In-gel proteomics: Optimisation of Coomassie Brilliant Blue staining as a fluorescent alternative for sensitive protein detection

A thesis submitted for the degree of
Doctor of Philosophy

by

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STATEMENT OF AUTHENTICITY

This thesis is submitted in fulfilment of the requirements for the Doctor of Philosophy at the University of Western Sydney; School of Medicine. The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not previously submitted this material, either in full or in part, for a degree at this or any other institution. Unless otherwise stated, all of the data and observations presented here are the results of my own work.

...........................................

Victoria Jayne Gauci

March 2012
ABSTRACT

Gel electrophoresis, particularly one-dimensional electrophoresis (1DE) and two-dimensional electrophoresis (2DE), remain among the most widely used methods for resolving and analysing a variety of proteomes. Detection of the resulting proteome map relies on the stain employed (i.e. Coomassie Brilliant Blue (CBB) or SYPRO Ruby (SR) in addition to many others). Fluorescent in-gel protein stains are generally preferred due to their higher sensitivity, reduced background interference, and wider dynamic range.

Although traditionally used densitometrically, CBB has been shown to possess fluorescent properties. Recently, it was noted that infrared detection of CBB stained proteins was similar to SR which suggested a competitive alternative for sensitive fluorescent staining. Systematic characterisation of numerous CBB formulations in the Coorssen Lab identified BioSafe (Bio-Rad) and the Neuhoff formulation (NG) as superior performers; however, application to native proteomes saw slightly poorer detection in comparison to SR. Sub-optimal performance of the CBB stains tested might well have been due to the standardised protocol applied at the time.

Using protein standards and 1DE, the protocol for both BioSafe and NG were optimised to improve selectivity without affecting sensitivity; the resulting linear dynamic range for BioSafe and NG were similar to that of SR. Although the capacity for detection was investigated using purified proteins, the ultimate goal of this research program is the improved detection of native proteomes. 2DE analyses of mouse brain and A. thaliana (leaf) proteomes indicate superior total spot detection using CBB, in particular NG, relative to SR and BioSafe. Thus, although SR is
widely employed for sensitive protein detection, the same or better analyses can be achieved using CBB, at a fraction of the cost.

NOTE: The bulk of this thesis concentrates on quantitative proteomics with the focus on optimising Coomassie Brilliant Blue staining for competitive sensitive fluorescent detection of proteins in-gel. However, initially my PhD candidature was focussed on yeast research, in particular investigating the stress regulated transcription factor complex Msn2/4p and to determine whether this complex is involved during zinc deficiency. This work was published in FEMS Yeast Research in 2009 and is presented in Chapter 4 of this thesis.
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Thank you.

Victoria Jayne Gauci

University of Western Sydney

March 2012
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>% C</td>
<td>Weight percent concentration of bis acrylamide per total acrylamide (w/w %)</td>
</tr>
<tr>
<td>% T</td>
<td>Total concentration of acrylamide (w/v %)</td>
</tr>
<tr>
<td>° C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>1DE</td>
<td>One-dimensional electrophoresis</td>
</tr>
<tr>
<td>2DE</td>
<td>Two-dimensional electrophoresis</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AS</td>
<td>Ammonium sulphate</td>
</tr>
<tr>
<td>BGAL</td>
<td>β-galactosidase from Escherichia coli (isolated protein standard)</td>
</tr>
<tr>
<td>BioSafe</td>
<td>BioSafe Coomassie Stain (commercially available from Bio-Rad)</td>
</tr>
<tr>
<td>BN-PAGE</td>
<td>Blue Native-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin (isolated protein standard)</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic Anhydrase from Bovine Erythrocyte (isolated protein standard)</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie Brilliant Blue G-250 (a dye for in-gel protein staining)</td>
</tr>
<tr>
<td>cCBB</td>
<td>Colloidal Coomassie Brilliant Blue (colloidal suspension of Coomassie dye)</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate</td>
</tr>
<tr>
<td>CN-PAGE</td>
<td>Clear Native-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton, unit of mass</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Deep Purple</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
</tr>
<tr>
<td>g</td>
<td>Units of gravity, units used to express relative centrifugal force</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>H₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>H₃PO₄</td>
<td>Phosphoric acid</td>
</tr>
<tr>
<td>HAc</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
</tbody>
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h
ICAT Isotope-coded affinity tags
ID Identification
IEF Isoelectric focussing
IMPS Integrated Measure of Practical Sensitivity
IPG Immobilised pH gradient
IPV Inter-Protein Variability (based on fluorescence signal intensity)
IRF InfraRed Fluorescence (detection method for Coomassie)
kDa Kilodalton, unit of mass
LDR Linear Dynamic Range
LLD Lowest Limit of Detection
LP Longpass
LPS Lowest practical sensitivity (the lowest concentration of a protein that could be statistically determined on the raw image)
LYS Lysozyme from Chicken Egg White (isolated protein standard)
M Molarity, an expression of concentration. Moles per litre.
m/z Mass to charge ratio: m represents the molecular mass and z the number of elementary charges carried by the ion
MALDI Matrix-Assisted Laser Desorption Ionisation
MeOH Methanol
min Minute(s)
MS Mass Spectrometry
MW Molecular Weight
NG Neuhoff Colloidal Coomassie Brilliant Blue Stain formulation (prepared in-house)
NH\textsubscript{3}HCO\textsubscript{3} Ammonium Bicarbonate
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
pH Measure of acidity or basicity of an aqueous solution
PHOSB Phosphorylase b from Rabbit Muscle (isolated protein standard)
pI Isoelectric point, when protein possesses a net charge of zero
PIC Protease inhibitor cocktail
PMT Photomultiplier Tube
RNA Ribonucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RuBPS</td>
<td>Ruthenium (II) tris (bathophenanthroline disulfonate)</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to noise ratio; can also be defined as Signal squared to noise ratio ($S^2/N$) to highlight contrast for low quantum yield dyes.</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SR</td>
<td>SYPRO Ruby (fluorescent dye for in-gel protein staining)</td>
</tr>
<tr>
<td>STI</td>
<td>Soybean Trypsin Inhibitor from Glycine Max (isolated protein standard)</td>
</tr>
<tr>
<td>TBP</td>
<td>Tributyl phosphine</td>
</tr>
<tr>
<td>TYRP</td>
<td>Trypsinogen, PMSF treated from Bovine Pancreas (isolated protein standard)</td>
</tr>
<tr>
<td>V</td>
<td>Voltage, the difference in electrical potential between two points</td>
</tr>
<tr>
<td>v/v %</td>
<td>Volume to volume</td>
</tr>
<tr>
<td>w/v %</td>
<td>Weight to volume</td>
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CHAPTER 1: Introduction to Proteomics

1.1. General Introduction

To understand the fundamental importance of accurate quantitative protein detection it is first necessary to understand the critical role proteins play in directing cell function, as well as the methods currently used to separate and detect these proteins in order to study their activity.

The cell is the functional unit of life and its components are composed of specific biomolecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, carbohydrates, lipids and their metabolites. It is the DNA within the nucleus that carries the primary genetic code, whereas mitochondrial DNA encodes genes essential for normal mitochondrial function. Generally the genetic code, regardless of origin, is transcribed into messenger RNA that is transported out of the nucleus and this code is translated by ribosomes in the cytoplasm to produce proteins. It was originally thought that one gene resulted in one polypeptide, but research over the years has revealed that this is not so \[1\]. RNA processing and post-translational modifications (i.e. including but not limited to phosphorylation, glycosylation, alternative splicing) can yield a number of modified proteins from the same gene and thus the number of proteins present within a single cell can be quite extensive. Though genomics and transcriptomics have allowed us to understand the structure and function of DNA and gene expression patterns, respectively, this information reveals little about the types of proteins produced from RNA processing, the specific post-translational modifications, the localisation of specific proteins within the cell, and generally nothing about the actual protein amount at the site of action.
Proteins are involved in numerous biological functions ranging from metabolism and signalling cascades to the transport of molecules across membranes. This entire complement of proteins expressed by a genome in a given biological sample, whether it be a whole organism, tissue, fluid, cell or organelle, is referred to as the proteome, a term introduced by Professor Marc Wilkins in 1994 at the 2-D Electrophoresis meeting held in Siena, Italy \cite{2}. The proteome can be affected by external and internal stimuli (i.e. hormones binding to membrane receptors or effects of reactive oxygen species, respectively), with changes in the expression of proteins ‘responding’ to the stimuli. The comprehensive study of these proteomes using a range of techniques – gel electrophoresis, mass spectrometry, x-ray crystallography and confocal microscopy, and protein/antibody arrays – to understand structure and function is broadly known as Proteomics.

1.2. Protein preparation, resolution and identification technologies

To understand the proteome we require numerous technologies. This section will explore a number of technologies that have enabled the field of proteomics and thus a better understanding of proteins and the proteome.

1.2.1. Protein sample preparation

There are various methods that can be used for the preparation of protein samples depending on sample type or the research objective (i.e. total protein profile vs. membrane only). Since even a single cell consists of a complex mixture of proteins, many isolation methods are based on simplifying the overall sample complexity to enhance the detection of proteins present at low concentrations, thus expanding proteome coverage.
The solubilisation of all proteins in a sample is critical if we wish to examine the proteome in its entirety. Currently, a number of strategies have been developed to solubilise proteins; however, it has not been clearly defined as to whether all protein types and/or the entire complement of any given protein have been completely solubilised under any condition.

Three important features of a solubilisation buffer must be considered to ensure efficient protein solubilisation. First, there must be a chaotropic agent which interferes with intra-molecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces, and hydrophobic effects. Second, a detergent is necessary since solubility is unfavourable when hydrophobic regions of a protein are exposed to aqueous solutions; clearly this is particularly true of membrane proteins. Lastly, a reducing agent is necessary to maintain thiol groups on cysteine residues in a reduced state. Using all these measures, it is highly likely that most if not all of the proteins present in that sample are represented thus resulting in a total protein extract.

The most common solubilisation method for protein samples to be resolved by one-dimension electrophoresis (1DE) is the Laemmli buffer system utilising sodium dodecyl sulfate (SDS) as the detergent, and heating to ensure denaturation \[3-4\]. A solubilisation buffer compatible with two-dimensional electrophoresis (2DE), which reduced artefacts due to issues of protein insolubility in the first dimension of resolution, was introduced in 1975 \[5\]. This buffer contained the chaotrope urea, detergent Nonidet P-40, and reducing agent $\beta$-mercaptoethanol. Since the introduction of this buffer system there have been exhaustive efforts to improve the capacity to solubilise proteins for 2DE analyses \[6-21\]. The major improvements most successful in increasing solubilising power have been substitution of Nonidet P-40
with the zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), and the additions of tributyl phosphine (TBP) and thiourea to complement β-mercaptoethanol/dithiothreitol (DTT) and urea, respectively [6, 10, 13, 15].

Although solubilisation of the protein sample is essential, it must be noted that if the method used for homogenisation is incomplete (i.e. heterogeneous, with material remaining as both small and larger pieces), this can have a dramatic impact on the efficiency of solubilisation and thus the subsequent resolved protein profile. It was shown by Butt and Coorssen [22] that using automated frozen disruption rather than conventional manual homogenisation resulted in a more uniform homogenisation, improved protein yield, preservation of protein integrity, and broad applicability to various tissues.

Although accessibility and solubilisation of the entire protein sample is important, the complexity of the sample does pose complications. To investigate the proteome at a reduced complexity, pre-fractionation of the total protein extract prior to analysis by 2DE and/or mass spectrometry is a common approach. This pre-fractionation can be achieved by using physical or chemical means. A widely used strategy to reduce sample complexity is to ultracentrifuge a total protein extract to obtain total soluble (supernatant) and membrane (pellet) fractions [11, 22-26]. However, membrane proteins as well as other hydrophobic proteins can also be isolated and concentrated by organic solvents or selective detergent extractions [27-29]. For further analysis of the membrane protein profile, washing the isolated membranes with a high pH solution (i.e. sodium carbonate, pH 11) yields samples containing peripheral membrane proteins (supernatant) and integral/transmembrane proteins (pellet) [11, 23, 30-31]. Separation of the total cellular lysate into subcellular fractions can be achieved
by differential centrifugation, whereby the initial lysate and subsequent supernatants are subjected to repeated centrifugations with increasing centrifugal force. Alternatively, density gradient centrifugation, in which cellular components are separated in a medium (usually sucrose) of increasing density, or differential detergent fractionation, where sequential extractions with different detergents result in fractions with specific subpopulations of proteins, can also be employed \cite{ref1, ref3, ref4, ref5}.

It is also of great interest to characterise sub-populations of the protein extract, which is achieved mainly with a technique that targets specific characteristics of a protein. To selectively enrich a sample with a particular subset of proteins the most common applications are variations on ‘liquid’ chromatography, including hydrophobic interaction, hydroxyapatite, heparin, lectin, and immobilised metal affinity chromatography \cite{ref10, ref13, ref14, ref15}. Although chromatographic techniques can be used to enrich a certain sub-population of proteins, the reverse is also true for depletion of high abundance proteins from a sample \cite{ref16, ref17, ref18}. However, it has been shown that depletion of these high abundance protein species does result in the co-depletion of other protein species that may interact with the target protein \cite{ref18}. Thus, these depletion techniques are not entirely benign in terms of potential effects on the subsequent assessments of the overall proteome.

