After storage in RNA later RNA Stabilization Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem. For easier disruption and homogenization, we recommend using 600 µl Buffer RLT.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with the TissueLyser LT, TissueLyser II, and rotor–stator homogenizers generally results in higher RNA yields than with other methods.

### Table 8. Volumes of Buffer RLT for tissue disruption and homogenization

<table>
<thead>
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<th>Amount of starting material (mg)</th>
<th>Volume of Buffer RLT (µl)</th>
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<tbody>
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<td>350 or 600*</td>
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<td>20–30</td>
<td>600</td>
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* Use 600 µl Buffer RLT for tissues stabilized in RNA later RNA Stabilization Reagent or for difficult-to-lyse tissues.

3a. Disruption and homogenization using a rotor–stator homogenizer:

Place the weighed (fresh, frozen, or RNA later stabilized) tissue in a suitably sized vessel. Add the appropriate volume of Buffer RLT (see Table 8). Immediately disrupt and homogenize the tissue using a conventional rotor–stator homogenizer until it is uniformly homogeneous (usually 20–40 s). Proceed to step 4.

3b. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer:

Immediately place the weighed (fresh, frozen, or RNA later stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see Table 8). Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Proceed to step 4.
3c. Disruption using a mortar and pestle followed by homogenization using a needle and syringe:
Immediately place the weighed (fresh, frozen, or RNA later stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen-cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.
Add the appropriate volume of Buffer RLT (see Table 8), and homogenize by passing the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. Proceed to step 4.

3d. Disruption and homogenization using the TissueLyser LT or TissueLyser II:
See the TissueLyser LT Handbook or the TissueLyser Handbook. Then proceed to step 4.

4. Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to a new microcentrifuge tube (not supplied). Use only this supernatant (lysate) in subsequent steps.

In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.

5. Add 1 volume of 70% ethanol* to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.

Note: The volume of lysate may be less than 350 µl or 600 µl due to loss during homogenization and centrifugation in steps 3 and 4.

Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.†

Reuse the collection tube in step 7.
If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.†

Optional: If performing optional on-column DNase digestion (see “Eliminating genomic DNA contamination”, page 21), follow steps D1–D4 (page 67) after performing this step.

* Using 50% ethanol (instead of 70% ethanol) may increase RNA yields from liver samples.
† Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.
7. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.**

*Discard the flow-through.*

Reuse the collection tube in step 8.

**Note:** After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

Skip this step if performing optional on-column DNase digestion (page 67).

8. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.**

Discard the flow-through.

Reuse the collection tube in step 9.

**Note:** Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).

9. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.**

The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

**Note:** After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.**

12. **If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.**

If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 6 for safety information.
Applied Biosystems™ High-Capacity cDNA Reverse Transcription Protocol
High-Capacity cDNA Reverse Transcription Kits

Catalog Numbers 4368813, 4368814, 4374966, and 4374967

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit uses the random primer scheme for initiating cDNA synthesis. Random primers ensure that the first strand synthesis occurs efficiently with all species of RNA molecules present, including mRNA and rRNA. The kit has been tested extensively and validated against various RNA templates, including G/C-rich and A/U-rich RNA species. The effect of relative mRNA abundance was also examined. An essential requirement for the relative quantitation of cDNA is that the reverse transcriptase reaction generates products in a manner directly dependent on the amount of input RNA template. In all cases, quantitative conversion of mRNA and 18S ribosomal RNA species was observed.

Available kits

<table>
<thead>
<tr>
<th>Kit</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-Capacity cDNA Reverse Transcription Kit, 200 reactions</td>
<td>4368814</td>
</tr>
<tr>
<td>High-Capacity cDNA Reverse Transcription Kit, 1000 reactions</td>
<td>4368813</td>
</tr>
<tr>
<td>High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor, 200 reactions</td>
<td>4374966</td>
</tr>
<tr>
<td>High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor, 1000 reactions</td>
<td>4374967</td>
</tr>
</tbody>
</table>

Contents and storage

<table>
<thead>
<tr>
<th>Contents</th>
<th>Cat. Nos. 4368813 and 4374967</th>
<th>Cat. Nos. 4368814 and 4374966</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT Buffer, 1.0 mL</td>
<td>2 tubes</td>
<td>1 tube</td>
<td>-25°C to -15°C</td>
</tr>
<tr>
<td>10X RT Random Primers, 1.0 mL</td>
<td>2 tubes</td>
<td>1 tube</td>
<td></td>
</tr>
<tr>
<td>25X dNTP Mix (100 mM)</td>
<td>1 tube, 1.0 mL</td>
<td>1 tube, 0.2 mL</td>
<td></td>
</tr>
<tr>
<td>MultiScribe™ Reverse Transcriptase, 50 U/μL</td>
<td>1 tube, 1.0 mL</td>
<td>2 tubes, 0.1 mL</td>
<td></td>
</tr>
<tr>
<td>RNase Inhibitor, 100 μL</td>
<td>10 tubes</td>
<td>2 tubes</td>
<td></td>
</tr>
</tbody>
</table>

[1] Included in Cat. Nos. 4374966 and 4374967 only.

Workflow

1. Prepare 2X reverse transcription master mix
2. Add RNA to reverse transcription reactions
3. Perform reverse transcription in a thermal cycler
4. Use the reverse transcription reactions (cDNA) directly for quantitative or other PCR applications
5. Store the reverse transcription reactions (cDNA) at:
   - 2°C to 6°C for short-term storage
   - -25°C to -15°C for long-term storage

Reverse transcription reaction guidelines

The kit contains reagents that, when combined, form a 2X reverse transcription (RT) master mix. An equal volume of RNA sample should be added. To avoid RNase contamination, RNase-free reagents and consumables must be used.

Prepare the 2X master mix

1. Allow the kit components to thaw on ice.
2. Calculate the volume of components needed to prepare the required number of reactions.

Note: Prepare the RT master mix on ice.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume with RNase Inhibitor</th>
<th>Volume without RNase Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT Buffer</td>
<td>2.0 μL</td>
<td>2.0 μL</td>
</tr>
<tr>
<td>25X dNTP Mix (100 mM)</td>
<td>0.8 μL</td>
<td>0.8 μL</td>
</tr>
<tr>
<td>10X RT Random Primers</td>
<td>2.0 μL</td>
<td>2.0 μL</td>
</tr>
<tr>
<td>MultiScribe™ Reverse Transcriptase</td>
<td>1.0 μL</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>1.0 μL</td>
<td>—</td>
</tr>
<tr>
<td>Nuclease-free H₂O</td>
<td>3.2 μL</td>
<td>4.2 μL</td>
</tr>
<tr>
<td>Total per reaction</td>
<td>10.0 μL</td>
<td>10.0 μL</td>
</tr>
</tbody>
</table>

IMPORTANT! Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

3. Place the 2X RT master mix on ice and mix gently.

For Research Use Only. Not for use in diagnostic procedures.
Prepare the reverse transcription reactions
1. Pipette 10 μL of 2X RT master mix into each well of a 96-well reaction plate or individual tube.
2. Pipette 10 μL of RNA sample into each well, pipetting up and down two times to mix.
3. Seal the plates or tubes.
4. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

Program the thermal cycling conditions
Program the thermal cycler using the conditions below.

<table>
<thead>
<tr>
<th>Settings</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp.</td>
<td>25°C</td>
<td>37°C</td>
<td>85°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Time</td>
<td>10 minutes</td>
<td>120 minutes</td>
<td>5 minutes</td>
<td>∞</td>
</tr>
</tbody>
</table>

Limited product warranty
Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies’ General Terms and Conditions of Sale found on Life Technologies’ website at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.

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Table 1 Revision history of Pub. No. 4375222

<table>
<thead>
<tr>
<th>Revision</th>
<th>Date</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>13 April 2006</td>
<td>Baseline for this revision history</td>
</tr>
<tr>
<td>C</td>
<td>21 March 2016</td>
<td>Format, style, and legal updates</td>
</tr>
</tbody>
</table>

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For support visit thermofisher.com/support or email techsupport@lifetech.com
ThermoFisher Scientific NanoDrop™ 2000 Spectrophotometer Operating Instructions
3. Applications

Overview

UV-VIS spectrophotometry is easy for micro-volume samples using NanoDrop 2000/2000c spectrophotometers. The NanoDrop 2000/2000c is ideally suited for measuring:

- Nucleic acid concentration and purity of nucleic acid samples ≤15,000 ng/µL (dsDNA) without dilution
- General UV-Vis spectrophotometry
- Pured protein analysis (A280)
- Fluorescent dye corporation for Micro array and Proteins & Labels applications
- Expanded spectrum measurement and quantitation of fluorescent dye labeled proteins, conjugates, and metalloproteins
- Bradford Assay analysis of protein
- Lowry Assay analysis of protein
- Pierce 660 nm Assay analysis of protein
- Microbial cell culture measurements
- Kinetic methods
- Custom methods

Quick Start

1. Double-click the software icon and select the software application of interest from the right pane.
   - Select Add to report prior to a measurement to save the sample data to a workbook.

2. Establish a blank using the appropriate buffer.
   - Pedestal Option: Pipette 1-2 µL of the appropriate blanking solution onto the bottom pedestal, lower the arm and click Blank.
   - Cuvette Option (Model 2000c only): Select Use Cuvette. Insert the cuvette noting the direction of the light path indicated by the etched arrow. The optical beam (2 mm) is directed 8.5 mm above the bottom of the cuvette. Refer to the cuvette manufacturer for volume recommendations.

   Note: The arm must be down for all measurements, including those made with cuvettes. It is recommended that cuvettes be removed from the instrument prior to making a pedestal measurement to ensure that the pedestal arm can move to the proper starting position.

3. Wipe away the blank from the measurement pedestals using a dry, lint free laboratory wipe. Enter the sample ID in the appropriate field. Pipette 1 µL of sample and click Measure.