More recently, there has been an increased interest in combinatorial hexapeptide ligand libraries, also known as ProteoMiner™ or Equalizer beads, as a fractionation tool for proteomic applications. As the name suggests, a hexapeptide ligand is synthesised and attached via a spacer onto polymethylmethacrylate beads, the solid carrier, and due to the nature of the synthesis each bead consists of only type of hexapeptide \cite{ref19, ref20}. Since there are 20 natural amino acids the number of
hexapeptide ligands reaches well over 60 million. When combined with a protein extract, all proteins bound to the solid-phase-ligands are collected by centrifugation and unbound protein washed away before recovery. For high abundance proteins the corresponding beads quickly saturate leaving the remaining unbound, whereas the low abundance proteins do not saturate the beads. Based on this saturation-overloading principle these combinatorial peptide ligand libraries have the advantage of limiting the presence of high abundance proteins while enriching the sample for low abundance proteins. Although this technique has been applied to many sample types for improved detection of low-abundant protein species [56, 58-65], it has been demonstrated that some protein species are no longer present after treatment with this peptide ligand library [58]. Thus, while such approaches can be useful, improvements are needed to ensure the recovery of all protein species. So it is important for comparable samples to both be treated with the beads, but if a protein is present in excess of the saturation point of the bead in one condition, the comparison does not truly represent the native density and can be somewhat misleading.

Other strategies to prepare protein samples include electrophoretic methods such as free-flow electrophoresis, gradiflow, multicompartment electrolyzers, three-layer sandwich gel electrophoresis, and sephadex isoelectric focusing (IEF) [38, 66-70]. Free flow electrophoresis separates samples based on electrophoretic mobility within a thin buffer stream and can be used to separate cells, organelles and membranes [66]. However, it is possible for multiple components to have the same electrophoretic mobility so an extension of free flow electrophoresis was developed by immunocomplexing the molecule with a specific antibody prior to free flow electrophoresis, thereby inducing a more negative charge on the molecule of interest [38].
The gradiflow method also makes use of a continuous buffer stream; however, the stream is divided into two by a separation membrane. In an electric field, charged molecules can be fractionated under non-denaturing conditions by migrating through the membrane based on their isoelectric point (pI) \[^{71-72}\]. It is also possible to fractionate proteins based on size using the Gradiflow technology \[^{71}\]. Sample prefractionation using the multicompartment electrolyzer or sephadex IEF both rely on the pI a protein for separation and result in numerous fractions \[^{68-69}\]. The main difference is that multicompartment electrolyzers consist of buffered gel membranes defining a certain pH range and thus fractionation of the protein sample \[^{68}\]. In contrast, with sephadex IEF, the sample is mixed together with the sephadex and pH gradient before producing a solid gel; an electric current is subsequently applied to effect protein fractionation \[^{69}\].

The three-layer sandwich gel electrophoresis was developed to provide a means for better protein sample cleanup prior to mass spectrometry since samples can be desalted and concentrated simultaneously \[^{70}\]. The bottom sealing layer, has a high acrylamide concentration (40%) to confine protein to the middle concentrating layer when it moves from the top agarose loading layer upon application of an electric field \[^{70}\]. Since this method does not resolve/fractionate proteins in any way, an additional step would be required if this was desired.

1.2.2. Gel-based resolving technologies

Early electrophoresis methods for the resolution of proteins involved many mediums including cellulose acetate and filter strips, agarose, agar, and starch gels \[^{73-77}\]. However, it wasn’t until the late 1950s that the advantages of using a polyacrylamide gel matrix were truly appreciated and quickly became the standard
medium of choice for protein resolution. These advantages include highly reproducible pore formation in the matrix, versatility in pore size controlled by changes in acrylamide concentration, and a transparent gel that is stable, flexible and insoluble in water once polymerised. Essentially this is a highly defined matrix for electrochromatography.

Not long after the widespread acceptance of the use of polyacrylamide for the separation and purification of proteins came the development of a method that enabled investigation of proteins as individual linear polypeptides: the Laemmli SDS polyacrylamide gel electrophoresis (PAGE) procedure (i.e. SDS-PAGE). The anionic headgroup of SDS interacts strongly with the cationic amino groups of the protein chain, and the hydrophobic tail with hydrophobic domains in proteins. By also using heat to ensure protein denaturation, the result is polypeptides that are uniformly coated with SDS molecules, with an overall negative charge of 1.4 g SDS/g protein. To ensure that the polypeptides are maintained as a single linear molecule, SDS is present in all aspects of the system – Laemmli protein solubilisation buffer (i.e. 1DE sample buffer), electrophoresis buffer, and the polyacrylamide gel matrix. Since each individual polypeptide present within a sample has a negative charge, all polypeptides will migrate toward the anode and their apparent molecular weights can be reproducibly estimated.

The Laemmli method for protein resolution in one dimension was thus widely accepted. However, 27 years later it was noticed that the solubilisation conditions were suboptimal for maintaining protein integrity. Upon heating, the original recipe with Tris at pH 6.8 would promote acidic conditions resulting in hydrolysis of the aspartyl–prolyl peptide bonds within proteins. This problem was overcome by preparing Tris at the higher pH of 8.8, so after heating sample pH would be neutral,
hence preserving protein integrity [4]. Although the method of Laemmli is excellent for the resolution of most proteins, it is however insufficient when resolving proteins with a molecular weight below ~10 kilodaltons (kDa) glycine migrates slower than these proteins impeding the resolution of low molecular weight proteins from the SDS zone [86]. This problem overcome by using tricine as the trailing ion instead of glycine since tricine migrates faster than glycine in the stacking gel despite its higher molecular mass and, together with lower acrylamide gel concentrations, high resolution of proteins within the range of 1-100 kDa was achieved [86]. The migration of large molecular weight proteins is unaffected by glycine migration since it will always migrates faster, but can be affected if the pore size of the gel is too small, yielding ill-defined bands and/or poor resolution between proteins of similar molecular weight; this can be easy rectified by reducing the acrylamide concentration, thus increasing pore size. Since the pore size can be controlled, gels having a fixed uniform concentration or a gradient of increasing acrylamide concentration (i.e. decreasing pore size) can be produced. Thus, proteins within a gradient gel also migrate until pore size impedes migration, but this format can often improve protein resolution, particularly in specific MW ranges.

Although most applications of 1DE utilise denaturing conditions (i.e. with the use of SDS and heating), it is also feasible to perform non-denaturing separations of proteins. Originally, Coomassie brilliant blue (CBB) was used for direct visualisation of polypeptides during electrophoresis with the added advantage of high protein recovery for downstream analysis since there was no fixation step [87]. Building on this approach, the same group developed the method known as blue native PAGE (BN-PAGE) specifically for the resolution of native membrane protein complexes [88]. For BN-PAGE, the protein sample is prepared with CBB, and this is also present
in the cathode buffer to ensure saturation of the system. Unlike SDS, the binding of CBB to the protein surface is not uniform, and is dependent on the amino acid sequence of a protein since CBB binds to basic, aromatic and hydrophobic residues [75, 89-91]. The CBB molecule conveys a negative charge and since the amount of CBB bound to each protein varies, the electrophoretic mobility for each protein is determined by the negative charges of the bound CBB.

Recently there have been efforts to improve reproducibility and reduce variation of BN-PAGE when used as the first dimension for two dimensional BN/SDS-PAGE by casting the gel onto a polyester support film (GelBond) [92]. Benefits of this approach include direct visualisation of separation during electrophoresis, reduced opportunity of protein aggregation due to negative surface charge, the approximate molecular weight of the resolved complexes can be determined, and the functional activity of the protein is often unaffected (i.e. enzymatically active proteins can be isolated following resolution) [88, 93]. Not long after the introduction of BN-PAGE came the development of clear native PAGE (CN-PAGE) [93]. This method for the separation of protein complexes is essentially the same as that for BN-PAGE but CBB is omitted from the sample and cathode buffer. Hence, protein resolution is based on native charge rather than a charge shift induced by CBB in BN-PAGE. The main advantage to using CN-PAGE is the preparation of highly pure native membrane protein complexes when used as the first dimension for two dimensional native PAGE (i.e. CN-PAGE followed by BN-PAGE) [93-94].

Resolving a protein mixture based on the molecular weights of constituent proteins (denatured or non-denatured) is useful to reduce, to some extent, the complexity of the original sample. However, there is one major drawback; it cannot
be assumed that a particular band in a 1DE gel represents only one type of polypeptide, since it is likely that more than one protein will have the same approximate molecular weight. The separation of proteins based on two physiochemical characteristics increases the confidence that a spot/band consists of only one type of polypeptide.

It was only five years after the introduction of denaturing 1DE (SDS-PAGE) that the first gel-based protein separations were accomplished utilising two independent physio-chemical characteristics – commonly referred to as 2DE \[5, 95-96\]. Using this procedure, proteins are first separated within a pH gradient based on their pI; during focusing, proteins migrate through the matrix to a pH region at which their net charge is zero. This is the fundamental principle of IEF. The IEF gel is then placed on top of a 1DE SDS gel and an electric current applied, resulting in the resolution of proteins according to molecular weight. Although the power of this technique was recognised at its infancy, it would be years before it was fully established as a reliable and reproducible technique for the separation of a complex protein sample, and improvements continue to be introduced \[5, 97\].

As mentioned previously (Section 1.2.1) there was much effort invested into improving the solubilisation of a protein sample, as well as pre-fractionation to enhance the capacity to detect more proteins when using 2DE. However, another critical aspect for refining 2DE was IEF. In the earliest experiments, IEF was conducted using soluble carrier ampholytes \[5\]. Under an electric field, the small ampholyte molecules migrate through the gel matrix and after a defined period of time establish the pH gradient from low to high, anode to cathode. Only once this pH gradient was established within the gel could the samples be applied.
Although the ampholytes are small, mobile and can create a pH gradient very rapidly, these same characteristics can consequently lead to rapid diffusion of the ampholytes resulting in a deterioration of the pH gradient and hence poor resolution of the protein sample. Since the ampholytes are completely mobile, focusing over long periods of time causes the gradient to drift toward the cathode as a result of electro-osmotic flow (the motion of liquid induced by an applied potential). This then, in a time dependent manner, causes protein to drift from their pI dependent position toward the cathode. These factors, thus, reduce the reproducibility and resolution of IEF.

It was noticed very quickly that the resolution of basic proteins was poor when IEF of a sample reached equilibrium and this led to the development of an alternative strategy for IEF [98]. This involved short electrophoresis times for the separation of proteins and was hence referred to as non-equilibrium pH gradient electrophoresis [98]. Using this method certainly improved the resolution of basic proteins but affected the resolution in the acidic region. Although high resolution of the basic region was achieved, it was not possible to apply one tactic to examine the entire range of proteins – equilibrium IEF [5] was suitable for the acidic region, and non-equilibrium pH gradient electrophoresis [98] only for the basic region. Thus, two entirely different gels were required [5, 98].

It would be another five years before the greatest improvement to IEF was accomplished. Unlike carrier ampholyte pH gradients in which ampholytes are mobile entities within the gel medium, the improved gel ‘strips’ consisted of immobilised pH gradients (IPG). This was achieved with the use of buffering acrylamide derivatives, Immobilines, that when added to the gel mixture become covalently linked to the matrix to generate a defined pH gradient [99]. The controlled
conditions for generating IPG provided for better protein resolution, reproducibility, uniform buffering, and higher loading capacity in comparison to carrier ampholyte IEF, as well as easily allowing the pH gradient to be linear or non-linear, and either broad or narrow, in addition to zoom and ultrazoom gels which range from 1-3 pH units [99-103].

Like all newly developed methods, problems were instantly recognised. Severe protein losses, in particular for membrane protein samples, were observed when using IPG for the first dimension of 2DE, and were suggested to be due to hydrophobic interactions with the IPG matrix [11, 104]. This drawback was quickly rectified with improvements to the protein solubilisation buffer used with IPG (i.e. the introduction of CHAPS and thiourea) [10, 15]. The other main problem was sample precipitation at the application site; however, this was overcome by introducing the protein sample during rehydration of IPG, resulting in greater control over protein load and sample volume [105-106].