   Note: A fresh aliquot of sample should be used for each measurement.

After the measurement:

- Simply wipe the upper and lower pedestals using a dry lint free-laboratory wipe and the unit is ready for the next sample.

- When using the cuvette option, remove the cuvette, rinse thoroughly and dry between samples.

Although it is not necessary to blank between each sample, it is recommended that a new blank be taken every 30 minutes when measuring many samples in one measurement session. After 30 minutes, the time since the last blank measurement will be displayed in the bottom status bar.
### Measurement Ranges

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Lower Detection Limit</th>
<th>Upper Detection Limit</th>
<th>Typical Reproducibility</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleic Acids</strong></td>
<td>2 ng/µL (pedestal) ≤ 15,000 ng/µL (dsDNA)</td>
<td>2 - 100 ng/µL: ± 2 ng/µL &gt;100 ng/µL: ± 2%</td>
<td></td>
</tr>
<tr>
<td>Microarray</td>
<td>2 ng/µL (pedestal) ≤ 750 ng/µL (dsDNA)</td>
<td>2 - 100 ng/µL: ± 2 ng/µL &gt;100 ng/µL: ± 2%</td>
<td></td>
</tr>
<tr>
<td>Protein A280</td>
<td>0.10 mg/mL (purified BSA - pedestal) 0.010 mg/mL (purified BSA-cuvette) 400 mg/mL (purified BSA)</td>
<td>0.10 - 10 mg/mL: ± 0.10mg/mL &gt;10 mg/mL: ± 2%</td>
<td></td>
</tr>
<tr>
<td><strong>Proteins &amp; Labels</strong></td>
<td>0.10 mg/mL (purified BSA - pedestal) 0.010 mg/mL (purified BSA-cuvette) 20 mg/mL (purified BSA)</td>
<td>0.10 - 10 mg/mL: ± 0.10 mg/mL</td>
<td></td>
</tr>
<tr>
<td><strong>BCA</strong></td>
<td>8.0 mg/mL (1:1 reagent:sample volume) 0.20 mg/mL (20:1 reagent:sample volume)</td>
<td>2% over entire range 0.01 mg/mL over entire range</td>
<td></td>
</tr>
<tr>
<td><strong>Modified Lowry</strong></td>
<td>8.0 mg/mL (1:5 reagent:sample volume) 4.0 mg/mL (50:1 reagent:sample volume)</td>
<td>2% over entire range</td>
<td></td>
</tr>
<tr>
<td>Bradford</td>
<td>100-500 µg/mL: ± 25 ug/mL 500-8000 µg/mL: ± 5%</td>
<td>100-500 µg/mL: ± 25 ug/mL 500-8000 µg/mL: ± 5%</td>
<td></td>
</tr>
<tr>
<td>Pierce 660 nm</td>
<td>0.25 mg/mL (7:1 reagent:sample volume) 0.125 mg/mL (15:1 reagent:sample volume) 25-125 mg/mL &gt; 125 mg/mL</td>
<td>sample range 0.20-4.0 pmol/µL: ± 0.20 pmol/µL sample range &gt; 4.0 pmol/µL: ± 2%</td>
<td></td>
</tr>
<tr>
<td>Cy3, Cy3.5, Alexa Fluor 555 and Alexa Fluor 660</td>
<td>0.2</td>
<td>100</td>
<td>sample range 0.12-2.4 pmol/µL: ± 0.12 pmol/µL sample range &gt; 2.4 pmol/µL: ± 2%</td>
</tr>
<tr>
<td>Cy5, Cy5.5 and Alexa Fluor 647</td>
<td>0.12</td>
<td>60</td>
<td>sample range 0.40-8.0 pmol/µL: ± 0.40 pmol/µL sample range &gt; 8.0 pmol/µL: ± 2%</td>
</tr>
<tr>
<td>Alexa Fluor 488 and Alexa Fluor 594</td>
<td>0.4</td>
<td>215</td>
<td>sample range 0.30-6.0 pmol/µL: ± 0.30 pmol/µL sample range &gt; 6.0 pmol/µL: ± 2%</td>
</tr>
<tr>
<td>Alexa Fluor 546</td>
<td>0.3</td>
<td>145</td>
<td>sample range 0.30-6.0 pmol/µL: ± 0.30 pmol/µL sample range &gt; 6.0 pmol/µL: ± 2%</td>
</tr>
</tbody>
</table>

**Note:** Measurement ranges noted for dyes in the above are specific to pedestal measurements.
Nucleic Acid

Overview

Nucleic acid samples can be easily checked for concentration and quality using the NanoDrop 2000/2000c spectrophotometer. To measure nucleic acid samples select the Nucleic Acid application from the home page.

Nucleic Acid Calculations

For nucleic acid quantification, the Beer-Lambert equation is modified to use a factor with units of ng-cm/microliter. The modified equation used for nucleic acid calculations is the following:

\[ c = \frac{A \times \varepsilon}{b} \]

- \( c \) = the nucleic acid concentration in ng/microliter
- \( A \) = the absorbance in AU
- \( \varepsilon \) = the wavelength-dependent extinction coefficient in ng-cm/microliter
- \( b \) = the pathlength in cm

The generally accepted extinction coefficients for nucleic acids are:

- Double-stranded DNA: 50 ng-cm/µL
- Single-stranded DNA: 33 ng-cm/µL
- RNA: 40 ng-cm/µL

When the pedestal mode is selected, the NanoDrop 2000/2000c spectrophotometer uses short pathlengths between 1.0 mm to 0.05 mm to enable measurement of concentrated samples without dilution.

**Note:** Absorbance data shown in reports is archived as displayed on the software screen. The Nucleic Acid application absorbance values are normalized to a 1.0 cm (10.0 mm) path for all pedestal and cuvette measurements.

Measurement Concentration Ranges

The NanoDrop 2000/2000c will accurately measure purified dsDNA samples \(<15,000\) ng/µL without dilution. The software automatically utilizes the optimal pathlength to measure the absorbance of each sample. Refer to "Measurement Ranges" for additional information.

The small sample volume option is available when samples have 10 mm equivalent absorbance values of 3.0 or higher (>150 ng/µL dsDNA.)
Unique Screen Features

The right pane displays features specific to the Nucleic Acid application. Task bars in the left pane not described below are described in "Software Overview."

The spectral display shows data for the current sample normalized to a 10 mm path for all measurements including measurements made with any cuvette pathlength.

The following features are to the right of the spectral display:

- **Sample ID** - field into which a sample ID is entered. The appropriate sample ID should be entered prior to each measurement.
- **Type** - a drop down list from which the user may select the (color-keyed) type of nucleic acid being measured. Options include DNA-50 for dsDNA, RNA-40 for RNA, and ssDNA-33 for single-stranded DNA. Additional options include Oligo DNA and Oligo RNA which utilize the appropriate extinction coefficient based upon user-defined base sequences. The Custom option allows the user to enter an extinction coefficient between 15 and 150.
- **Conc** - concentration based on absorbance at 260 nm and the default or user defined extinction coefficient. Concentration units may be selected from the adjacent drop-down box. Refer to "Nucleic Acid Calculations" for more details.
- **A260** - displays absorbance at 260 nm normalized to a 10 mm pathlength.
- **A280** - displays absorbance at 280 nm normalized to a 10 mm pathlength.
- **260/280** - ratio of absorbance at 260 nm and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. See "260/280 Ratio" in "Diagnostics and Troubleshooting" for more details on factors that can affect this ratio.
Section 3 - Applications

- **260/230** - ratio of absorbance at 260 nm and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for a "pure" nucleic acid are often higher than the respective 260/280 values and are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

- **Baseline correction** - if selected, the default wavelength for the bichromatic normalization is 340 nm. The user can manually enter a different wavelength for the bichromatic normalization of the absorbance data. In either case, the baseline is automatically set to the absorbance value of the sample at the selected wavelength. All wavelength data will be referenced off this value.

  Note: If a baseline correction is not selected, the spectra may be offset from the baseline and the calculated concentration will change accordingly.

### Making Nucleic Acid Measurements

1. Select the **Nucleic Acid** application from the main menu. If the wavelength verification window appears, ensure the arm is down and click **OK**.

2. Select the type of sample to be measured from the **Type** drop-down list. The default setting is **DNA-50**.

3. Choose the concentration units from the drop-down list adjacent to the color coded concentration box. The default units are ng/µL.

4. A default wavelength of 340 nm is automatically used for a bichromatic normalization. Select an alternative reference wavelength or choose not to have the spectrum normalized by de-selecting the **baseline correction** box.

   - Select the file drop-down option **Use current settings as default** as a convenient way to limit set-up time for each new workbook.

5. Select **Add to report** to automatically include all measurements in the current report. The **Add to report** checkbox must be selected prior to a measurement to save the sample data to a workbook.

6. Select **Overlay spectra** to display multiple spectra at a time.

7. Establish a blank using the appropriate buffer. The blank solution generally is the buffer that the molecule of interest is suspended or dissolved in. This solution should be the same pH and of a similar ionic strength as the sample solution.

   - **Pedestal Option**: Pipette 1-2 µL of the appropriate blanking solution onto the bottom pedestal, lower the arm and click the **Blank** button.

   - **Cuvette Option (Model 2000c only)**: Insert the cuvette noting the direction of the light path indicated by the etched arrow. The optical beam (2 mm) is directed 8.5 mm above the bottom of the cuvette. Refer to the cuvette manufacturer for volume recommendations.

   Note: The arm must be down for all measurements, including those made with cuvettes. It is recommended that cuvettes be removed from the instrument prior to making a pedestal measurement to ensure that the pedestal arm can move to the proper starting position.

8. Enter a Sample ID in the appropriate field, load the first sample as described for the blank above and click **Measure**.

   Note: A fresh aliquot of sample should be used for each measurement.
After the measurement:

- Simply wipe the upper and lower pedestals using a dry laboratory wipe and the instrument is ready to measure the next sample.
- When using the cuvette option, remove the cuvette, rinse thoroughly and dry between samples.

Oligo Calc

Oligo Calc is used to calculate molecular weights, extinction coefficients, concentration factors and melting points for specific nucleic acid sequences. Selecting this Task bar will bring up two tabs:

- **Oligo Calc** - used to enter sequences of interest and select appropriate sample type variables.
- **Melting Points** - displays the calculated melting points of a DNA strand. This tab is available only for DNA sequences.

To use the Oligo Calc:

1. Use one of the following options to enter in a base sequence:
   - The buttons below the Base Sequence display.
   - The keyboard. (Only the A, C, G, T, and U keys will work to enter bases.)
   - Copy and paste a base sequence into the display from another application. (Only the letters A, C, G, T, and U can be pasted into the Base Sequence display.)
   - To clear the base sequence display, click **Clear** to the right of the display. Individual bases can only be manually deleted.
2. Select degree of phosphorylation, if applicable: Mono-phosphate for DNA, mono or tri-phosphate for RNA.
3. Select **Double-Stranded** if applicable. The complementary base sequence will be included in the analysis.
4. From the drop down list, select the type of nucleic acid to be analyzed. The default is DNA.
5. For additions to the base sequence select **Modification** and enter the molecular weight associated with the addition.

Oligo Calc analysis result fields include:

- **Molecular Weight** - displays calculated base sequence molecular weight
- **Extinction Coefficient** - displays the 260 nm wavelength-dependent extinction coefficient in ng-cm/µL.
- **Concentration Factor** - the constant, based on the extinction coefficient that is used to calculate the concentration of the base sequence.
- **Number of bases** - displays how many bases have been entered.
- **% GC** - shows the percentage of the total number of bases made up by guanine and cytosine.
To calculate the melting point of a DNA sequence:

1. Enter the base sequence as described above. If a base sequence has already been entered in the Oligo Calc Tab, then the base sequence box in the Melting Points tab will be auto-populated with that sequence.

2. Enter the appropriate values in the each of the boxes as described below:
   - **Oligo Molarity** - enters the oligo molarity of the sample. A default value of 10 uM is displayed, but can be changed to a value more appropriate for the sample.
   - **Cation Molarity** - enters the concentration of cations in the sample. A default value of 50 mM is displayed, but can be changed to a value more appropriate for the sample.
   - **% Formamide** - enters the percentage concentration of formamide in the sample. A default value of 0.00% is displayed, but can be changed to a value more appropriate for the sample.

Melting Point analysis result fields include:

- **Salt-Adjusted** - calculates the melting point of the base sequence without accounting for the effect of interaction between neighboring bases.
- **Nearest-Neighbor** - displays the melting point of the base sequence when the effect of interaction between neighboring bases is taken into account.
GLT-1d Specific qPCR Primer Evaluation
### Appendix N: GLT-1d Specific qPCR Primer Evaluation

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence Data</th>
<th>Length</th>
<th>Select</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-mGLT-1(1703)</td>
<td>CAACACCGAATGCAGGAAGAC</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>F-&lt;s/rGLT-1(1593/1524)</td>
<td>TGGACTGGCTGAGATAGAA</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Rev GLT-1d (R1)</td>
<td>GAAACAGAGAACCAGGTGCCCT</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>For GLT-1d (R1) (Mouse/Rat)</td>
<td>AGGGCACCTGGTCTCTGTTC</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Rev GLT-1d (R2) (Mouse/Rat)</td>
<td>ATGAAGAGACTGGATTTATGGAAGCA</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

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### Appendix N: GLT-1d Specific qPCR Primer Evaluation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Na+ (Molar)</th>
<th>Ct µM</th>
<th>Go Temp (°C)</th>
<th>Threshold Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>27.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Primer Dimer Results**

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Matches Score</th>
<th>Est. Tm (°C)</th>
<th>ΔG(@ 37.0 °C) (kcal/mol)</th>
<th>Lineup</th>
</tr>
</thead>
<tbody>
<tr>
<td>For GLT-1d (R1)(Mouse/Rat) AGGGCACCTGGTTCTCTGTTC vs R-m/rGLT1d (R1) GAACAGAGAACCAGGTGCCCT</td>
<td>21</td>
<td>21</td>
<td>62.1</td>
<td>-21.5</td>
</tr>
<tr>
<td>For GLT-1d (R1)(Mouse/Rat) AGGGCACCTGGTTCTCTGTTC vs R-m/rGLT1d (R1) GAACAGAGAACCAGGTGCCCT</td>
<td>21</td>
<td>21</td>
<td>62.1</td>
<td>-21.5</td>
</tr>
</tbody>
</table>

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Appendix N: GLT-1d Specific qPCR Primer Evaluation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Na+ (Molar)</th>
<th>Ct µM</th>
<th>ΔG Temp (°C)</th>
<th>Threshold Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.081</td>
<td>1.0</td>
<td>37.0</td>
<td>7</td>
<td></td>
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</table>

Hairpin Results

<table>
<thead>
<tr>
<th>Sequence Match</th>
<th>Matches</th>
<th>Score</th>
<th>Est. Tm (°C)</th>
<th>ΔG(@ 37.0 °C) (kcal/mol)</th>
<th>Lineup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Download CSV</td>
<td>Download Excel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Return To Primer Tools

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### Appendix N: GLT-1d Specific qPCR Primer Evaluation

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>Ex @ 260nm (cm⁻¹*M⁻¹)</th>
<th>Mass (g/mol)</th>
<th>fGC</th>
<th>ΔG(@ 37.0 °C) (kcal/mol)</th>
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</thead>
<tbody>
<tr>
<td>F-mGLT-1 (1703)</td>
<td>CAACACCGAATGCAGGAAGAC</td>
<td>21</td>
<td>59.9</td>
<td>214200</td>
<td>6442.3</td>
<td>0.52</td>
<td>-20.8</td>
</tr>
<tr>
<td>R-mGLT-1 (1703/1724)</td>
<td>TGATTCGCTCTTGGCAGGA</td>
<td>22</td>
<td>62.7</td>
<td>226600</td>
<td>6526.3</td>
<td>0.52</td>
<td>-20.8</td>
</tr>
<tr>
<td>For GLT-1d (K1) (Mouse/Rat)</td>
<td>AAGGAGTTCTCCTTTGTCGTC</td>
<td>22</td>
<td>62.7</td>
<td>187900</td>
<td>6404.3</td>
<td>0.57</td>
<td>-21.5</td>
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<tr>
<td>Rev GLT-1d (R1) (Mouse/Rat)</td>
<td>AGGGCACCTGGTTCTCTGTTC</td>
<td>21</td>
<td>62.1</td>
<td>274400</td>
<td>8107.4</td>
<td>0.38</td>
<td>-22.8</td>
</tr>
</tbody>
</table>

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BIOTAQ™ DNA Polymerase
BIOTAQ™ DNA Polymerase

**Features**
- Premium Taq polymerase suited to a wide range of applications
- Amplifies fragments ≤5kb
- Available as ready-to-use 2x reaction mixes (BioMix™/BioMix Red)

**Applications**
- Routine PCR applications
- TA cloning

**Description**
BIOTAQ™ is widely used by molecular biologists that have come to depend upon the robust performance of this reagent.

BIOTAQ is a highly purified thermostable DNA polymerase offering very high yield over a wide range of PCR templates, and is the ideal choice for most assays. BIOTAQ is a robust preparation and consistently delivers high yields with minimal background. BIOTAQ possesses 5-3' exorribonuclease activity and leaves an 'A' overhang such that the PCR product is suitable for effective integration into TA cloning vectors.

BIOTAQ is supplied with 10x NH₄-based reaction buffer, which provides optimal conditions for most experiments. Additional MgCl₂ is provided to allow reaction conditions to be adjusted to suit the template. The specificity and performance of BIOTAQ can be further improved with the use of 2x PolyMate Additive (Cat No. BIO-37041), which is designed for GC- or AT-rich DNA, "dirty" templates or sequences with a high level of secondary structure.

**Components:**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>500 Units</th>
<th>2500 Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOTAQ DNA Polymerase</td>
<td>1 x 100µl</td>
<td>5 x 100µl</td>
</tr>
<tr>
<td>10x NH₄ Reaction Buffer</td>
<td>2 x 1.2ml</td>
<td>10 x 1.2ml</td>
</tr>
<tr>
<td>50mM MgCl₂ Solution</td>
<td>1 x 1.2ml</td>
<td>5 x 1.2ml</td>
</tr>
</tbody>
</table>

**General Considerations:**
The optimum concentration of Mg²⁺ is 3mM and should only be increased above this if absolutely necessary. For first tests, use no less than 2.5 units of BIOTAQ in a 50µl reaction.

**PCR Reaction Conditions (for a 50µl reaction)**
- 10x NH₄ Reaction Buffer: 5µl
- 50mM MgCl₂ Solution: 1.5 - 4.0µl
- 100mM dNTP Mix (see below): 0.5 - 1.0µl
- Template and primers: As required
- BIOTAQ: 0.5 - 1µl
- Water (ddH₂O): Up to 50µl

**Notes:**
- Bioline operates under ISO 9001 Management System. BIOTAQ and its components are extensively tested for activity, processivity, efficiency, sensitivity, absence of nucleoside contamination and absence of nucleic acid contamination prior to release.
- Notes: Research use only. BIOTAQ, BioMix and HyperLadder are Trademarks of Bioline.

**Storage and stability:**
The BIOTAQ is shipped on dry/blue ice. On arrival store at -20°C for optimum stability. Repeated freeze/thaw cycles should be avoided.

**Expiry:**
When stored under the recommended conditions and handled correctly, full activity of the kit is retained until the expiry date on the outer box label.