Overall, there have been serious efforts to optimise 2DE, with most enhancements apparent in the neutral pH region. However, it has also been shown that post-fractionation, by an additional electrophoretic separation of the stacked regions at the pH extremes of a 2DE gel improves resolution, with similar results for the front and areas of the gel ‘obscured’ by hyper-abundant proteins [97]. Even though a technique when developed can yield great results, there is always room for improvement to obtain as much information as possible. Attention to such details always appears to be at the heart of the ‘best’ proteomic analyses. This section focussed on gel resolving technologies, however, in-gel protein quantitation will be addressed in detail in Section 1.3 below.
1.2.3. Mass spectrometry

A number of factors need to be taken into consideration when investigating the proteome, including characterisation of the resolved proteins. Initially proteins were characterised using methods involving their sequential degradation at the N-terminus (i.e. Edman degradation), or at the C-terminus\textsuperscript{[107-110]}. However, these methods were somewhat limited in that they require milligram quantities of relatively high purity protein, and are time consuming. In addition, Edman sequencing can also be affected by N-terminal blocks like pyroglutamate or N-acetylated/N-formylated amino acids and/or other amino acid modifications including phosphorylation, glycosylation, palmitoylation, myristoylation and oxidation.

Since the development of ionisation methods compatible with large, non-volatile biomolecules, mass spectrometry (MS) has become the most useful technique for protein characterisation, identification, and quantitation. Briefly, the mass spectrometer consists of three elements – the ion source, which converts gas phase molecules into ions; the mass analyser, which sorts the ions by mass using electromagnetic fields; and a detector, which records the charge or current produced by passing ions. Generally, a sample is introduced into the ionisation source, either directly or following some type of ‘upfront’ chromatography, and ionised to produce positively (gain of proton – $\text{[M+H]}^+$) or negatively (loss of proton – $\text{[M-H]}^-$) charged sample ions depending upon the proton affinity of the sample. For protein and peptide measurements, positive mode is almost always employed. These ions are then separated according to their mass-to-charge ratios (m/z) by one or more mass analysers and detected signals are sent to a data system to store m/z ratios together with abundance to present this data as a m/z spectrum. The resulting mass spectrum
can then be searched against a database of theoretical spectrums based on protein or genomic information in order to identify the protein being analysed.

The two ionisation methods used mainly for peptide analyses are electrospray ionisation (ESI) or matrix-assisted laser desorption ionisation (MALDI). When using ESI, the sample (native protein or peptides after trypsin digestion) is passed through a capillary held at high voltage, resulting in highly charged droplets. Since proteins and peptides are usually analysed using positive ionisation conditions, positive droplets are produced. These charged droplets are flushed with an inert gas to help evaporate the solvent, thereby releasing gaseous charged ions under atmospheric pressure. Nanospray-ESI is a low flow rate version of ESI, employing lower voltages for sample ionisation and very low flow rates (i.e. nL/min), thereby consuming less volume of sample. A feature of ESI is the production of multiply charged ions for molecular mass fragments greater than 1200 Da. This feature makes it possible to analyse high molecular weight samples even on a mass spectrometer with a limited m/z range. ESI is susceptible to the presence of non-volatile buffer and other additives which can have an effect on ionisation and therefore sensitivity. Physical clogging of the capillary can also be a problem.

MALDI involves mixing the protein sample with ultraviolet-absorbing matrix molecules and a droplet of this mixture is dried onto a target platform. When this solid matrix containing sample is irradiated with a laser, the matrix molecules absorb the energy and transform this into excitation energy which results in the sublimation of matrix and sample ions from the target surface. Since the protein sample is surrounded by the matrix molecules, decomposition of sample by direct contact with the laser energy is minimised. Unlike ESI, MALDI ionisation can be performed under vacuum or atmospheric pressure. The matrix molecules most suitable for
MALDI are usually acidic in nature (to act as a proton source encouraging ionisation of the sample), absorb in the ultraviolet or infra-red region, have polar groups, and are of low molecular weight but do not evaporate. The most common matrix used for peptide analysis is alpha-cyano-4-hydroxycinnamic acid, whereas sinapinic acid is used for intact protein analysis.

To analyse the mass of the ions produced after ESI or MALDI, they are electrostatically directed into the mass analyser, under high vacuum to ensure the ions travel throughout the instrument without any hindrance from air molecules, and separated according to their m/z. The method by which the ions are produced (i.e. a continuous stream by ESI or pulses by MALDI), determines the type of mass analyser used. Time of flight analysers use an electric field to accelerate ions and measure the time taken for these ions to reach the detector. Sector field mass analysers use electric and/or magnetic fields to affect the path trajectory of the charged ions. Quadrupole analysers are composed of four parallel metal rods, and a voltage is applied to each opposing rod pair. Depending on the voltage applied to these rods, ions of a particular m/z will travel through the quadrapole to reach the detector, while others have unstable trajectories and collide with the rods. Ion trap instruments essentially work on the same principles as quadrupole analysers, except the ions are contained within the quadrupole and sequentially ejected onto a detector. Fourier transform ion cyclotron resonance MS determines m/z of ions based on the cyclotron frequency of the ions in the presence of a fixed magnetic field. There are also a number of important characteristics to consider when choosing the type of mass analyser. The mass resolving power is the ability of the mass analyser to yield distinct signals for two ions with a small m/z difference. The mass accuracy is the
ratio of the m/z measurement error to the true m/z, and the mass range limit is that range of m/z amenable to analysis.

There are two strategies for the identification of proteins by MS. One approach is to introduce an intact protein into the mass spectrometer \[115-118\]. A mass spectrometrist refers to this as top-down MS and is not to be confused with top-down proteomics which refers to the investigation of proteins/proteomes obtained from organisms, tissues, cells and/or organelles. This technique was initially restricted to analysis of isolated protein standards, but can now be used to characterise single proteins isolated from a native sample \[118\].

The other approach involves the digestion of proteins, either as a single species or as a mixture of species, with a protease (i.e. trypsin), to yield a sample containing large numbers of smaller peptides. When introduced into the mass analyser, this peptide mixture yields a mass ion spectrum often referred to as a fingerprint. The experimental peptide mass fingerprint generated can then be searched against a database consisting of theoretical peptide mass fingerprints (i.e. created by ‘digesting’ protein sequences in silico) \[119-122\]. It is possible to obtain a protein identification (ID) from this search, however, if the starting protein sample is a mixture or is contaminated the confidence in the ID obtained is considerably reduced since it is quite possible that the masses detected are not unique. To have more confidence in a protein ID, it is better to fragment as many peptide precursor ions as possible and perform database searching using the m/z of the precursor ions along with the corresponding fragment ion spectrum \[123\]. This fragment spectrum enables determination of the amino acid sequence for that particular peptide ion since each amino acid has a specific mass. Although we can identify a protein from database searching of the mass and fragment information obtained from MS, it is also
important to keep in mind where in the protein these sequences are located. To ensure the best possible protein ID, it is most advantageous if these sequences are distributed throughout the protein rather than clustered at the N- or C- terminus. A crucial restriction to database searches is if the protein of interest is not present in the database(s) interrogated or the organism of origin has no information available for its genome. Nonetheless, proteins with critical functions in fundamental cellular mechanisms tend to be highly conserved, so often high quality IDs are possible regardless of the organism.

‘Shotgun’ proteomics involves the tryptic digestion of a complex protein mixture (i.e. an extracted proteome) and the separation of the resulting peptides by single or multidimensional liquid chromatography prior to MS analysis. Using this strategy there is no need for prior resolution of the original protein sample, since peptides are separated using strong cation exchange and reverse phase chromatography before analysis by MS. However, given that the sample is so complex, there is a potential problem for the thoroughness of proteolytic digestion. The possibility of finding a peptide from a low abundance protein is so small due to the presence of higher abundance peptides, which are preferred for ionisation and thus fragmentation. There is also difficulty when assigning a peptide sequence to a particular protein since this sequence may be present in multiple different isoforms or proteins within the sample. Additionally, analysis by ‘shotgun’ takes approximately 24 hours (h) per sample, or even longer if iterative methods are used in an effort to improve the overall extent/quality of coverage (i.e. the peptide population is scanned repetitively in an attempt to find additional ‘unique’ peptides); in an experiment with multiple controls and samples, and utilising multiple analyses per sample to ensure analytical rigour, this amounts to a significant amount of
dedicated instrument time, during which the MS is not available for any other analyses. In contrast, a peptide sample derived from a gel band/spot only takes one h/sample when analysed by MS. The ‘shotgun’ approach also produces a large volume of MS/MS data for one single experiment, so there is a considerable demand for computing power and storage as well as a challenge for the analytical software to assign the correct peptide to a correct protein of origin.

Mass spectrometry data can also be used to quantitate proteins by methods that utilise peptide-labelling or those that are label-free. Quantitation by MS can be defined as either relative or absolute. Relative quantitation involves the comparison of peak intensities of the same analyte in one sample relative to those in another. One of the most used label-free methods is spectral counting \[128\]. This is based on the frequency of peptide precursor ions being selected for fragmentation, and this information is used as a measure of protein abundance \[124-125, 129-132\]. Spectral counting often utilises the frequency of the top six precursor ions which are selected for fragmentation, usually the most abundant ions are selected within each scan, so the chances to quantitate a protein of low abundance is limited in addition, this approach assumes that all peptides are ionised with equal efficiency and that there are no effects of ion suppression arising from competing ionisation processes. The data for spectral counting are readily available, so there is no need for additional experimental samples; however, since there is no internal standard quantitation is relative. Although relative quantitation is the most common approach for investigating the amount of protein, methods for absolute quantitation have also been developed \[133-135\].

Absolute quantitation requires known concentrations of synthetic isotope-labelled peptides to be added into the experimental sample at known quantities and
from the ratio of the target peptide in the experimental sample and the labelled peptide, the concentration of the protein of interest can be determined. However, the main limitation to this strategy is that the peptide and/or gene sequence must be known in order to synthesise the standard peptide. As mentioned previously, a peptide sequence can be present within multiple proteins, so to have the most honest and reliable quantitation, this standard peptide should to be unique or multiple standards should be used for a single protein.

For direct comparison between two or more samples (i.e. control vs. cancer cells) a labelling strategy is preferred since the samples can be labelled with different isotopes mixed together prior to MS. Some labelling approaches can utilise the metabolism of cells grown in culture, where amino acids of a different isotope are added to media, taken up by the cells and incorporated into the proteins, commonly referred to as Stable isotope labelling of amino acids in cell culture (SILAC) \[^{136-138}\]. Metabolic labelling of proteins is a straightforward measure for quantitation but is limited by the requirement of live cultures to be grown in defined media. If metabolic labelling is not feasible an alternative is to directly tag the cysteine residues of the protein sample (Isotope-coded affinity tags-ICAT) \[^{127, 139-140}\]. Although two samples can be quantitatively compared due to labelling with different isotopes, analysis is limited to peptides containing (accessible) cysteine residues and thus fails to detect any changes in proteins that have no cysteine residues or those that are poorly accessible, and can only assess the population of purified ICAT labelled peptides. Since the cysteine composition between proteins is varied there can also be non-uniform labelling between peptides containing more than one cysteine residue.

Another strategy for quantitation is the use of isobaric amine-reactive tags (referred to as iTRAQ) \[^{141}\], where up to eight independent digests can be labelled
with different isobaric tags and combined prior to MS/MS analysis. Since amines are present within every peptide, no information is lost by selective enrichment, as with ICAT – although this assumes no accessibility issues and uniformity of the labelling reaction. However, the concentration of the peptide sample restricts how much can be used, since equal amounts of each sample are mixed together. This strategy not only restricts how much sample is used but reduces the chances that a low abundance peptide will be selected for analysis.

There are many qualitative and/or quantitative approaches in which a protein sample can be examined by MS, and its application is not limited by sample type. Overall, mass spectrometry has become an integral part of proteomics for the identification, characterisation and quantitation of proteins, thus highlighting MS as an indispensable tool for large scale biology.

1.2.4. Other protein characterisation methods

The characterisation approaches mentioned above are widely employed for analysing the proteome on a global scale without prior knowledge of the components within the sample. However, there are a plethora of other techniques available to examine proteins and proteomes and some will be covered briefly in this section.

The quantitative determination of an antigen with the use of antibodies was first developed by Eva Engvall and Peter Perlman\textsuperscript{142-143} and was referred to as an enzyme-linked immunosorbent assay (ELISA). Here the antigen is adsorbed onto a solid phase and then incubated with antiserum. After washing, the antibody targeting the antigen will remain bound and recognised by an enzyme-conjugated anti-immunoglobulin (indirect ELISA). If the enzyme is conjugated to the antigen recognising antibody this is referred to as direct ELISA. A colourless substrate for
the enzyme (i.e. alkaline phosphatase), is added and a colour change upon reaction with the enzyme occurs. This colour change is proportional to the amount of enzyme and hence antigen present, and so can be used to quantitatively determine the concentration of the protein of interest. It is also possible to have the antibody adsorbed onto the solid phase to detect antigen in a sample and then detection delivered by indirect and/or direct ELISA, and this is called sandwich ELISA. Alternatives to ELISA have also been developed and include enzyme immunoassays, which rely on the same principle as ELISA but do not employ a solid phase to immobilise conjugates but rather use density gradient centrifugation for separation of these conjugates \[144\], and the solid-phase enzyme-linked immunospot assay which was designed to detect antibody secreting cells \[145\].