**Safety precautions:**
Please refer to the material safety data sheet for further information.

**Unit definition:**
One unit is defined as the amount of enzyme that incorporates 10nmoles of dNTPs into acid-insoluble form in 30 minutes at 72°C.

**Quality control specifications:**
Bioline operates under ISO 9001 Management System. BIOTAQ and its components are extensively tested for activity, processivity, efficiency, sensitivity, absence of nucleoside contamination and absence of nucleic acid contamination prior to release.

**Notes:**
Research use only. BIOTAQ, BioMix and HyperLadder are Trademarks of Bioline.

**Citations:** ([http://www.bioline.com/h_scholar.asp](http://www.bioline.com/h_scholar.asp))


**Associated Products:**

<table>
<thead>
<tr>
<th>Product</th>
<th>Pack size</th>
<th>Cat. No.</th>
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<tbody>
<tr>
<td>dNTP Set</td>
<td>4 x 25µmol</td>
<td>BIO-39025</td>
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<tr>
<td>dNTP Mix</td>
<td>500µl</td>
<td>BIO-39028</td>
</tr>
<tr>
<td>2x PolyMate Additive</td>
<td>2 x 1.2ml</td>
<td>BIO-37041</td>
</tr>
<tr>
<td>HyperLadder™ 1kb</td>
<td>200 Lanes</td>
<td>BIO-33025</td>
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</table>
ThermoFisher Scientific Sybr\textsuperscript{TM} Gold Nucleic Acid Gel Stain
**SYBR® Gold Nucleic Acid Gel Stain**

**Table 1. Contents and storage information.**

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
<th>Concentration</th>
<th>Storage</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR® Gold nucleic acid gel stain</td>
<td>500 µL</td>
<td>Solution in high-quality, anhydrous DMSO *</td>
<td>≤–20°C</td>
<td>When stored as directed, stain stock solution is stable for 6 months to 1 year.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Desiccate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protect from light</td>
<td></td>
</tr>
</tbody>
</table>

* DMSO stock solution is a 10,000X concentrate.

**Number of labelings:** Sufficient dye is provided to stain at least 100 agarose or polyacrylamide minigels.

**Approximate fluorescence excitation/emission maxima:** 300, 495/537 nm, bound to nucleic acid

---

**Introduction**

Molecular Probes SYBR® Gold nucleic acid gel stain is the most sensitive fluorescent stain available for detecting double- or single-stranded DNA or RNA in electrophoretic gels, using standard ultraviolet transilluminators—surpassing even the sensitivity of our SYBR® Green gel stains in this application.¹ SYBR® Gold stain is a proprietary unsymmetrical cyanine dye that exhibits >1000-fold fluorescence enhancement upon binding to nucleic acids and has a high quantum yield (~0.6) upon binding to double- or single-stranded DNA or to RNA.¹ Excitation maxima for dye–nucleic acid complexes are at ~495 nm in the visible and ~300 nm, in the ultraviolet (Figure 1). The emission maximum is ~537 nm. SYBR® Gold stain is >10-fold more sensitive than ethidium bromide for detecting DNA and RNA in denaturing urea, glyoxal, and formaldehyde gels, even with 300 nm transillumination.¹ For detecting glyoxalated RNA, SYBR® Gold stain is 25–100 times more sensitive than ethidium bromide (Figure 2) and is by far the most sensitive stain available for this application.¹ SYBR® Gold stain has also been shown to be much more sensitive than SYBR® Green II stain for detecting single strand conformation polymorphism (SSCP) products.² SYBR® Gold stain penetrates thick and high percentage agarose gels rapidly, and even formaldehyde agarose gels do not require destaining, due to the low intrinsic fluorescence of the unbound dye. The presence of the dye in stained gels at standard staining concentrations does not interfere with restriction endonucleases, T4 DNA ligase, Taq polymerase, or with Southern or Northern blotting.³ Dye is readily removed from nucleic acids by ethanol precipitation, leaving pure templates available for subsequent manipulation or analysis.
Before You Begin

Materials Required but Not Provided

- TE, TBE, or TAE buffer
- SYBR® photographic filter (S7569)
- Ethanol
- Sodium acetate or ammonium acetate

Working with the SYBR® Gold Gel Stain

Before opening, each vial should be allowed to warm to room temperature and then briefly centrifuged in a microfuge to deposit the DMSO solution at the bottom of the vial. Be sure the dye solution is fully thawed before removing an aliquot.

Staining reagent diluted in buffer can be stored protected from light either at 4°C for several weeks or at room temperature for three or four days. Staining solutions prepared in water are less stable than those prepared in buffer and must be used within 24 hours to ensure maximal staining sensitivity. In addition, staining solutions prepared in buffers with pH below about 7.0 or above 8.5 are less stable and show reduced staining efficacy. We recommend storing aqueous working solutions in plastic rather than glass, as the stain may adsorb to glass surfaces.

Figure 1. Excitation and emission spectra of SYBR® Gold nucleic acid gel stain bound to double-stranded DNA.

Figure 2. Comparison of glyoxalated RNA stained with SYBR® Gold stain and with ethidium bromide. Identical twofold dilutions of glyoxalated E. coli 16S and 23S ribosomal RNA were separated on 1% agarose minigels using standard methods and stained for 30 minutes with SYBR® Gold stain in TBE buffer (A) or 0.5 µg/mL ethidium bromide in 0.1 M ammonium acetate (B). Both gels were subjected to 300 nm transillumination and photographed with Polaroid 667 black-and-white print film, through a SYBR® photographic filter (S7569) for the gel stained with SYBR® Gold dye and through an ethidium bromide gel stain photographic filter for the gel stained with ethidium bromide.
Caution
No data are available addressing the mutagenicity or toxicity of SYBR® Gold nucleic acid gel stain. Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and handled with appropriate care. The DMSO stock solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

Disposal
As with all nucleic acid reagents, solutions of SYBR® Gold stain should be disposed of in accordance with local regulations.

Experimental Protocol

The protocol below describes how to stain minigels with SYBR® Gold stain after electrophoresis. To stain agarose gels or polyacrylamide minigels, immerse the entire gel in staining solution. To stain large or extremely fragile polyacrylamide gels, leaving the gel on one of the gel plates and overlaying the gel with dye is probably a more practical procedure. When employing the dye overlay procedure, be sure to turn the stained gel upside down on the transilluminator prior to photography, as most glass plates will block at least some of the ultraviolet light, resulting in poor excitation of dye–nucleic acid complexes. Casting gels containing SYBR® Gold stain is not recommended, as the dye causes severe electrophoretic mobility retardation of nucleic acids in the gel.

Staining Minigels with SYBR® Gold Stain

1.1 Dilute the stock SYBR® Gold stain 10,000-fold to make a 1X staining solution.

- Dilute into TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5–8.0), TBE (89 mM Tris base, 89 mM boric acid, 1 mM EDTA, pH 8.0), or TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.5–8.0) buffer.

- Staining with SYBR® Gold stain is somewhat pH sensitive. For optimal sensitivity, verify that the pH of the staining solution at the temperature used for staining is between 7.0 and 8.5.

1.2 Incubate the gel in 1X staining solution for 10–40 minutes.

- Place the gel in the staining container, such as a petri dish, the lid of a pipet-tip box, or a polypropylene container.

- Add enough staining solution to completely cover the gel. A 50 mL volume is generally sufficient for staining most standard minigels. To stain large agarose gels, scale up the volume of staining solution in proportion to the increased gel volume and ensure that the entire gel is fully immersed during staining.

- Protect the staining solution from light by covering it with aluminum foil or by placing it in the dark.

- Prewashes of gels are not required, even for gels containing urea, formaldehyde, or glyoxalated samples. Removal of the glyoxal is also not necessary.

1.3 Agitate the gel gently at room temperature.

- The optimal staining time is typically 10–40 minutes, depending on the thickness of the gel and the percentage of agarose or polyacrylamide.
Viewing and Photographing the Gel

2.1 Illuminate the stained gel.

- Blue-light transilluminators, such as Invitrogen’s Safe Imager™ blue-light transilluminator also show excellent sensitivity with SYBR® Gold stained gels.
- Stained gels may also be viewed with 300 nm ultraviolet or 254 nm epi- or transillumination.
- Stained gels may also be visualized and analyzed with laser scanners. Maximum visible-light excitation is 495 nm.

2.2 Photograph the gel.

- Gels may be photographed using Polaroid 667 black-and-white print film and a SYBR® photographic filter (S7569). When using Polaroid film and this filter, we find that when exciting gels at 300 nm using the FOTO/UV 450 transilluminator (FotoDyne, Inc., Hartland, WI), a 0.5-1.0 second exposure with an f-stop of 5.6 is generally optimal. Optimal photographic conditions should be determined empirically for other light sources.
- With 254 nm epi-illumination, exposures of ~1 minute may be required for maximal sensitivity when using Polaroid film and the SYBR® filter.
- Generally, optimal exposure times for SYBR® Gold dye-stained gels are shorter than those required for identical gels stained with the SYBR® Green gel stains, due to the higher quantum yield of SYBR® Gold stain.
- Gels stained with the SYBR® Gold dye can also be documented using CCD cameras or laser scanner systems equipped with appropriate optical filters. Generally filters designed for use with the SYBR® Green gel stains are adequate. Optimal exposure times or other instrument settings will have to be determined empirically.

Removing SYBR® Gold Stain from Nucleic Acids

The SYBR® Gold stain can be efficiently removed from nucleic acids by simply precipitating the DNA or RNA with ethanol. More than 97% of the dye is removed by a single precipitation step. More than 99% of the dye is removed if ammonium acetate is used as the salt in the precipitation procedure.

3.1 Add one of the following salts to the nucleic acid sample, to the indicated final concentration: 200 mM NaCl, 300 mM sodium acetate (pH 5.2) or 2.0 M ammonium acetate. Mix gently.