Perhaps the most common technique exploiting the advantage of antibodies for protein detection is Western blotting. A protein sample resolved by gel electrophoresis can be electrophoretically transferred to a nitrocellulose (or polyvinylidene fluoride) membrane, yielding a blot of the original gel \[146\text{-}147\]. This blot is then incubated with primary antibodies for detection of a specific protein. Like ELISA the primary antibody can be conjugated to a label (e.g. a radioisotope, fluorophore or enzyme) to aid visualisation and/or quantitation, or a secondary antibody which recognises the primary antibody bound to the target can be used. It was originally noted that sensitive detection using Western blotting could be achieved down to nanogram quantities of the target, more recently, immunodetection has been shown to be quantitative down to the femto- and even attomole levels \[148\]. Another useful approach is the application of a mixture of monoclonal antibodies to a blot for simultaneous detection of a distinct set of proteins within a sample \[149\].
Protein chips have over the years received a great deal of attention since there is a high capacity to evaluate protein interactions on a large scale. Since proteins have such diverse roles in cells there has been much effort to examine protein-protein, protein-nucleic acid, protein-ligand, protein-lipid and protein-carbohydrate interactions [150-156]. Fundamentally the strategy behind protein chips is the use of a solid support (i.e. a glass slide [151-154], nitrocellulose membrane [150], gel pad [155] or poly(dimethylsiloxane) microwells [156]), to which the protein/enzyme or small molecule is immobilised. After incubation with an interaction partner or substrate, a coloured or fluorescent spot reveals an interaction.

The genome of every cell, tissue and organism harbours the information for every primary protein sequence. There have been many studies devoted to deciphering the genes constituting the entire genome [157-164]. With this genetic information readily available, the examination of protein localisation is made possible. In 1994, a green fluorescent protein (GFP) originally identified in the jellyfish Aequorea victoria, was introduced and expressed in Escherichia coli and Caenorhabditis elegans and possessed the same fluorescent properties [165]. It wasn’t until later that year that GFP was tagged to a protein via fusion of the GFP gene to the gene sequence of the target protein to visualise the localisation of the tagged protein in Drosophila [166]. These tagged proteins have also been used in other cell types including fungi and mammalian [167-169]. There has been much effort to improve the fluorescent properties of GFP; one in particular is the mutation of the original GFP sequence to prevent plant cells from processing the messenger RNA resulting in inefficient expression of GFP [170-172]. Since the discovery of GFP, a number of other fluorescent proteins have been identified and cover a large range of the light spectrum, far-red, red, orange, yellow-green, green, cyan and ultraviolet wavelengths.
to enhance biological imaging \[^{173-175}\]. Together, these enable a number of proteins to be examined simultaneously in a single cell due to the differences in spectral properties. Fluorescent proteins have even been used to produce ‘cameleons’, which are fusion proteins consisting of two GFPs, blue or cyan and green or yellow separated by a calmodulin \[^{176}\]. With this fluorescent fusion protein, changes in calcium concentration can be evaluated in specific subcellular locations \[^{176}\].

1.2.5. Summary

It is clear that the vast array of methods available for the characterisation and ID of proteins each have their own advantages and disadvantages. However, it is important to keep in mind that the method(s) used will depend greatly on the questions to be answered. Thus, gel electrophoresis (1DE and 2DE) and MS will continue to be among the leading global assessment methods for proteome analysis, while methods such as ELISA, protein chips and GFP are exceptionally valuable when trying to understand the function, interaction partners, and localisation of a particular protein.

1.3. Detection of proteins in-gel

The detection of proteins in-gel is mostly commonly achieved with the use of protein stains. Currently there are a number of protein stains available, but their use is limited by their sensitivity and dynamic range. The more sensitive the stain the more information we can obtain, especially in regard to those proteins present in lower abundance that may be involved in physiological dysfunctions. This section will primarily focus on the most common protein stains used in proteomics.
1.3.1. Densitometric stains

Densitometry involves measuring the optical density (i.e. absorbance) of a substance by shining light on it and measuring the resulting light transmission. Thus, on illumination, proteins detected with densitometric stains are visualised as black bands or spots. Amido black, one of the oldest protein stains, was used primarily for qualitative assessment of proteins in-gel. Although detection of proteins was useful for obtaining information about molecular weight, the question of how much protein was present was also important. It wasn’t until the 1960s that one of the first quantitative assessments of proteins in-gel was made using the textile wool dye, CBB \[75, 177\]. The mode that CBB uses to bind to proteins is not clearly understood but it has been suggested to be through non-covalent forces such as electrostatic interactions between the sulfonic groups of CBB and side groups of basic amino acid residues \[75, 89-91\] as well as interactions with aromatic residues and hydrophobic contacts \[89-91\].

The introduction of CBB as the preferred stain for quantitative assessment of proteins in-gel saw a rise in the number of studies dedicated to improving the staining protocol – solvent composition, stain concentration, stain type (CBB G-250 vs. CBB R-250), combination stain formulations, and different strategies for staining protein and/or destaining the gel matrix \[178-197\]. However, none of these approaches were able to improve the detection limit of CBB stained proteins below ~30 ng \[183, 186, 194, 198\]. The major problem with the CBB stain in an organic solvent was the resulting high background in the polyacrylamide gel matrix; this was initially addressed with laborious destaining procedures using organic solvents.

An elegant study by Neuhoff and colleagues revealed that by changing the solvent formulation, CBB could form colloids \[199\]. Unlike the CBB molecules in an
organic solvent which were all free to penetrate the gel completely, the colloidal state of the CBB stain reduced the number of free CBB molecules able to penetrate the gel to stain protein since the colloidal particles were too large, resulting in reduced background staining \[199-200\]. The improved colloidal CBB (cCBB) not only addressed the background problem associated with traditional CBB formulations but also enhanced protein detection sensitivity from \(\sim 30 \text{ ng}\) to \(\sim 1 \text{ ng}\); in practice, detection sensitivity down to \(\sim 4-8 \text{ ng}\) is more commonly achieved if one considers a range of proteins. Additional advantages to this method of CBB staining, apart from reduced background staining, is the simple procedure, low cost, and reduced need for use of organic solvents.

With the progress of research and the desire to detect as much of the proteome as possible, in particular those proteins of lower abundance, stains with greater detection sensitivity are needed. With this in mind came the birth of silver stain in 1979, which was claimed to be \(\sim 100\)-fold more sensitive than CBB \[201\]. Like CBB, there was also a period of research concerned with improvement of the silver stain protocol \[202-215\]. Although detection sensitivity using silver stain was improved in comparison to CBB there were issues that need to be considered: 1) The method is laborious and tedious, with no definitive endpoint; 2) Non-uniform staining of proteins (i.e. high inter-protein variability); 3) A narrow range of protein concentration in which staining is linear (i.e. poor linear dynamic range); and 4) Staining of other biomolecules such as lipopolysaccharides and nucleic acids \[210, 216-222\]. Additionally, the original silver stain protocol was incompatible with MS \[223\], requiring modifications but at the cost of detection sensitivity \[212, 224-225\]. The literature shows that a number of other densitometric stains were explored for their
potential as quantitative proteomic stains; however, quantitative characterisation was minor and/or sensitivity did not surpass the capacity of CBB and silver stain \(^{[226-239]}\).

Complementary to the stains described above, which bind to protein for visualisation and quantitation, negative staining results in clear bands due to selective staining of the matrix (i.e. non-protein areas of the polyacrylamide gel) \(^{[240-243]}\). The most common negative stain utilises the heavy metal zinc (as zinc chloride) that forms a precipitate in the presence of imidazole and SDS \(^{[244-245]}\). Zinc staining has been shown to yield sensitivity of \(\sim 1-4\) ng of protein \(^{[217, 246]}\), but there is some doubt since the signal can only be an approximation due to image inversion. Additionally, this staining procedure is not specific to protein and, like silver stain has no exact endpoint \(^{[247-248]}\).

1.3.2. Fluorescent stains

More recently, fluorescent stains have been favoured over densitometry. Fluorescence occurs when a molecule has absorbed a certain wavelength of light and releases this energy by emitting photons. Since quantitative assessment using fluorescent stains relies on the emission of light, rather than absorbance, these stains are generally more sensitive and are less affected by background interference.

SYPRO Ruby (SR) was first introduced in 2000 \(^{[217]}\), and since then has been considered the gold standard for quantitative proteomics. Detection sensitivity of SR for proteins in-gel is by far better than any of the currently available protein stains. Using broad range molecular mass standards (Bio-Rad) SR was shown to detect protein at \(\sim 1-2\) ng, and to have a wide linear dynamic range with little inter-protein variability \(^{[217, 249]}\). However, variations in these measures by different researchers have been noted, although usually attributed to slight differences in the staining...
procedure and/or the types of proteins analysed [249-250]. Nonetheless, if some of this variability arises from assessing different proteins, it suggests that further, more detailed characterisation of SR as a detection tool is necessary. Additionally, despite the perceived advantages of using SR for quantitative proteomics, the high cost puts a strain on most research budgets. There have been attempts to economise the use of SR by dilution with water and/or reuse, but the limited quantitative evaluations used to assess these economies raise uncertainty [251-252].

Beyond the fact that SR contains ruthenium, the chemical structure is proprietary. A ruthenium based dye known as Ruthenium (II) tris (bathophenanthroline disulfonate) (RuBPS) was developed and suggested to be a sensitive and cheaper alternative to SR [253]. Although the manufacturer of SR noted no advantage of RuBPS over the original and optimised SR formulations [254], there has been one independent study showing similar quantitative capacity between the two stains [255]. To date the fluorescent alternative with detection close to that of SR is Deep Purple (DP). This dye consists of the naturally fluorescent compound, epicocconone, derived from the fungus, Epicoccum nigrum [256]. It has been noted that protein detection can be achieved at picogram levels of protein [257], however, 2DE analysis of native proteomes by DP was shown to be poorer in comparison to SR [258-259]. Additionally, it was also observed that the signal to noise ratio for DP was closer to that of densitometric CBB than SR, as well as inferior detection of acidic, membrane and low molecular weight proteins [259]. Also, a well known disadvantage to the use of DP is its photoinstability, with six minutes of light exposure reducing DP signal by half, unlike SR for which three-fold more time was needed [260]. Additionally the staining protocol requires much more hands on time and there is no difference in cost relative to SR [261]. There have been a number of
fluorescent stains proposed as alternatives to SR but limited quantitative evaluation would appear to have somewhat marginalised their use in proteomic applications [249, 262-274].

The stains mentioned above have all been used for in-gel protein detection post-electrophoresis. One of the main issues though with comparisons between related samples is inter-gel variation, as no two gels will run 100% identically regardless of preparation. A strategy developed to address this issue was difference gel electrophoresis [275]. This approach uses reactive dyes to covalently pre-label proteins prior to electrophoresis, which then allows the user to simultaneously resolve one sample (i.e. one gel) consisting of two proteomes labelled with different reactive dyes.

The reactive fluorescent dyes, Cyanine 3 (Cy3) and Cyanine 5 (Cy5), were specifically designed to convey the same charge on the protein residue they modify and the same molecular weight change but possess different spectral characteristics. The initial trials involved labelling all lysine residues to saturation within a total protein extract; however, this proved detrimental since the proteins became insoluble [275]. In order to avoid problems associated with insolubility, it was determined that minimal labeling of lysine residues (a predicted 1-2% of all lysines) was optimal. An alternative target for labeling is cysteine; because the frequency of cysteine compared to lysine is less common, this reaction can be completed to saturation. Amersham Biosciences thus designed the Cy3 and Cy5 dyes with maleimide groups for labeling of cysteine residues.

Evaluation of sensitivity for minimal (lysine) and saturation (cysteine) labeling CyDyes revealed that saturation labeling was slightly more sensitive, when using the 2920-2D Master Imager for analysis (Amersham Biosciences) [276]. This level of
sensitivity was also observed to be superior when compared to SR using this imaging system. It was also noted that sensitivity of the same dyes using the Fujifilm FLA-3000 series imager (Fuji Photo, Kanagawa, Japan) was inferior to the 2920-2D Master Imager [276]. Dynamic range was also shown to be at least three orders of magnitude, but this was only tested on the Cy5 minimal and saturation dyes for three standard proteins (bovine serum albumin, ovalbumin and myoglobin) [276].

Although using dyes to label proteins prior to electrophoresis may be useful as it theoretically decreases labour time and subjects samples to the same electrophoretic conditions, some issues need to be considered. Due to the specific nature of the labeling process it is not unusual for large variations in labelling to occur since the number of lysine or cysteine residues is not constant between proteins and hence a broad degree of inter-protein variability cannot be avoided. In addition, any post-translational modifications present will also reduce the capacity to label these residues. With regards to cysteine labeling, not all proteins contain this amino acid, and thus there will be a portion of the proteome that will go undetected. As the name suggests, minimal labeling only labels a fraction of the sample with the balance being undetectable. Since comparisons between samples can be achieved on the same gel, the amount of protein loaded per sample is reduced (i.e. due to the limits on protein loading per gel) and this affects protein detection, in particular those that are of lower abundance assuming that labelling is optimal. Although the number of gels can be reduced by running multiple samples together, it is also important to have numerous replicates to account for biological differences. There is also the fact that the minimal dyes are incompatible with MS, which actually results in an increased workload since the location of the unlabeled proteins needs to be estimated based on the subtraction of the mass of the dye or additional gels to be prepared in parallel for
spot picking. Thus it is important that these problems are considered when using DIGE for proteomic analyses.

1.3.3. Target specific stains

Originally proteomics was primarily concerned with detection of the total proteome. However, years of research has shown not only the important roles proteins have within the cell but also the importance of their post-translation modifications. One of the most important protein modifications is phosphorylation, which controls protein activity, whether it is turning a protein “on” or “off” and/or regulating interactions with other biomolecules. Detection of this sub-proteome, referred to as the phosphoproteome, was originally accomplished by entrapping liberated phosphate within the gel; under alkaline conditions the phosphoester bond was hydrolysed and the presence of calcium ions promoted the formation of insoluble calcium phosphate \[277\]. This complex was then treated with ammonium molybdate in nitric acid and detection of this blue complex enhanced by staining with methyl green. This densitometric stain was specific to phosphoproteins, with detection sensitivity of ~ 3 µg (i.e. 1 nmol of protein-bound phosphate) \[277\]. Another method used aluminium ions in an acidic CBB solution to promote the formation of a bridge between the dye and phosphate residue \[278\]; detection of 40 ng of apo-phosvitin was attained (i.e. 0.13 nmol of phosphate).

Since there are a number of limitations to using densitometric stains for detection, a fluorescent alternative was developed. Pro-Q Diamond preferentially binds to phosphoproteins, detecting phospho-serines, -tyrosines and -threonines with similar sensitivity \[279-280\]. As is to be expected, sensitivity does rely on the number of phosphate residues; β-casein (five residues) and pepsin (one residue) were detected at
1-2 ng and 8 ng, respectively \cite{279}. Although Pro-Q Diamond was shown to be highly specific for phosphoproteins there was a weak non-specific detection of non-phosphorylated proteins especially those of a basic nature \cite{279}. It was suggested, however, that by using a fixing solution containing 50% methanol (MeOH)/10% trichloroacetic acid rather than 45% MeOH/5% acetic acid (HAc) non-specific staining could be eliminated \cite{279}. Advantages of Pro-Q Diamond include compatibility with MS and potential for reduced cost as it can be diluted with water without compromising sensitivity, signal intensity, and dynamic range \cite{279, 281}.

Glycosylation is another important protein modification and detection of these proteins was first successful in the 1960s \cite{282-283}. Modifications to the periodic acid-Schiff stain were investigated \cite{284-289}, but use of this method was limited since there was a requirement of a minimum of 1 µg carbohydrate content for detection \cite{287}. Based on this periodic Schiff mechanism, in which the glycols are oxidised to aldehydes using periodic acid and then reacted with the dye to form a conjugate, the fluorescent dye Pro-Q Emerald 300 was developed \cite{290}. Although effective for detection of glycosylated proteins it was noted that there was non-specific binding to lipopolysaccharides and incompatibility with laser-based bed scanners \cite{290}. This resulted in a new fluorescent glycoprotein stain, Pro-Q Emerald 488 \cite{291}. As mentioned for phosphorylated protein, detection sensitivity was also dependent on carbohydrate content \cite{291}.

A big advantage to the use of phospho- and glyco-protein stains is that a single proteome can be examined for these specific proteins in conjunction with the assessment of the total protein profile \cite{279, 291-294}. Examination of the three proteomes – phospho, glyco and total – can be successful when performed sequentially in this order to avoid quenching of the fluorophores \cite{294}. This multiplex staining approach
facilitates the characterisation of the proteome on a global scale as well as increasing our knowledge of the contributions of proteins with multiple post-translational modifications.

1.3.4. Characteristics of quantitative protein stains

Gel-based proteomics relies on stains for protein detection and it is thus critical that these have the capability to detect as much of the proteome as possible. There are a number of characteristics a stain must possess in order to be used for reliable quantitative proteomics. The lowest limit of detection (LLD) is referred to as the lowest amount of protein detected that is three standard deviations greater than the measured background \(^{295}\). This measurement provides information on the detection sensitivity for a stain; thus, the smaller this value the more sensitive the stain is. Although this is informative, it doesn’t always reflect what can be seen on the image. Practical sensitivity, though not a common characteristic, is defined in this thesis as a measure used to statistically determine whether the protein signal is distinguished from background based on the raw image. This is a less stringent measure of sensitivity in comparison to LLD but also important in determining the overall usefulness of a stain.

The proteome itself consists of a number of proteins which are all present in a range of concentrations. To ensure that a stain accurately reflects the amount of a protein one must define as the linear dynamic range (LDR) \(^{239}\). This measure indicates the range of protein concentrations in which the relationship between stain signal intensity and protein abundance is linear; this is thus the range of reliable quantitation. It is also valuable to characterise inter-protein variability (IPV) - how well the stain interacts from protein to protein \(^{239}\). Although IPV is, to a degree,
unavoidable due to the complex nature of the proteome, it can provide an important performance comparison between stains since a lower IPV is clearly preferable. The signal to noise ratio (S/N) is used to assess how well a stain can discriminate protein from background \[^{239}\]. However, if the signal of a given stain is weak though sensitive, S/N for this stain may not highlight the difference between protein signal and background adequately, the signal squared to noise ratio (i.e. S^2/N ratio) is often used as an alternative measurement to highlight this contrast.

It is also important to consider the stability of the stain-protein interaction. Most stains bind to proteins via non-covalent interactions, so if the molecule can be easily stripped off protein while sitting in solution (e.g. waiting time before imaging) an image taken immediately after destaining may have sufficiently different signal intensities when compared to a gel that has been sitting and destaining prior to imaging. This can have serious implications by increasing error between replicates, or can provide misleading information when comparing different samples. Other important characteristics for the use of a stain that are not related to quantitation are: ease of use, low cost, researcher and environmental safety, and compatibility with mass spectrometry techniques.
HYPOTHESIS AND AIMS

Coomassie brilliant blue, in particular BioSafe (BioRad) and Neuhoff cCBB formulations have been shown to be sensitive fluorescent protein stains [296]. However, the protocol employed was standardised and may have been sub-optimal for detection of proteins in-gel. Thus, here I test the hypothesis that optimisation of the CBB staining protocol will further improve fluorescent in-gel protein detection.

To test this hypothesis, the following aims were addressed:

1. Determine the optimal variables for stain time, destain time, destain solution for BioSafe and Neuhoff cCBB.
2. Evaluate the impact of storage conditions and duration on detection sensitivity.
3. Determine the LDR for optimised BioSafe and Neuhoff cCBB protocols.
4. Determine compatibility of the optimised BioSafe and Neuhoff cCBB protocols with MS (ESI and MALDI).
5. Apply optimised BioSafe and Neuhoff cCBB protocols to native proteome detection in comparison with SR.
6. Determine the applicability of optimised BioSafe and Neuhoff cCBB protocols to tall gel formats.
7. Examine the effect of imagers on detection using the optimised BioSafe and Neuhoff cCBB staining protocols.
CHAPTER 2: Optimisation of Coomassie Brilliant Blue staining as a competitive fluorescent alternative for sensitive in-gel protein detection

2.1. Introduction

Proteomics seeks to examine the entire complement of proteins expressed by a genome in a given biological sample (whole organism, tissue fluid, cell or organelle) using numerous techniques. These are extensive and include gel electrophoresis, liquid chromatography, mass spectrometry, protein chips and many others. However, the application of newly developed proteomic methods is accompanied with a cost. As with any technique, confidence and reproducibility are important features for application and success.

Polyacrylamide gel electrophoresis (PAGE) for the resolution of proteins has been a well-established method since the late 1950s. Its continued use is attributed to its stability, flexibility, ease of use, reproducibility, and high resolution. The most common method for one-dimensional PAGE (1DE), is SDS-PAGE. SDS possesses a strong negative charge masking the native charge of the protein, making this approach useful not only for the resolution of polypeptide chains but also for determining molecular weight (MW) with great accuracy. Although SDS-PAGE on its own is useful for resolving polypeptides within a mixture, the main concern is that a single band may include a number of co-resolved polypeptides since separation solely relies on MW and no other biochemical characteristic. It is likely that more than one protein will possess the same MW, but less likely that several proteins will possess the same MW and isoelectric point (pI).
but does, however, depend on the properties of the gel. These two independent protein biochemical properties thus provide the foundation of resolution by 2DE. First proteins are resolved by isoelectric focusing (IEF), during which proteins pass through a pH gradient and are immobilised when the pH conveys a net charge of zero (i.e. pI). Focussed proteins are then subjected to SDS-PAGE. Over the years 2DE has been and is continuously being enhanced to produce reliable and reproducible protein resolution. In a single analysis, 2DE presents a visual window of the proteome of any biological sample based on protein charge, MW, abundance and post-translational modifications.

Although 2DE has been shown to be highly reproducible, an ongoing issue faced by this well established technique is one of protein detection (i.e. stain sensitivity). Quantitative proteomics is dependent on the protein stain and it is essential that a stain (i) provides reproducible detection of low amounts of protein (LLD); (ii) delivers a linear relationship between the amount of protein and staining intensity (LDR); (iii) has low staining variability between different proteins (IPV); (iv) delivers high signal to noise ratio; (v) is compatible with downstream microchemical characterisation techniques such as mass spectrometry, and, if possible, (vi) is of low cost. A recent review assessing the quantitative aspect of protein detection methods highlighted the need for more rigorous and routine characterisation of protein stains for standardised assessment and comparison.

Textiles dyes, including Amido black, Coomassie Brilliant Blue (CBB) and Fast green were used for densitometric detection of proteins in-gel. However, quantitative detection of proteins by densitometric measures was superior with the use of CBB and this largely became the stain of choice. Many improvements to CBB solvent composition, dye concentration/type and alternate
strategies for staining protein/destaining gel matrix were investigated to achieve higher levels of sensitivity \[178-181, 183, 185, 187, 189-193, 197\]. However, there was limited success and CBB detection sensitivity could not be enhanced below \(\sim 30\) ng of protein \[183, 186, 194, 198\].

A detailed systematic study focusing on the chemistry of stain solvent composition, developed three strategies for enhanced protein detection with low background staining \[199\]. By altering the organic solvent to include phosphoric acid and ammonium sulfate the formation of colloidal CBB (cCBB) particles reduced the number of free CBB molecules available to penetrate the gel and stain protein while reducing background. The solvent composition of cCBB was subsequently optimised and detection sensitivity of proteins in-gel was improved \[200\]. Some reports suggested that changes to the formulation of the original cCBB delivered sensitivity below 8 ng of protein; however, limited quantitative characterisation of the new formulations may have limited their use \[197, 303-304\]. The use of CBB in combination with other dyes has also been investigated, but with little quantitative characterization; in some cases, failure to provide any quantitative advantage over CBB alone has limited the use of these combined formulations in proteomics \[194, 196, 305\].

Densitometric detection of protein relies on the absorbance of light; so if the stain also binds strongly to matrix (i.e. where no protein is present), this poses a problem for quantitation since protein signal may be indistinguishable from background. Recently fluorescent stains have gained more popularity, since background interference can (to an extent) be overcome as the stains primarily fluoresce when bound to protein. Additional advantages of fluorescent protein stains
include reproducible sensitivity, broad linear response and compatibility with mass spectrometry, which are often stated in literature but not always extensively assessed.

There are a number of fluorescent protein stains available on the market, including SYPRO Ruby (SR), Deep Purple (DP), Flamingo, Rubeo, Pro-Q Diamond and many others. The most widely used is SR, a luminescent metal chelate stain composed of ruthenium as part of an organic complex that interacts non-covalently with proteins on membranes (nitrocellulose/polyvinylidene difluoride) and in polyacrylamide gels \[217, 254, 306\]. The use of SR for staining proteins in-gel is simple like cCBB staining, however, detection sensitivity is superior with the lowest amount of protein detected between ~1-2 ng and wide LDR \[217, 249, 271\]. The superior performance of SR was also observed for two dimensional applications of various protein samples, when compared to other common protein stains (silver stain, cCBB, DP) \[258, 307\]. Although SR has all these advantages, its use is accompanied with a high cost. To economise the use of SR there have been some suggestions in the literature for re-use or dilution but these attempts were largely unproductive since signal intensity and protein detection were affected \[251-252\]. Another approach to reduce cost, was the development of alternate ruthenium stains based on the known chemical aspects of SR, these include RuBPS, ASCO_Ru and Rubeo (G Biosciences) but none of these at present have been able to surpass the sensitivity of SR \[253-255, 259, 308-309\].