3.2 Add two volumes of ice-cold absolute ethanol and mix well. Incubate at 0°C (on ice) for 30 minutes.

3.3 Pellet nucleic acids by centrifuging for at least 15 minutes at 10,000–12,000 × g.

3.4 Remove the supernatant and wash the pellet with 70% ethanol.

3.5 Centrifuge again to pellet nucleic acids.

3.6 Allow the pellet to air dry and resuspend as desired.
References


Product List

Current prices may be obtained from our website or from our Customer Service Department.

<table>
<thead>
<tr>
<th>Cat #</th>
<th>Product Name</th>
<th>Unit Size</th>
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<tr>
<td>S11494</td>
<td>SYBR® Gold nucleic acid gel stain <em>10,000X concentrate in DMSO</em></td>
<td>500 µL</td>
</tr>
<tr>
<td>S7569</td>
<td>SYBR® photographic filter</td>
<td>each</td>
</tr>
</tbody>
</table>

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

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Phone: (541) 465-8300
Fax: (541) 335-0504
probesorder@invitrogen.com

Toll-Free Ordering for USA:
Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

Technical Service:
8:00 am to 4:00 pm (Pacific Time)
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Toll-Free: (800) 438-2209
Fax: (541) 335-0338
probestech@invitrogen.com

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Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

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GoTaq™ qPCR Master Mix
GoTaq® qPCR Master Mix

Instructions for Use of Products

A6001 and A6002
GoTaq® qPCR Master Mix

GoTaq® qPCR Master Mix is a reagent system for quantitative PCR (qPCR). The system contains a new fluorescent DNA-binding dye that often exhibits greater fluorescence enhancement upon binding to double-stranded DNA (dsDNA) than SYBR® Green I.

GoTaq® qPCR Master Mix is provided as a simple-to-use, stabilized 2X formulation that includes all components for qPCR except sample DNA, primers and water. This formulation, which includes a proprietary dsDNA-binding dye, a low level of carboxy-X-rhodamine (CXR) reference dye (identical to ROX™ dye), GoTaq® Hot Start Polymerase, MgCl₂, dNTPs and a proprietary reaction buffer, produces optimal results in qPCR experiments. A separate tube of CXR Reference Dye is included for use with instruments that require a higher level of reference dye than that in the GoTaq® qPCR Master Mix.

Advantages of the GoTaq® qPCR Master Mix

**Dye:** The proprietary dye provides brighter dsDNA-dependent fluorescence than SYBR® Green I, with less PCR inhibition than SYBR® Green. The dye enables efficient amplification, resulting in earlier quantification cycle (Cq) values and an expanded linear range using the same filters and settings as SYBR® Green I. The CXR reference dye can be detected using the same filters and settings as those used for ROX™ dye.

**Polymerase/Buffer Formulation:** GoTaq® Hot Start Polymerase contains full-length Taq DNA polymerase bound to a proprietary antibody that prevents polymerase activity at room temperature. Thermal activation is achieved by incubating the assembled reaction at 95°C for 2 minutes. The proprietary polymerase/buffer formulation accommodates extended cycle numbers (45–50 cycles) and is compatible with thermal cycling programs that require extended activation (95°C for 10 minutes).

**Performance:** You can expect reliable performance with minimal lot-to-lot variation: efficient, sensitive and linear qPCR amplification over a wide dynamic range.
2. Product Components and Storage Conditions

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
<th>Cat.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq® qPCR Master Mix</td>
<td>200 reactions</td>
<td>A6001</td>
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</table>

For Research Use Only. Not for use in diagnostic procedures. Includes:

- 5 × 1ml GoTaq® qPCR Master Mix, 2X
- 100µl CXR Reference Dye
- 2 × 13ml Nuclease-Free Water

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
<th>Cat.#</th>
</tr>
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<tbody>
<tr>
<td>GoTaq® qPCR Master Mix</td>
<td>1,000 reactions</td>
<td>A6002</td>
</tr>
</tbody>
</table>

For Research Use Only. Not for use in diagnostic procedures. Includes:

- 25 × 1ml GoTaq® qPCR Master Mix, 2X
- 5 × 100µl CXR Reference Dye
- 10 × 13ml Nuclease-Free Water

**Storage Conditions:** GoTaq® qPCR Master Mix is shipped at –20°C. Upon arrival, store all components at –20°C, protected from light. For immediate use, components may be stored at 2–8°C, protected from light, for up to 3 months.
3. General Considerations

3.A. Spectral Properties
The proprietary dye in the GoTaq® qPCR Master Mix has spectral properties similar to those of SYBR® Green I: Excitation at 493nm and emission at 530nm. Instrument optical settings established for SYBR® Green I assays should be used with GoTaq® qPCR Master Mix. The CXR reference dye has the same spectral properties as ROX™: Excitation at 580nm and emission at 602nm. Use the instrument settings for ROX™ dye for reactions containing GoTaq® qPCR Master Mix.

3.B. Magnesium Chloride Concentration
The MgCl₂ concentration of the GoTaq® qPCR Master Mix has been determined to be optimal for performance. If desired, the MgCl₂ concentration may be adjusted using a PCR-grade stock solution (not provided).

3.C. Instrument Compatibility
GoTaq® qPCR Master Mix can be used with any real-time instrument capable of detecting SYBR® Green I or FAM™ dye. GoTaq® qPCR Master Mix contains a low level of CXR reference dye.

If you are using any of the following instruments, supplement the GoTaq® qPCR reaction mix with 0.5µl of CXR Reference Dye per 50µl reaction.

- Applied Biosystems 7000 Sequence Detection System
- Applied Biosystems 7300 Real-Time PCR System
- Applied Biosystems 7700 Sequence Detection System
- Applied Biosystems 7900HT Real-Time PCR System

4. GoTaq® qPCR Master Mix Protocol
If you are currently performing dye-based qPCR, the GoTaq® qPCR Master Mix can simply be substituted for your current master mix. For consistency within an experimental set, prepare a sufficient volume of reaction mix without template DNA for the DNA standard reactions and experimental sample reactions.

The protocol for a 50µl reaction is outlined below. Component volumes may be scaled as appropriate. This protocol assumes that 20% of the reaction volume is DNA template (e.g., 10µl of DNA template added to 40µl of reaction mix). If the volume of DNA template is more or less than 10µl, adjust the volume of Nuclease-Free Water accordingly so that the final reaction volume is 50µl.
4. GoTaq® qPCR Master Mix Protocol (continued)

Materials to Be Supplied by the User

- qPCR primers
- DNA template, positive control template standards
- barrier pipette tips
- sterile, nuclease-free, DNA-free tubes for reaction mix setup
- optical multwell reaction plates and adhesive film covers
- real-time thermal cycler
- optional: sterile MgCl₂ stock solution
- alternative normalization dye, if required (e.g., fluorescein for BioRad instruments)

1. Prepare the standard DNA dilution series and experimental samples in nuclease-free water. Store on ice until use. Carefully add 10µl of template (or water for no-template control reactions) to the appropriate wells of the reaction plate. Store plate at room temperature or on ice.

2. Thaw the GoTaq® qPCR Master Mix at room temperature. Gently vortex to ensure it is adequately mixed. Take care to avoid foaming or extended exposure to light. Store on ice until use.

3. Prepare the reaction mix, without template DNA, by combining the reagents in the order listed in Table 1. See Notes 1 and 2. Gently vortex to mix. Take care to avoid foaming.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per 50µl Reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq® qPCR Master Mix, 2X</td>
<td>25µl</td>
<td>1X</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>to a final volume of 40µl</td>
<td></td>
</tr>
<tr>
<td>upstream and downstream PCR primers</td>
<td>___µl</td>
<td>0.2µM or 0.05–0.9µM each</td>
</tr>
</tbody>
</table>

Notes:

a. See Section 3.C for a list of instruments that require addition of the CXR Reference Dye.

b. Some instruments such as the BioRad instruments require addition of a normalization dye (e.g., fluorescein).

4. Carefully add the appropriate volume of reaction mix prepared in Step 3 (e.g., 40µl of reaction mix for a 50µl reaction) to the appropriate wells of the reaction plate prepared in Step 1. Take care to avoid cross-contamination.

5. Seal the reaction plate, and centrifugate at low speed for 1 minute to bring all reaction components together and eliminate air bubbles.
6. Program the thermal cycler as per the manufacturer's instructions using the following guidelines:
   a. Select SYBR® or FAM™ as the detection dye for the entire plate.
   b. Select the ROX™ channel to detect CXR as the reference dye for the entire plate.
   c. Select a standard or fast, two-step, 40-cycle qPCR and dissociation program. Please note that the cycling parameters given below are offered as a guideline and may be modified as necessary.

<table>
<thead>
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<th># Cycles</th>
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<th>Fast Cycling Program</th>
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<tr>
<td>Hot-Start Activation</td>
<td>1</td>
<td>95°C for 2 minutes</td>
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<td>Denaturation</td>
<td>40</td>
<td>95°C for 15 seconds</td>
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<td>Annealing/Extension</td>
<td></td>
<td>60°C for 60 seconds</td>
</tr>
<tr>
<td>Dissociation</td>
<td>1</td>
<td>60–95°C</td>
</tr>
</tbody>
</table>

d. Designate that data will be collected during the annealing step of each cycle.

7. Place the plate into the instrument, and press "Start".
   When the run is complete, analyze the data using your usual procedures.
### 5. Related Products

#### DNA Purification

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
<th>Cat.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wizard® Genomic DNA Purification Kit</td>
<td>100 isolations × 300µl</td>
<td>A1120</td>
</tr>
<tr>
<td></td>
<td>500 isolations × 300µl</td>
<td>A1125</td>
</tr>
<tr>
<td></td>
<td>100 isolations × 10ml</td>
<td>A1620</td>
</tr>
<tr>
<td>Wizard® SV Genomic DNA Purification System</td>
<td>50 preps</td>
<td>A2360</td>
</tr>
<tr>
<td></td>
<td>250 preps</td>
<td>A2361</td>
</tr>
<tr>
<td>MagneSil® Blood Genomic, Max Yield System</td>
<td>1 × 96 preps</td>
<td>MD1360</td>
</tr>
<tr>
<td>MagneSil® ONE, Fixed Yield Blood Genomic System</td>
<td>1 × 96 preps</td>
<td>MD1370</td>
</tr>
<tr>
<td>MagneSil® Genomic, Fixed Tissue System</td>
<td>100 samples</td>
<td>MD1490</td>
</tr>
<tr>
<td>MagneSil® Genomic, Large Volume System</td>
<td>48 preps</td>
<td>A4082</td>
</tr>
<tr>
<td>Maxwell® 16 Blood DNA Purification Kit</td>
<td>48 preps</td>
<td>AS1010</td>
</tr>
<tr>
<td>Maxwell® 16 Cell DNA Purification Kit</td>
<td>48 preps</td>
<td>AS1020</td>
</tr>
<tr>
<td>Maxwell® 16 Tissue DNA Purification Kit</td>
<td>48 preps</td>
<td>AS1030</td>
</tr>
<tr>
<td>PureYield™ Plasmid Miniprep System</td>
<td>100 preps</td>
<td>A1223</td>
</tr>
<tr>
<td></td>
<td>250 preps</td>
<td>A1222</td>
</tr>
<tr>
<td>PureYield™ Plasmid Midiprep System</td>
<td>25 preps</td>
<td>A2492</td>
</tr>
<tr>
<td></td>
<td>100 preps</td>
<td>A2495</td>
</tr>
<tr>
<td></td>
<td>300 preps</td>
<td>A2496</td>
</tr>
<tr>
<td>PureYield™ Plasmid Maxiprep System</td>
<td>10 preps</td>
<td>A2392</td>
</tr>
<tr>
<td></td>
<td>25 preps</td>
<td>A2393</td>
</tr>
</tbody>
</table>

#### Accessory Products

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
<th>Cat.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maxwell® 16 Instrument</td>
<td>1 each</td>
<td>AS2000</td>
</tr>
<tr>
<td>Maxwell® 16 SEV Hardware Kit</td>
<td>1 each</td>
<td>AS1200</td>
</tr>
<tr>
<td>Maxwell® 16 LEV Hardware Kit</td>
<td>1 each</td>
<td>AS1250</td>
</tr>
</tbody>
</table>
Appendix Q: GoTaq™ qPCR Master Mix

RNA Purification

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
<th>Cat. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PureYield™ RNA Midiprep System</td>
<td>10 preps</td>
<td>Z3740</td>
</tr>
<tr>
<td></td>
<td>50 preps</td>
<td>Z3741</td>
</tr>
<tr>
<td>SV Total RNA Isolation System</td>
<td>10 preps</td>
<td>Z3101</td>
</tr>
<tr>
<td></td>
<td>50 preps</td>
<td>Z3100</td>
</tr>
<tr>
<td></td>
<td>250 preps</td>
<td>Z3105</td>
</tr>
<tr>
<td>Maxwell® 16 Total RNA Purification Kit</td>
<td>48 preps</td>
<td>AS1050</td>
</tr>
<tr>
<td>Maxwell® 16 Tissue LEV Total RNA Purification Kit</td>
<td>48 preps</td>
<td>AS1220</td>
</tr>
<tr>
<td>Maxwell® 16 Cell LEV Total RNA Purification Kit</td>
<td>48 preps</td>
<td>AS1225</td>
</tr>
</tbody>
</table>

For Laboratory Use.

Reverse Transcription System

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
<th>Cat. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription System</td>
<td>100 reactions</td>
<td>A3500</td>
</tr>
<tr>
<td>AMV Reverse Transcriptase</td>
<td>300u</td>
<td>M5101</td>
</tr>
<tr>
<td>M-MLV Reverse Transcriptase</td>
<td>10,000u</td>
<td>M1701</td>
</tr>
<tr>
<td></td>
<td>50,000u</td>
<td>M1705</td>
</tr>
<tr>
<td>M-MLV Reverse Transcriptase, RNase H Minus</td>
<td>10,000u</td>
<td>M5301</td>
</tr>
<tr>
<td>M-MLV Reverse Transcriptase, RNase H Minus, Point Mutation</td>
<td>2,500u</td>
<td>M3681</td>
</tr>
<tr>
<td></td>
<td>10,000u</td>
<td>M3682</td>
</tr>
<tr>
<td></td>
<td>50,000u</td>
<td>M3683</td>
</tr>
<tr>
<td>ImProm-II™ Reverse Transcription System</td>
<td>100 reactions</td>
<td>A3800</td>
</tr>
<tr>
<td>ImProm-II™ Reverse Transcriptase</td>
<td>10 reactions</td>
<td>A3801</td>
</tr>
<tr>
<td></td>
<td>100 reactions</td>
<td>A3802</td>
</tr>
<tr>
<td></td>
<td>500 reactions</td>
<td>A3803</td>
</tr>
</tbody>
</table>
6. Summary of Changes

The following changes were made to the 6/14 revision of this document:

1. Expired legal disclaimers were removed.
2. Related products section (Section 5) was updated.
3. Document design was updated.


(b) NOTICE TO PURCHASER: LIMITED LICENSE

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All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.
Invitrogen™ TRIzol™ Reagent Protocol
TRIzol™ Reagent

Catalog Numbers 15596026 and 15596018

Contents and storage

<table>
<thead>
<tr>
<th>Contents</th>
<th>Cat. No. 15596026 (100 reactions)</th>
<th>Cat. No. 15596018 (200 reactions)</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIzol™ Reagent</td>
<td>100 mL</td>
<td>200 mL</td>
<td>15–30°C</td>
</tr>
</tbody>
</table>

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. MLS: Fisher Scientific (fisherscientific.com) or other major laboratory supplier.

Table 1 Materials required for RNA, DNA, and protein isolation

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Equipment</strong></td>
<td></td>
</tr>
<tr>
<td>Centrifuge and rotor capable of reaching 12,000 × g and 4°C</td>
<td>MLS</td>
</tr>
<tr>
<td><strong>Tubes</strong></td>
<td></td>
</tr>
<tr>
<td>Polypropylene microcentrifuge tubes</td>
<td>MLS</td>
</tr>
<tr>
<td><strong>Reagents</strong></td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>MLS</td>
</tr>
</tbody>
</table>

Table 2 Materials required for RNA isolation

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Equipment</strong></td>
<td></td>
</tr>
<tr>
<td>Water bath or heat block at 55–60°C</td>
<td>MLS</td>
</tr>
<tr>
<td><strong>Reagents</strong></td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>MLS</td>
</tr>
<tr>
<td>Ethanol, 75%</td>
<td>MLS</td>
</tr>
<tr>
<td>RNase-free water of 0.5% SDS</td>
<td>MLS</td>
</tr>
<tr>
<td>Optional/RNase-free glycogen</td>
<td>MLS</td>
</tr>
</tbody>
</table>

Table 3 Materials required for DNA isolation

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagents</strong></td>
<td></td>
</tr>
<tr>
<td>Ethanol, 100%</td>
<td>MLS</td>
</tr>
<tr>
<td>Ethanol, 75%</td>
<td>MLS</td>
</tr>
<tr>
<td>0.1 M sodium citrate in 10% ethanol</td>
<td>MLS</td>
</tr>
<tr>
<td>8 mM NaOH</td>
<td>MLS</td>
</tr>
<tr>
<td>HEPES</td>
<td>MLS</td>
</tr>
</tbody>
</table>

Table 4 Materials required for protein isolation

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Equipment</strong></td>
<td></td>
</tr>
<tr>
<td>Optional/Dialysis membranes</td>
<td>MLS</td>
</tr>
<tr>
<td><strong>Reagents</strong></td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>MLS</td>
</tr>
<tr>
<td>Ethanol, 100%</td>
<td>MLS</td>
</tr>
<tr>
<td>0.3 M Guanidine hydrochloride in 75% ethanol</td>
<td>MLS</td>
</tr>
<tr>
<td>1% SDS</td>
<td>MLS</td>
</tr>
</tbody>
</table>

For Research Use Only. Not for use in diagnostic procedures.
Input sample requirements

**IMPORTANT!** Perform RNA isolation immediately after sample collection or quick-freeze samples immediately after collection and store at –80°C or in liquid nitrogen until RNA isolation.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Starting material per 1 mL of TRIzol Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissues[^1]</td>
<td>50–100 mg of tissue</td>
</tr>
<tr>
<td>Cells grown in monolayer</td>
<td>1 × 10⁶–1 × 10⁷ cells grown in monolayer in a 3.5-cm culture dish [^1] (mL)</td>
</tr>
<tr>
<td>Cells grown in suspension</td>
<td>5–10 × 10⁶ cells from animal, plant, or yeasty origin or 1 × 10⁷ cells of bacterial origin</td>
</tr>
</tbody>
</table>

[^1] Fresh tissues or tissues stored in RNAlater® Stabilization Solution (Cat. No. AM7020).

Procedural guidelines

- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- Use cold TRIzol™ Reagent if the starting material contains high levels of RNase, such as spleen or pancreas samples.
- Use disposable, individually wrapped, sterile plastic ware and sterile disposable RNase-free pipettes, pipette tips, and tubes.
- Wear disposable gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin; change gloves frequently, particularly as the protocol progresses from crude extracts to more purified materials.
- Always use proper microbiological aseptic techniques when working with RNA.
- Use RNaseZap® RNase Decontamination Solution (Cat. No. AM9780) to remove RNase contamination from work surfaces.