Traditional use of cCBB for protein detection is poor by densitometric measures; however, recent research has demonstrated cCBB as a competitive alternative for sensitive protein detection via infra-red fluorescence (IRF) \[230, 259, 296\]. Preliminary assessment of cCBB-IRF of native proteomes, soluble and membrane, not only indicated similar spot detection but more importantly, an improved signal to
noise (S/N) ratio in comparison to SR. A thorough extension of this revealed that protein detection via CBB-IRF can match and even exceed the detection performance of SR. In that study 14 formulations of CBB, organic and colloidal states, were examined in detail, providing substantial evidence that indicated the superior performance of Bio-Safe™ Coomassie Stain (Bio-Rad; Hercules, CA) and the Neuhoff cCBB formulation (NG) in terms of detection sensitivity, selectivity, LDR and IPV.

Although the information obtained from 1DE analyses of standard proteins is valuable, the real application of a stain is the sensitive detection of proteins in complex native samples. Assessment of murine brain samples resolved by 2DE (i.e. both total soluble and total membrane proteomes), comparing cCBB-IRF and SR, revealed that SR provided only a small advantage over cCBB-IRF for overall spot detection (0.6%). The approach in this study, however, utilised a standardised method of CBB staining in order to accommodate the parallel comparison of the 14 stain formulations. Is it possible that the unified CBB staining protocol was sub-optimal for CBB-IRF protein detection? This question is addressed here.

Here we used the FLA-9000 imaging platform (FUJIFILM Corporation, Tokyo Japan) to examine the extent of cCBB-IRF protein sensitivity. As originally supplied, this instrument was not optimal for cCBB-IRF detection of proteins, and a number of emission filters were tested (740 nm and 750nm long pass filters). The unified cCBB protocol was tested using the FLA-9000 to ensure a comparable sensitivity relative to SR, since the Odyssey imaging platform (LiCor, Lincoln, NB) was originally used. Since it was shown that Bio-Safe™ Coomassie Stain and Neuhoff cCBB were the best performers for quantitative proteomics, method optimisation was carried out only for these two stains. Optimisation tests explored
three critical features of cCBB staining to enhance detection sensitivity – (i) stain duration; (ii) destain duration and solution; (iii) storage conditions for cCBB stained gels. To determine which variables yielded optimal staining, quantitative assessments of sensitivity (LLD), selectivity ($S^2/N$), IPV and LDR were carried out in addition to MS compatibility.
2.2. Methods

2.2.1. Chemicals

All consumables were of electrophoresis grade or higher quality. Electrophoresis consumables including acrylamide, tris-glycine SDS buffer, Coomassie Brilliant Blue G-250 dye were purchased from Ameresco Inc. (Solon, OH). A commercially available, ready-made CBB formulation, Bio-Safe™ Coomassie Stain, and SYPRO Ruby protein gel stain were purchased from Bio-Rad Laboratories (Hercules, CA). All other consumables, including a number of isolated protein standards (Table 2-1) were purchased from Sigma (St. Louis, MO). Double distilled water was used for all reagents prepared throughout the work presented in this study.

2.2.2. Native protein sample preparation

2.2.2.1. Tissue homogenisation

Mouse brains were harvested fresh, rinsed thrice with ice cold 1× phosphate buffered saline (PBS) containing 1× protease inhibitor cocktail (PIC; 2 µg/mL aprotinin, pepstatin and leupeptin), snap frozen in liquid nitrogen, and stored at -80°C until needed. Mouse brains thawed at room temperature, were sliced into 5×5 mm cubes and rinsed thrice with ice cold 1× PBS/1× PIC.

Deep frozen tissue was placed in a chilled self-sealing teflon chamber (2 mL internal volume) containing a 9 mm steel ball bearing. The sealed chamber was placed horizontally in locking callipers of the Mikro-dismembrator S (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and the tissue was homogenised for one minute (min) at 2000 rpm. Powdered sample was transferred into a chilled tube
(i.e. in liquid nitrogen), and the process repeated until the entire sample was processed before storage at -80 °C.

2.2.2.2. Pre-fractionation of powdered protein sample

Powdered sample (Section 2.2.2.1) was suspended in three volumes of ice cold hypotonic lysis buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 with 1× PIC] and vortexed vigorously for three min. Isotonicity was restored with an equal volume of ice cold 2× PBS/2× PIC and placed on ice for three min. If insoluble material was visible after lysis, the homogenate was centrifuged at 100 g for 10 min at 4 °C. The supernatant was transferred to ultracentrifuge tubes to pellet cellular membranes at 124 436 g for three hours (h) at 4 °C.

After ultracentrifugation the supernatant was collected, snap frozen in liquid nitrogen and stored at -80 °C (Total soluble fraction I). The membrane pellet was suspended in a small volume of ice cold 1× PBS/1× PIC and centrifuged at 124 436 g for 3 h at 4 °C. The supernatant was collected, snap frozen in liquid nitrogen and stored at -80 °C (Total soluble fraction II). The membrane pellet was solubilised in 2DE solubilisation buffer (8 M urea, 2 M thiourea, 4% CHAPS, 1× PIC), aliquoted, and stored at -80 °C (Total membrane protein).

Total soluble fractions I and II thawed at room temperature, were pooled and kept on ice prior to concentration to a minimal volume using a Nanosep centrifugal concentration device with a 3 kDa size exclusion filter (Pall Life Sciences, East Hills, New York). The total soluble sample buffer was exchanged with 4 M urea/1× PIC thrice to remove residual PBS by centrifugation at the maximal force tolerated by the Nanosep unit (4000 g); this was carried out at 4 °C, with numerous 20 min spins.
required to reduce the volume to 1/8 of the original volume (4 mL). The final concentrate was combined with 2DE solubilisation buffer, aliquoted and stored at -80 °C (Total soluble protein).

NOTE: in this thesis “Total soluble protein” and “Total membrane protein” refer to the total extractable protein enabled by the extraction process used.

2.2.2.3. Protein concentration assay

Total membrane and soluble samples were measured against a Bovine serum albumin (BSA) standard using the EZ-Q protein quantitation assay (Molecular Probes, Eugene, Oregon). Briefly, 2 µL aliquots were blotted on filter paper in triplicate for each sample and air dried. The blot was fixed with 100% MeOH for five min with agitation; fixing solution was discarded and the blot air dried. While protected from light, the blot was stained with EZ-Q protein stain for 30 min with continuous agitation. The blot was destained, 3×25 s, with 10% MeOH/7% HAc, before imaging using the LAS-4000 imager and quantitation using Multi Gauge software v3.0 (FUJIFILM Corporation, Tokyo Japan).

2.2.3. Two-dimensional gel electrophoresis (2DE)

To ensure that gels used for 2DE were not overloaded, 100 µg total protein loads were used for mini-SDS-gels. Sample protein in 2DE solubilisation buffer was combined 1:1 with 2DE ampholyte buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1× PIC, 1% broad range carrier ampholytes (pH 3-10) and 0.25% each of four narrow range ampholytes (pH 3-5, 6-8, 7-9 and 8-10); the final ampholyte concentration was 1% (w/v). The sample was first reduced with 2.6 mM TBP/45 mM DTT and then alkylated with 230 mM acrylamide, sequentially, for incubations of
one h each at 25 °C. Sample was passively hydrated onto a 7-cm non-linear pH = 3-10 IPG strip (Bio-Rad Laboratories, Hercules, California) overnight at room temperature (no longer than 16 h).

Isoelectric focussing (IEF) was carried out at 20 °C using the Bio-Rad Protean IEF cell (Bio-Rad Laboratories, Hercules, California). A constant current of 50 µA/gel was applied during all phases - ramping up to 250V maximum for 15 min (desalting) and linear ramping to 4000V maximum over 2 h. Electrode wicks were changed every 30 min during linear ramping to ensure effective removal of trace salts and to ensure smooth ramping. Once attained, a constant voltage of 4000V was applied for 37500 volt-hours. Following IEF, IPG strips were equilibrated in IPG equilibration buffer (6 M urea, 20% glycerol, 0.375 M tris, 2% SDS) supplemented with 2% DTT for the first wash and then replaced with the same buffer supplemented with 2.5% acrylamide for the second wash. Each equilibration step was carried out at room temperature for 10 min without agitation.

IPG strips were placed on top of SDS-PAGE gels consisting of 12.5% T, 2.6% C acrylamide/bis-acrylamide resolving gel with a 5% T, 2.6% C stacking gel. Both resolving and stacking gels were buffered with 375 mM tris-HCl (pH 8.8) and contained 0.1% SDS. IPG strips were sealed to stacking gel with an agarose solution (0.5% low-melting agarose, 375 mM tris-HCl (pH 8.8), 0.1% SDS) before electrophoresis was carried out in a cold room (4 °C) at 150V during migration into the stacking gel and then resolved at 90V to completion. 2DE gels were either stained using the cCBB protocol developed by Butt [296] or with SR. Gels were fixed in 10% MeOH/7% HAc for 4 h with continuous rocking. Fixed gels were washed 10 times with water, 20 min per wash. Gels were stained with NG for 4 h with continuous rocking, and destained with a total of 10 water washes, 5×5 min and 5×20
min. Gels stained with SR were stained in fresh SR for 12 h and destained with 10% MeOH/7% HAc (3×20 min) and H₂O (3×20 min).

2.2.4. Standard protein sample preparation

Isolated protein samples purchased from Sigma (St. Louis, MO) were dissolved in distilled water (H₂O). Any insoluble material was removed by centrifugation; 2000 g for five min. Soluble protein was aliquoted into microfuge tubes and stored at -30 °C, or -80 °C for longer term storage. Using the Protein Knowledgebase (http://www.uniprot.org/), characteristics provided by Sigma for each of the isolated protein standards were searched and appropriately selected using the ProtParam Tool to obtain the extinction co-efficient for each protein, and these and are listed in Table 2-2. Extensive searches of the literature for extinction co-efficient of each protein corroborated the calculated ProtParam Tool values. The only exceptions were Soybean trypsin inhibitor (STI) and trypsinogen (TRYP) due to limited literature on their extinction co-efficients [310-352].

The concentration of each protein was determined using the Beer-Lambert Law, \( A = \varepsilon c l \). Where \( A \) is absorbance, \( \varepsilon \) is the molar absorptivity in units M⁻¹ cm⁻¹, \( c \) is the concentration of the protein in solution expressed as M and \( l \) the path length of the sample, cm. Absorbance of protein samples was assessed at 280 nm using the POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany) with in-built 1 cm path length correction selected prior to readings. For each protein three independent replicates were prepared. The concentration of each protein determined by this method is listed in Table 2-3.
2.2.5. One-dimensional gel electrophoresis (1DE)

1DE was carried out essentially according to Laemmli [3] with some minor modifications according to Cannon-Carlson and Tang [4], and Coorssen et al. [148], unless specified otherwise. 1DE separations were carried out using the mini-format (6 cm tall). All gels were prepared in triplicate. Unless stated, all steps were carried out at room temperature.

Isolated protein standards described in Table 2-3 were diluted serially as a protein mixture with 1DE sample buffer (2% SDS, 25 mM tris (pH = 8.8), 12.5 mM DTT and 5% glycerol), within the range of 119 – 0.08 ng (dilution factor = 2.5, nine dilutions in total). All proteins in the mixture were present at the same concentration. This dilution series was aliquoted and stored at -80 °C before use.

Prior to electrophoresis, samples were boiled (100 °C) for five min and cooled to room temperature for five min, before loading into three millimetre wide wells on standard one millimetre thick SDS-PAGE gel (mini-format – 6 cm tall). Isolated protein standards were resolved on 15% T, 2.6% C acrylamide/bis-acrylamide for optimal resolution. Stacking gel consisted of 5% T, 2.6% C. Both resolving and stacking gels were buffered with 375 mM tris-HCl (pH 8.8) and contained 0.1% SDS. Electrophoresis was carried out in a cold room (4 °C) at 150V during migration into the stacking gel and resolved at 90V to completion.

2.2.6. Management of 1DE and 2DE gels post electrophoresis

2.2.6.1. Fixation

After electrophoresis all gels were fixed in 10% MeOH/7% HAc (v/v) overnight, with continuous rocking. Fixed gels were washed three times with
distilled water, 20 min per wash, to minimise potential HAc contamination. All gels were agitated (60 rpm) at room temperature unless stated otherwise.

2.2.6.2. Stain preparation

Two formulations of cCBB were examined. One formulation was commercially sourced from Bio-Rad Laboratories (Hercules, CA), Bio-Safe™ Coomassie Stain (BioSafe) and the other stain was prepared in-house according to Neuhoff et al. [199-200] (NG). Briefly, ammonium sulphate (AS) was added to phosphoric acid (H₃PO₄) with continuous stirring (10% v/v and 2% v/v, respectively). To this CBB G-250 was added to yield a final concentration of 0.1% v/v, followed by MeOH added to a final concentration of 20% (v/v). The remaining volume was made up with water and the stain solution was mixed thoroughly before use. This in-house prepared stain was observed to form precipitates over extended periods of storage and so was prepared fresh just before use. A 10-fold volume of stain was required for one mini-SDS-gel (i.e. 50 mL).