Lyse samples and separate phases

1. Lyse and homogenize samples in TRIzol™ Reagent according to your starting material.
   - **Tissues:**
     - Add 1 mL of TRIzol™ Reagent per 50–100 mg of tissue to the sample and homogenize using a homogenizer.
   - **Cell grown in monolayer:**
     - a. Remove growth media.

Isolate RNA

1. **Precipitate the RNA**
   - **(Optional)** If the starting sample is small (<10⁶ cells or <10 mg of tissue), add 5–10 μg of RNase-free glycogen as a carrier to the aqueous phase.
   - **Note:** The glycogen is co-precipitated with the RNA, but does not interfere with subsequent applications.
   - a. Add 0.3–0.4 mL of TRIzol™ Reagent per 1 × 10⁶–10⁷ cells directly to the culture dish to lyse the cells.
   - b. Add 0.75 mL of TRIzol™ Reagent per 0.25 mL of sample (5–10 × 10⁶ cells from animal, plant, or yeasty origin or 1 × 10⁷ cells of bacterial origin) to the pellet.
   - **Note:** Do not wash cells before addition of TRIzol™ Reagent to avoid mRNA degradation.
   - c. Pipet the lysate up and down several times to homogenize.
   - **Cells grown in suspension:**
     - a. Pellet the cells by centrifugation and discard the supernatant.
   - **Note:** The RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C.

2. **Wash the RNA**
   - a. Resuspend the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis.
   - **Note:** The RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C.
   - b. Vortex the sample briefly, then centrifuge for 5 minutes at 7500 × g at 4°C.
   - c. Discard the supernatant with a micropipettor.
   - **Vacuum or air dry the RNA pellet for 5–10 minutes.**

**IMPORTANT!** Do not dry the pellet by vacuum centrifuge. Do not let the RNA pellet dry to ensure total solubilization of the RNA. Partially dissolved RNA samples have an A230/280 ratio <1.6.
3 Solubilize the RNA

a. Resuspend the pellet in 20–50 µL of RNase-free water, 0.1 mM EDTA, or 0.5% SDS solution by pipetting up and down.

IMPORTANT! Do not dissolve the RNA in 0.5% SDS if the RNA is to be used in subsequent enzymatic reactions.

b. Incubate in a water bath or heat block set at 55–60°C for 10–15 minutes.

Proceed to downstream applications, or store the RNA at −70°C.

3 Determine the RNA yield

Determine the RNA yield using one of the following methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>1. Dilute sample in RNase-free water, then measure absorbance at 260 nm and 280 nm. 2. Calculate the RNA concentration using the formula A260 × dilution + 40 µg RNA/mL. 3. Calculate the A260/A280 ratio. A ratio of ~2 is considered pure. RNA samples can be quantified by absorbance without prior dilution using the NanoDrop™ Spectrophotometer. Refer to the instrument’s instructions for more information.</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>• Quantity RNA yield using the appropriate Qubit™ or Quant-iT™ RNA Assay Kit (Cat. Nos. Q32852, Q10210, Q33140, or Q10213). Refer to the kit’s instructions for more information.</td>
</tr>
</tbody>
</table>

Table 5 Typical RNA (A260/280 > 1.8) yields from various starting materials

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Quantity</th>
<th>RNA yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial cells</td>
<td>1 × 10^6 cells</td>
<td>8–15 µg</td>
</tr>
<tr>
<td>New tobacco leaf</td>
<td>—</td>
<td>73 µg</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>1 × 10^6 cells</td>
<td>5–7 µg</td>
</tr>
<tr>
<td>Skeletal muscles and brain</td>
<td>1 mg</td>
<td>1–1.5 µg</td>
</tr>
<tr>
<td>Placenta</td>
<td>1 mg</td>
<td>1–4 µg</td>
</tr>
<tr>
<td>Liver</td>
<td>1 mg</td>
<td>6–10 µg</td>
</tr>
<tr>
<td>Kidney</td>
<td>1 mg</td>
<td>3–6 µg</td>
</tr>
</tbody>
</table>

4 Isolate DNA

Isolate DNA from the interphase and the lower phenol-chloroform phase saved from “Lyse samples and separate phases” on page 2.

1 Precipitate the DNA

a. Remove any remaining aqueous phase overlying the interphase.

This is critical for the quality of the isolated DNA.

b. Add 0.3 mL of 100% ethanol per 1 mL of TRIzol™ Reagent used for lysis.

c. Cap the tube, mix by inverting the tube several times.

d. Incubate for 2–3 minutes.

e. Centrifuge for 5 minutes at 2000 × g at 4°C to pellet the DNA.

f. Transfer the phenol-ethanol supernatant to a new tube.

The supernatant is used for protein isolation (see “Isolate proteins” on page 40, if needed, and can be stored at −70°C for several months.

g. Resuspend the pellet in 1 mL of 0.1 M sodium citrate in 10% ethanol, pH 8.5, per 1 mL of TRIzol™ Reagent used for lysis.

h. Discard the supernatant with a micropipettor.

i. Repeat step 2a–step 2d once.

Note: Repeat step 2a–step 2d twice for large DNA pellets (>200 µg).

j. Resuspend the pellet in 1.5–2 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis.

k. Incubate for 10–20 minutes, mixing occasionally by gentle inversion.

Note: The DNA can be stored in 75% ethanol at several months at 4°C.

l. Centrifuge for 5 minutes at 2000 × g at 4°C.

m. Discard the supernatant with a micropipettor.

n. Vacuum or air dry the DNA pellet for 5–10 minutes.

IMPORTANT! Do not dry the pellet by vacuum centrifuge.
3 Solubilize the DNA

a. Resuspend the pellet in 0.3–0.6 mL of 8 mM NaOH by pipetting up and down.

Note: We recommend resuspending the DNA in a mild base because isolated DNA does not resuspend well in water or Tris buffer.

b. Centrifuge for 10 minutes at 12,000 × g at 4°C to remove insoluble materials.

c. Transfer the supernatant to a new tube, then adjust pH as needed with HEPES.

Proceed to downstream applications, or store the DNA at 4°C overnight. For longer-term storage at −20°C, adjust the pH to 7–8 with HEPES and add 1 mM EDTA.

4 Determine the DNA yield

Determine the DNA yield using one of the following methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Procedure</th>
</tr>
</thead>
</table>
| Absorbance   | 1. Dilute sample in water or buffer (pH >7.5), then measure absorbance at 260 nm and 280 nm.  
|              | 2. Calculate the DNA concentration using the formula A260 × dilution × 50 = µg DNA/mL.  
|              | 3. Calculate the A260/A280 ratio. A ratio of ~1.8 is considered pure. DNA samples can be quantified by absorbance without prior dilution using the NanoDrop™ Spectrophotometer. Refer to the instrument’s instructions for more information. |
| Fluorescence | 1. Prepare a wash solution consisting of 0.3 M guanidine hydrochloride in 95% ethanol.  
|              | 2. Resuspend the pellet in 2 mL of wash solution per 1 mL of TRIzol™ Reagent used for lysis.  
|              | 3. Incubate for 20 minutes. The proteins can be stored in wash solution for at least 1 month at 4°C or for at least 1 year at −20°C.  
|              | 4. Centrifuge for 5 minutes at 7500 × g at 4°C.  
|              | 5. Discard the supernatant with a micropipettor.  
|              | 6. Repeat step 2b–step 2e twice.  
|              | 7. Add 2 mL of 100% ethanol, then mix by vortexing briefly.  
|              | 8. Incubate for 20 minutes.  
|              | 9. Centrifuge for 5 minutes at 7500 × g at 4°C.  
|              | 10. Discard the supernatant with a micropipettor.  
|              | 11. Air dry the protein pellet for 5–10 minutes. Do not dry the pellet by vacuum centrifuge. |

Table 6 Typical DNA (A_{260/280} of 1.6–1.8) yields from various starting materials

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Quantity</th>
<th>DNA yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts</td>
<td>1 × 10⁶ cells</td>
<td>5–7 µg</td>
</tr>
<tr>
<td>Cultured cells, mammal</td>
<td>1 × 10⁶ cells</td>
<td>5–7 µg</td>
</tr>
<tr>
<td>Skeletal muscles and brain</td>
<td>1 mg</td>
<td>2–3 µg</td>
</tr>
<tr>
<td>Placenta</td>
<td>1 mg</td>
<td>2–3 µg</td>
</tr>
<tr>
<td>Liver</td>
<td>1 mg</td>
<td>3–4 µg</td>
</tr>
<tr>
<td>Kidney</td>
<td>1 mg</td>
<td>3–4 µg</td>
</tr>
</tbody>
</table>

Isolate proteins

Isolate the proteins from the phenol-ethanol supernatant saved from “Precipitate the DNA” on page 3 using either “Precipitate the proteins” on page 4 or “Dialyse the proteins” on page 5.

1 Precipitate the proteins

a. Add 1.5 mL of isopropanol to the phenol-ethanol supernatant per 1 mL of TRIzol™ Reagent used for lysis.

b. Incubate for 10 minutes.

c. Centrifuge for 10 minutes at 12,000 × g at 4°C to pellet the proteins.

d. Discard the supernatant with a micropipettor.

2 Wash the proteins

a. Prepare a wash solution consisting of 0.3 M guanidine hydrochloride in 95% ethanol.

b. Resuspend the pellet in 2 mL of wash solution per 1 mL of TRIzol™ Reagent used for lysis.

c. Incubate for 20 minutes.

Note: The proteins can be stored in wash solution for at least 1 month at 4°C or for at least 1 year at −20°C.

d. Centrifuge for 5 minutes at 7500 × g at 4°C.

e. Discard the supernatant with a micropipettor.

f. Repeat step 2b–step 2e twice.

g. Add 2 mL of 100% ethanol, then mix by vortexing briefly.

h. Incubate for 20 minutes.

i. Centrifuge for 5 minutes at 7500 × g at 4°C.

j. Discard the supernatant with a micropipettor.

k. Air dry the protein pellet for 5–10 minutes.