2.2.6.3. Optimisation of cCBB staining parameters

In the available literature there are various strategies for fixing, washing and staining with cCBB. Here the most common strategies have been investigated for optimisation of cCBB staining, using both the commercial and in-house prepared cCBB formulations. All staining was performed under standard lab conditions; there was no need to protect CBB from the light since there was no statistical difference in protein intensity (i.e. fluorescence signal) and selectivity in the presence or absence of light (APPENDIX A: Figure A-2).
2.2.6.4. Duration of cCBB staining

It has been suggested that staining in cCBB for 15 h or less is adequate to completely stain protein bands, with staining approaching maximum after 30 h \[^{200}\]. To determine a practical time for delivering optimal protein staining with BioSafe and NG, fixed and washed gels underwent 4 h or overnight staining (20 h) at room temperature, with continuous rocking.

2.2.6.5. Destaining of cCBB stained gels

The minimum amount of destaining for cCBB stained gels was four consecutive 15 min water washes (i.e. total of 1 h destain time). It was possible that this destain protocol may have been insufficient to destain background and thus enhance the signal of low abundance proteins. So in addition to the four consecutive 15 min washes, another four consecutive 15 min water washes were performed with images taken after each to determine the optimal time course for destaining cCBB-stained gels.

Although staining with cCBB results in reduced staining of non-protein regions of a polyacrylamide gel, referred to as background, the level of CBB present may still mask the presence of low abundance proteins. Therefore, it is vital to reduce background staining as much as possible without affecting the staining of proteins. Alternate destain solutions tested in this study were shown to be potential candidates for cCBB-stained proteins, especially for low abundance proteins (APPENDIX A: Figures A-3 and A-4). Destain solutions tested were (i) water, which is commonly used to destain cCBB stained gels; (ii) 0.5 M sodium chloride (NaCl); and (iii) 1-5% \(H_3PO_4\). The 0.5 M NaCl and 1% \(H_3PO_4\) solutions were found to be advantageous for improved sensitivity of NG and BioSafe stained protein respectively.
2.2.6.6. Storage of cCBB stained protein

One issue with cCBB stained gels when stored in water for prolonged periods of time, is the strong tendency for CBB to detach from protein. This reduces the capacity to locate; and even re-assess resolved proteins in-gel; this can be problematic when proteins of lower abundance must be excised from the gel for further analysis (i.e. mass spectrometry). It has been suggested in the literature that a 20-25% AS solution stabilises CBB, maintaining protein staining [199-200]. Gels stained with BioSafe or NG were destained with 1% H₃PO₄ and 0.5 M NaCl, respectively, imaged, and stored in their respective destain solution, 5% AS, or 20% AS. Gels were stored motionless at 4 °C with images taken after 1 h, one day, two days, and one month, for all storage conditions.

2.2.6.7. Detection

When using the FLA-9000 imager for CBB-IRF detection, contamination is unavoidable due to residual cCBB left on the imaging surface and interferes with future gel scans. Therefore, based on experience with the stain, scanner and visual examination of the gel it was determined that 1 h washing as a sequence of four consecutive 15 min water washes, was the minimum requirement for destaining before use on the scanner to avoid CBB contamination issues with the instrument. Due to the nature of some of the chemical reagents used, gels underwent a brief water rinse before placing on the imaging surface of the FLA-9000. To further reduce potential interference resulting from CBB contamination, the glass imaging surface was cleaned with water followed by 90% ethanol before and after each gel scan. A good indication that the imaging surface is clean is if the cleaning tissue (i.e. Kim wipe) is free of any visible blue colour. Although the scanning plate for the
FLA-9000 can accommodate numerous gels to be imaged simultaneously, it has been shown that cCCB stained gels in particular must be imaged individually since background fluorescence is influenced by location on the imaging surface, since lower concentrations of dye should have reduced background signal which is the case when imaged individually (APPENDIX A: Figure A-1).

Prior to the imaging of CBB-stained gels, they were rinsed for a minimum of six times with distilled water to remove any CBB potentially coating the gel surface as this can affect the quality of the image. To ensure that a high resolution image was obtained, the gel was rinsed for a minimum of six times and covered with a thin even layer of water; this was found to reduce the potential for any dark shading during imaging. Using the FLA-9000, Coomassie stained gels were scanned in the infra-red using excitation at 685 nm; emission was collected in the 750 channel (i.e. >750 nm), unless stated otherwise. Images were acquired at 100 µm pixel resolution and a Photomultiplier Tube (PMT) setting of 600V, unless stated otherwise. The FLA-9000 was set to create 16-bit root images. The settings utilised for imaging cCBB stained gels were pre-determined from an analysis that established the Emission Filter and PMT voltage combination that would deliver optimal sensitivity (Figures 2-1 and 2-2, Tables 2-4 and 2-5). For CBB optimisation and further analyses images were taken at 600V as it was shown that there was no advantage to increasing the PMT setting beyond 600V (Results: Section 2.3.1 and APPENDIX A: Figures A-6 – A-9; Tables A-1 – A-4). There was a concern that imaging the gels in this sequential fashion (i.e. 600, 700, and 800V), might influence the results so the reverse was also assessed (i.e. 800, 700, and 600V), establishing that there was no statistical difference in the data (APPENDIX A: Figure A5). SR stained gels imaged with the FLA-9000 were excited using the 473 nm laser and fluorescence collected with the
LPG (O575) filter, with a PM set to 350V; this PMT setting was chosen as it yielded consistent sub-saturation signal intensity for the highest quantity of protein seen in standard 2D gels (V. Gauci, Coorssen lab, unpublished observations).

2.2.6.8. 1DE image analysis

All 16-bit root images were analysed using Multi Gauge V3.0 (FUJIFILM Corporation, Tokyo Japan). The fluorescent signal from protein bands in 1DE gels were calculated as volumes where: Volume = ε (pixel height * pixel area). That is, the volume is the integral sum of the volume of all pixels that make up that band. Background was calculated by selecting a non-protein region adjacent to the protein band examined and extrapolated to yield a background volume equivalent to the area for that protein band specifically. Only background subtracted signal volumes are reported here, thus a fluorescent volume equal to zero indicates signal equal to background. A significant difference from background for all fluorescent volumes was established using Student’s T-test (i.e. all below p < 0.05; n= 3). Fluorescent volumes, S^2/N ratios, and IPV values presented here were established using only protein volumes determined to be significantly different from background based on the raw image; the lowest concentration of a protein that could be statistically differentiated from background on the raw image is referred to here as the lowest practical sensitivity (LPS).

2.2.6.9. 2DE image analysis

Analysis of 2DE gel images was carried out using Delta2D 4.0 (DECODON GmbH, Greifswald, Germany), essentially according to company instructions with some modifications. Briefly, before being imported into the Delta2D software, gel
images (16-bit tiff) were cropped in Multigauge V3.0 (FUJIFILM Corporation, Tokyo Japan) so that only that portion of the image corresponding to the resolving area of the gel remained (APPENDIX B: Figure B1). Once imported, the total spot numbers were determined on the raw gel images.

2.2.6.10. Assessing detection sensitivity, selectivity and inter-protein variability by 1DE analyses

In order to assess cCBB stained gels used in the process of optimising the staining protocol, four critical parameters were used.

1. The lowest limit of detection (LLD): considered as the lowest quantity of protein that can be statistically distinguished from background \(^{295}\). This parameter is particularly important to determine the ability of a staining/destaining/storage variable to discriminate low abundance proteins (i.e. sensitivity). Value derived from \(3 \times \) background error (standard error of the mean) and slope based on protein fluorescent volumes. (APPENDIX A: Practical Examples of Quantitative Characteristics).

2. Signal squared to noise \((S^2/N)\) ratio: this ratio provides a quantitative assessment of the level of contrast (i.e. selectivity) provided by a given staining/destaining/storage variable. The protein fluorescent volumes and background error (standard deviation) were used to calculate these ratios. (APPENDIX A: Practical Examples of Quantitative Characteristics).
3. **Integrated Measure of Practical sensitivity (IMPS):** A mathematical formula to incorporate LLD and LPS. The IMPS is defined as: \[ \text{IMPS} = (\text{LLD} \times \text{LPS})^{-1} \] The greater the IMPS, the more sensitive the detection.

4. **Inter-protein variability (IPV):** Amount of variation in fluorescent signal yielded by the same quantity of different protein. With this parameter we can determine the effect of a variable on fluorescent intensity between different proteins. IPV is expressed as a percentage of the maximum fluorescent volume and was calculated as:
   \[ \text{IPV} = \frac{\text{Largest Fluorescent Volume} - \text{Smallest Fluorescent Volume}}{\text{Largest Fluorescent Volume}} \times 100 \]

   For each staining/destaining/storage variable tested, IPV was calculated for 8, 19 and 48 ng protein loads, and averaged, unless stated otherwise. Since, the tests described in Section 2.4.1 did not yield consistent protein detection at 8ng/band; IPV was based on 19, 48 and 119 ng band loads.

2.2.7. **LDR of cCBB and SYPRO Ruby**

   Isolated protein standards described in Table 2-3 were diluted serially as individual proteins with 1DE sample buffer, within the range of 25 000 – 0.38 ng (dilution factor = 4, nine dilutions in total). This dilution series was aliquoted and stored at -80 °C. Electrophoresis carried out and the gels fixed and stained as described in Sections 2.2.3 and 2.2.4. Gels were then destained with the optimised methods for BioSafe and NG as determined in this study (5×15 min, 1% H₃PO₄ and 0.5 M NaCl respectively), or for SR as mentioned above (Section 2.2.3). Quantitative
image analysis of SR stained gels was performed in the same manner as for those stained with CBB.

To determine the widest linear dynamic range, referred to as $R^2$, a Student’s T-test was performed ($n=3$), $p > 0.05$ to compare against the regression of the entire data set ($R^1$). By sequentially eliminating the highest data point, this yielded numerous $R$ values for smaller groups of the one data set (i.e. $R^21$ – all data points to $R^27$-data point above and including LPS). By statistical comparison of $R^21$-$R^27$, the widest LDR was determined and denoted $R^2$ (i.e. $R^22$-7 with a regression value statistically greater than $R^21$). (APPENDIX A: Practical Examples of Quantitative Characteristics).

2.2.8. Mass spectrometry analysis of isolated protein standards

2.2.8.1. Protein excision and digestion

One hundred micrograms of β-galactosidase was resolved by 1DE (Section 2.2.5). The resolved protein bands were excised from BioSafe, NG and SR stained gels, destained by washing twice with 50% acetonitrile (ACN)/50 mM ammonium bicarbonate (NH$_4$HCO$_3$), pH 9, for 10 min with vortexing. The gel bands were then dehydrated with 100% ACN for 10 min and subsequently rehydrated in 20 µL of NH$_4$HCO$_3$, pH 9 containing 12.5 ng/µL of trypsin (Promega). After incubating at 4°C for 30 min, 25 µL of NH$_4$HCO$_3$, pH 9 was added and samples were incubated overnight at 37°C. The digest solution was transferred to a new tube; 30 µL of 50% ACN/2% formic acid was added to the tube containing the gel piece and this was incubated for 10 min in a sonicating water bath at full power. The resulting solution was pooled with the digest solution and lyophilised to 15 µL. Either 2 µL (plus 13 µL of MS loading solvent) or 15 µL was transferred to an autosampler vial.
2.2.8.2. LC/MS/MS.

Using an Eksigent AS-1 autosampler connected to a Tempo nanoLC system (Eksigent, USA), 10 µL of the sample was loaded at 20 µL/min with MS loading solvent (2% ACN/0.2% trifluoroacetic acid) onto a C8 trap column (Michrom Biosciences, USA). After washing the trap for three min, peptides were washed off at 300 nL/min onto a PicoFrit column (75 µm × 100 mm) packed with Magic C18AQ resin (Michrom Biosciences, USA). Peptides were eluted from the column and into the source of a QSTAR Elite hybrid quadrupole-time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex) using the following program: 5-50% MS solvent B (98% ACN/0.2% formic acid) over 8 min, 50-80% MS buffer B over five min, 80% MS buffer B for two min, 80-5% MS buffer B for three min. MS solvent A (2% ACN/0.2% formic acid) prior to sample loading. The eluting peptides were ionised at 2300V with a 75 µm ID emitter tip that tapered to 15 µm (New Objective). An Intelligent Data Acquisition (IDA) experiment was performed; within a mass range of 375-1500 Da, peptides of charge state 2⁺-5⁺ with an intensity of more than 30 counts/s were continuously scanned for. Selected peptides were fragmented and the product ion fragment masses measured over a mass range of 100-1500 Da. The mass of the precursor peptide was then excluded for 15 s.