IMPORTANT! Do not dry the pellet by vacuum centrifuge.

3 Solubilize the proteins

a. Resuspend the pellet in 200 µL of 1% SDS by pipetting up and down.

Note: To ensure complete resuspension of the pellet, we recommend that you incubate the sample at 50°C in a water bath or heat block.

b. Centrifuge for 10 minutes at 10,000 × g at 4°C to remove insoluble materials.

c. Transfer the supernatant to a new tube.

Proceed directly to downstream applications, or store the sample at −20°C.
• Measure protein concentration by Bradford assay.

Note: SDS concentration must be <0.1%.

Dialyse the proteins
1. Load the phenol-ethanol supernatant into the dialysis membrane.
   Note: The phenol-ethanol solution can dissolve some types of dialysis membranes (cellulose ester, for example). Test dialysis tubing with the membrane to assess compatibility before starting.
2. Dialyze the sample against 3 changes of 0.1% SDS at 4°C. Make the first change of solution after 16 hours, the second change 4 hours later (at 20 hours), and the final change 2 hours later (at 22 hours).
   Note: A SDS concentration of at least 0.1% is required to resolubilize the proteins from the pellet. If desired, the SDS can be diluted after solubilization.
3. Centrifuge the dialysate for 10 minutes at 10,000 × g at 4°C.
4. Transfer the supernatant containing the proteins to a new tube.
5. (Optional) Solubilize the pellet by adding 100 µL of 1% SDS and 100 µL of 8 M urea.
   Proceed directly to downstream applications, or store the sample at –20°C.

Troubleshooting

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>A lower yield than expected is observed</td>
<td>The samples were incompletely homogenized or lysed.</td>
<td>Decrease the amount of starting material.</td>
</tr>
<tr>
<td>The sample is degraded</td>
<td>The pellet was incompletely solubilized</td>
<td>Increase the solubilization rate by pipetting the sample repeatedly, and heat the sample to 50–60°C.</td>
</tr>
<tr>
<td>The RNA or DNA is contaminated</td>
<td>The interphase/organic phase is pipetted up with the aqueous phase.</td>
<td>Do not attempt to draw off the entire aqueous layer after phase separation.</td>
</tr>
<tr>
<td>The DNA A&lt;sub&gt;260/280&lt;/sub&gt; ratio is low</td>
<td>The aqueous phase is incompletely removed.</td>
<td>Remove remnants of the aqueous phase prior to DNA precipitation.</td>
</tr>
<tr>
<td>The RNA A&lt;sub&gt;260/280&lt;/sub&gt; ratio is low</td>
<td>The DNA pellet is insufficiently washed with 0.1 M sodium citrate in 10% ethanol.</td>
<td>Make sure pellet is washed with 0.1 M sodium citrate in 10% ethanol.</td>
</tr>
<tr>
<td>The RNA A&lt;sub&gt;260/280&lt;/sub&gt; ratio is low</td>
<td>Sample was homogenized in an insufficient volume of TRIzol™ Reagent.</td>
<td>Add the appropriate amount of TRIzol™ Reagent for your sample type.</td>
</tr>
<tr>
<td>The DNA A&lt;sub&gt;260/280&lt;/sub&gt; ratio is low</td>
<td>The organic phase is incompletely removed.</td>
<td>Do not attempt to draw off the entire aqueous layer after phase separation.</td>
</tr>
<tr>
<td>Phenol was not sufficiently removed from the DNA preparation.</td>
<td>Wash the DNA pellet one additional time in 0.1 M sodium citrate in 10% ethanol.</td>
<td></td>
</tr>
</tbody>
</table>

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References
Appendix R: TRIzol™ Reagent Protocol

The information in this guide is subject to change without notice.

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Revision history: Pub. No. MAN00001271

<table>
<thead>
<tr>
<th>Revision</th>
<th>Date</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>A.0</td>
<td>09 November 2016</td>
<td>Added references to Phasemaker™ Tubes</td>
</tr>
<tr>
<td>Baseline</td>
<td>13 December 2012</td>
<td>Baseline for revision</td>
</tr>
</tbody>
</table>

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Invitrogen™ SuperScript™ III Reverse Transcriptase Protocol
SuperScript™ III Reverse Transcriptase

**Description**

SuperScript™ III Reverse Transcriptase is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability. The enzyme is purified to near homogeneity from *E. coli* containing the modified *pol* gene of Moloney Murine Leukemia Virus (1,2). The enzyme can be used to synthesize first-strand cDNA at temperatures up to 55°C, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases. It can generate cDNA from 100 bp to >12 kb.

**Component**

<table>
<thead>
<tr>
<th>Component</th>
<th>2,000 U Kit</th>
<th>10,000 U Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>SuperScript™ III RT (200 U/µl)</td>
<td>10 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>5X First-Strand Buffer*</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>500 µl</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

*{250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM MgCl₂}*

**Unit Definition**

One unit incorporates 1 nmol of dTTP into acid-precipitable material in 10 min at 37°C using poly(A)•oligo(dT)₂⁵ as template-primer (3).

**Storage Buffer**

20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% (v/v) NP-40, 50% (v/v) glycerol

**Storage**

Store all components at -20°C (non-frost-free). Thaw 5X First-Strand Buffer and 0.1 M DTT at room temperature just prior to use and refreeze immediately.

Part no. 18080.pps

Rev. date: 7 Dec 2004
First-Strand cDNA Synthesis
The following 20-µl reaction volume can be used for 10 pg–5 µg of total RNA or 10 pg–500 ng of mRNA.

1. Add the following components to a nuclease-free microcentrifuge tube:
   - 1 µl of oligo(dT)$_{20}$ (50 µM); or 200–500 ng of oligo(dT)$_{12-18}$; or 50–250 ng of random primers; or 2 pmol of gene-specific primer
   - 10 pg–5 µg total RNA or 10 pg–500 ng mRNA
   - 1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH)
   - Sterile, distilled water to 20 µl

2. Heat mixture to 65°C for 5 minutes and incubate on ice for at least 1 minute

3. Collect the contents of the tube by brief centrifugation and add:
   - 4 µl 5X First-Strand Buffer
   - 1 µl 0.1 M DTT
   - 1 µl RNaseOUT™ Recombinant RNase Inhibitor (Cat. no. 10777-019, 40 units/µl). Note: When using less than 50 ng of starting RNA, the addition of RNaseOUT™ is essential.
   - 1 µl of SuperScript™ III RT (200 units/µl)*
   - *If generating cDNA longer than 5 kb at temperatures above 50°C using a gene-specific primer or oligo(dT)$_{20}$, the amount of SuperScript™ III RT may be raised to 400 U (2 µl) to increase yield.

4. Mix by pipetting gently up and down. If using random primers, incubate tube at 25°C for 5 minutes.

5. Incubate at 50°C for 30–60 minutes. Increase the reaction temperature to 55°C for gene-specific primer. Reaction temperature may also be increased to 55°C for difficult templates or templates with high secondary structure.

6. Inactivate the reaction by heating at 70°C for 15 minutes.

The cDNA can now be used as a template for amplification in PCR. However, amplification of some PCR targets (those >1 kb) may require the removal of RNA complementary to the cDNA. To remove RNA complementary to the cDNA, add 1 µl (2 units) of *E. coli* RNase H and incubate at 37°C for 20 minutes.
PCR Reaction
The following example reaction is recommended as a starting point:

1. Add the following to a PCR reaction tube:
   10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl] 5 µl
   50 mM MgCl₂* 1.5 µl
   10 mM dNTP Mix 1 µl
   Sense primer (10 µM) 1 µl
   Antisense primer (10 µM) 1 µl
   Taq DNA polymerase (5 U/µl) 0.4 µl
   cDNA (from first-strand reaction) 2 µl
   Autoclaved, distilled water to 50 µl
2. Mix gently and layer 1–2 drops (~50 µl) of silicone oil over the reaction. (Note: The addition of silicone oil is unnecessary in thermal cyclers equipped with a heated lid.)
3. Heat reaction to 94°C for 2 minutes to denature.
4. Perform 15–40 cycles of PCR. Annealing and extension conditions are primer and template dependent and must be determined empirically. *Optimal concentration of MgCl₂ needs to be determined empirically for each template-primer pair.

Quality Control
This product has passed the following quality control assays: SDS-polyacrylamide gel analysis for purity; functional absence of endodeoxyribonuclease, 3’ and 5’ exodeoxyribonuclease, and ribonuclease activities; yield and length of cDNA product.

References
Related Products

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo(dT)$_{20}$ Primer (50 µM)</td>
<td>50 µl</td>
<td>18418-020</td>
</tr>
<tr>
<td>Oligo(dT)$_{12-18}$ Primer</td>
<td>25 µg</td>
<td>18418-012</td>
</tr>
<tr>
<td>Random Primers</td>
<td>A$_{260}$ units</td>
<td>48190-011</td>
</tr>
<tr>
<td>Custom Gene-Specific Primers</td>
<td>visit <a href="http://www.invitrogen.com/oligos">www.invitrogen.com/oligos</a></td>
<td></td>
</tr>
<tr>
<td>10 mM dNTP Mix</td>
<td>100 µl</td>
<td>18427-013</td>
</tr>
<tr>
<td>DEPC-treated Water</td>
<td>4 × 1.25 ml</td>
<td>10813-012</td>
</tr>
<tr>
<td>RNAseOUT™ Recombinant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease Inhibitor (40 U/µl)</td>
<td>5,000 units</td>
<td>10777-019</td>
</tr>
<tr>
<td>RNase H</td>
<td>30 units</td>
<td>18021-014</td>
</tr>
<tr>
<td>Platinum® Taq DNA Polymerase</td>
<td>100 units</td>
<td>10966-018</td>
</tr>
</tbody>
</table>

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