2.2.8.3. 5800 MALDI TOF/TOF

Tryptic peptides were desalted using ZipTip C18 (Millipore) according to the manufacturer’s instructions. Peptides were eluted with 10µL of 50 % ACN/0.1% trifluoroacetic acid. 1 µL of peptide sample was mixed in a 0.65 mL tube with 1 µL of 5 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in 50%ACN/0.1% trifluoroacetic acid/10 mM monoammonium phosphate. 1 µL of the mixed sample
was then transferred onto a clean 386-well OptiTOF target plate (AB Sciex) and allowed to dry. 0.5 μL of diluted TOF/TOF standards (AB Sciex) was added to the relevant calibration target spots (APPENDIX A: Table A-5).

Peptide samples were then analysed using a 5800 MALDI-TOF/TOF mass spectrometer in positive ion reflector mode. Laser intensity (349nm Nd:YAG laser) was set to 2600 for MS parent ion scans and 3000 for MS/MS fragmentation ion scans. 400 laser shots were averaged for MS scans and up to 1250 shots were averaged for MS/MS scans with the Dynamic Exit algorithm selected, which monitors spectral quality and stops shot accumulation if a user defined threshold is met. MS parent ion scans were calibrated using the TOF/TOF standards (APPENDIX A: Table A6) while MS/MS fragmentation ion scans were calibrated using the fragments of Glu-Fibrinopeptide B present in the TOF/TOF standards mixture. The resulting MS and MS/MS spectral data were then converted to Mascot Generic Format using TOF/TOF Extractor v2.1 (Michigan Proteome Consortium, 2003) and the data searched with the UNITE interface (APCF).

2.2.8.4. Data analysis

The MS/MS data files produced by the QSTAR were searched using Mascot Daemon (version 2.2.2, provided by the Australian Proteomics Computational Facility, http://www.apcf.edu.au/, and searched against the LudwigNR database [which is comprised of the UniProt, plasmoDB and Ensembl databases (vQ209.8785680 sequences, 3087386706 residues)] with the following parameter settings: (i) fixed modifications: none; (ii) variable modifications: propionamide, oxidised methionine; (iii) enzyme: trypsin; (iv) number of allowed missed cleavages: 3; (v) peptide mass tolerance: 100 ppm; (vi) MS/MS mass tolerance: 0.2 Da; (vii) charge
state: 2\(^+\) and 3\(^+\). The results of the search were then filtered by including only protein hits with at least one unique peptide (Bold Red) and excluding peptide hits with a p-value greater than 0.05. Peptides identified by Mascot were further validated by manual inspection of the MS/MS spectra for the peptide to ensure the b- and y-ion series were sufficiently extensive for an accurate protein ID.

NOTE: ESI and MALDI-MS experimentation after protein band excision was performed by Dr Matt Padula, University of Technology, Sydney (UTS) (i.e. Sections 2.2.8.2 – 2.2.8.4).
2.3. Results

2.3.1. FLA-9000 optimisation for cCBB-IRF

Before images could be taken on the FLA-9000 for sensitive cCBB-IRF in gel detection of protein, the optimal filter and PMT setting combination needed to be determined. Initially five emission collection filters; 725, 733, 740, 750 and 800 nm longpass (LP), were examined qualitatively and resulting images for the 740 and 750 nm LP filters (740 LP and 750 LP) were deemed suitable for quantitative analysis (i.e. no grainy appearance). Since method optimisation relies on optimal cCBB-IRF detection the Coorssen lab cCBB staining approach was used. All gels underwent standard fixing and washing steps (Section 2.2.7), overnight staining and four consecutive 15 min water destain washes before images were captured using the 740 and 750 LP filters at PMT settings – of 600, 700, 800, 900 and 1000V.

For BioSafe and NG-IRF the detection of protein standards with increasing PMT settings yielded increases in fluorescent volume and $S^2/N$ ratios, but no changes in IPV (Figures 2.1 & 2.2). Results for the 740 LP and 750 LP for BioSafe-IRF were quite similar, however, practical sensitivity (i.e. fluorescent volumes statistically discriminated from background) for the 750 LP was remarkably better. The 750 LP filter showed uniform detection of BioSafe stained protein down to 19 ng/band across most PMT settings in comparison to the 740 LP (Figure 2.1: T-V). In addition, six of the seven standard proteins, at 8 ng/band, were detected with the lowest PMT setting (i.e. 600V), and BSA was detected to 3 ng (Figure 2-1: W & X). In comparison to 740 LP, detection of protein at 8 and 3 ng/band was possible with increased PMT setting but notably only for higher molecular weight (MW) proteins (i.e. > 66 kDa). Sensitivity was thus improved with the use of the 750 LP filter for all PMT settings tested (Table 2-4). Therefore, all subsequent testing of potential
improvements for sensitive protein detection were accomplished with the use of the 750 LP filter for BioSafe-IRF.

Capture of NG-IRF using the 740 and 750 LP filters resulted in remarkable differences (Figure 2-2). It was clear that 740 LP was insufficient for protein detection since STI was the only protein standard detected consistently for all PMT settings (119 and 48 ng/band) (Figure 2-2: O & P), with three more proteins detected at one or two of the available PMT settings. However, the 750 LP filter yielded outstanding improvements to practical sensitivity, and consistent detection of all protein standards down to 19 ng/band at all PMT settings (Figure 2-2: V). At 8ng/band, six of seven proteins were detected and three of seven at 3ng/band (BGAL, BSA, CA) when the lowest PMT setting was used (Figure 2-2: W & X).

Since practical sensitivity of NG stained proteins using the 740 LP filter was only 48 ng/band, IPV could not be calculated. However, IPV for 750 LP was unchanged regardless of the PMT setting (Figure 2-2: Y). Comparison of IPV between NG and BioSafe for the 750nm LP showed better IPV with BioSafe, however, NG did display large variation in this pilot test to determine the best imaging parameters. Detection sensitivity (i.e. LLD) for all protein standards when using the 750 LP revealed no statistical differences regardless of the PMT setting (Table 2-5). The few LLD values derived from data for PHOSB, TYRP, STI and LYS when using the 740 LP were approximately four to eight-fold lower relative to those values determined when using the 750 LP filter (Table 2-5).

One objective of this work was to detect small quantities of protein; from the data it was obvious that the 740 LP filter was insufficient for the assessment of BioSafe- and NG-IRF. Although increased PMT settings yielded an increase in signal intensity (i.e. fluorescent volume) and selectivity (i.e. $S^2/N$), there was no
improvement to IPV and sensitivity. Thus, all subsequent analyses used only the 750 LP filter and a PMT setting of 600V to capture IRF from both BioSafe and NG stained proteins.

2.3.2. cCBB protocol optimisation

2.3.2.1. Assessment of cCBB-IRF protocol on FLA-9000

After the optimal FLA-9000 imaging parameters were determined for optimal IRF detection of cCBB-stained protein, it was necessary to identify how the general staining protocol \cite{296} would perform on this instrument, relative to SR, as the detection apparatus employed in that initial study of CBB-IRF was the scanner-based Odyssey Imaging System (LiCor, Lincoln, NB). To test this, a total membrane sample derived from mouse brain was used and cCBB-IRF was compared to SR. Even though SR detected a greater number of total spots relative to CBB (546 vs. 487 respectively), this was not significant as determined by a Student’s T-test (\( p > 0.05, n=3 \)) (Figure 2-3), suggesting that the FLA-9000 imaging system was suitable for the detection of cCBB-IRF. Thus, using this system, potential improvements to the staining protocol for protein detection by cCBB-IRF could be tested.

2.3.2.2. Assessment of optimal stain and destain time

Stain time is one critical step for any staining method, especially when applied post-electrophoretically for in-gel protein detection. The staining step relies on time as the limiting factor, and this may affect the ability of the stain to detect low abundant proteins. To investigate this, a stain time of 4 h was chosen since this has been suggested as the minimal time for saturation of protein bands \cite{200} and was also the stain time utilised in the initial characterisation of cCBB-IRF \cite{296}. A 20 h stain
time was also chosen as a representative of standard overnight staining protocols. After staining for a specific time, gels were destained with water using four consecutive fifteen min (4×15 min) washes. These gels were imaged and then destained with water for another 4×15 min washes, with images taken after each wash step in order to determine the most favourable destain cycle for optimal sensitivity of protein detection.

Fluorescent volumes for BioSafe and NG exhibited similar signal intensity between 4 and 20 h staining times, (Figures 2-4 and 2-5: A-G). Selectivity (i.e. $S^2/N$) between the two stain times was not significantly different for all BioSafe stained proteins; however, the $S^2/N$ ratio for LYS stained with NG for 20 h was significantly greater relative to 4 h (Figure 2-5: N). The most obvious advantage to increasing the stain time to 20 h was improvement to practical sensitivity. For BioSafe there was an improvement to CA and TYRP practical sensitivity (Figure 2-4: K & L). When stained with NG, improvement to practical sensitivity was seen for BSA, TYRP and LYS (Figure 2-5: J, L & N). Although there were no significant differences between the LLD values after 4 and 20 h stain times (BioSafe and NG), there was a trend of lower LLD values after 20 h of staining relative to 4 h, especially for NG stained proteins (Tables 2-6 and 2-7).

Examining the lowest practical sensitivity (LPS), the lowest concentration of a protein that could be statistically discriminated from background and LLD as individual characteristics to define stain sensitivity is valuable, but by integrating these measures it highlights the relationship between LPS and LLD. Thus, it is desirable for a stain to deliver both low LPS and LLD values which is represented by large values of the integrated measure of practical sensitivity - IMPS. There was no difference in the IMPS for BioSafe between 4 or 20 of staining (Figure 2.4: O-U);
however, when stained with NG, BSA and LYS had higher IMPS values after 20 h of staining (Figure 2.5: Q & U). In addition, IPV for NG was significantly reduced after 20 h stain time (Figure 2-5: V). Taking into consideration the results for all protein standards, it was not clear that the application of one stain time over the other was superior with the standard sequence of destain washes (i.e. 4×15 min) for both BioSafe and NG.

Although the standard destain wash sequence was sufficient to achieve sensitivity greater than seen using densitometric assessment of cCBB, non-protein regions of gels still retained a moderate blue colour. Thus, it was considered that with increased water washes sensitivity would be improved by reducing background staining. However, there were no significant changes for either BioSafe and NG, when using more than 5×15 min water washes (APPENDIX A: Figures A-6 – A-9 and Tables A1-A4), hence Figures 2-6 and 2-7 display results only for 4×15 and 5×15 min water washes.

Destaining of BioSafe stained gels revealed similarities in fluorescent volumes for all seven protein standards between 4×15 and 5×15 min water washes for both stain the 4 and 20 h stain intervals, and this also true of the IPV (Figure 2-6). Although $S^2/N$ ratios between 4 and 20 h for both 4×15 and 5×15 min water washes were similar, practical sensitivity for two proteins (BGAL and STI) was improved with 20 h of staining and five consecutive washes for destaining (Figure 2-6: V & AA). Sensitivity of BioSafe staining did not differ significantly between 4 and 20 h, but interestingly five proteins after 4 h staining and 5×15 min destaining had poorer LLD values in comparison to 4×15 min (Table 2-8). For 20 h staining LLD values did not change between destain washes of 4×15 and 5×15 min, however, a slight improvement was seen for low MW proteins (> 29 kDa) for 5×15 min.
For NG stained gels fluorescent protein volumes were similar for destain washes of 4 and 20 h stain intervals, and IPV was maintained regardless of increased washing (Figure 2-7). Practical sensitivity for five proteins was maintained with a subsequent wash (5×15min) after 20 h staining; however, this was not the case after 4 h. Even though there were no significant trends in sensitivity between destain washes, it is interesting to note that the LLD values after 20 h of staining were consistently better for all proteins after both destaining conditions (i.e. 4×15 and 5×15 min) in comparison to 4 h. For both BioSafe and NG, IMPS does not clearly indicate any obvious advantage between any stain time and/or destain sequence.

To optimise the cCBB staining protocol, it was clear that a 20 h stain time would be a suitable option when pursuing alternate destain conditions (i.e. other than water for destaining cCBB gels), since destaining gels with water after 4 h of staining suffered greater losses to practical sensitivity, which is the foundation for the assessment criteria – sensitivity (LLD), selectivity ($S^2/N$), IMPS and IPV. Thus, for all subsequent analyses, 20 h staining with BioSafe or NG was applied with only 4×15 and 5×15 min washes tested to examine the effect of destaining with alternate reagents.

2.3.2.3. Assessment of alternate destain conditions

The most common strategy to optimise cCBB for sensitive protein detection was to reduce background by using reagents other than water for destaining. A number of studies have employed a range of harsh reagents for destaining which may not be environmentally friendly and/or also impart a cost for solvent disposal. In addition there has been little quantitative characterisation to support abandoning the use of water altogether for the destaining of CBB stained gels. Sodium
chloride (NaCl) at 0.5 M may be a potential alternate destain since the risks to the user and environment are negligible, but no quantitative information was provided regarding this approach to destaining [191]. Preliminary analysis revealed that NaCl destaining of NG stained protein under the standard destain conditions of 4×15 min, yielded detection of 10 ng BSA, with selectivity significantly greater than seen after destaining with water (APPENDIX: A: A-3). However, after destaining BioSafe stained protein with NaCl, 10 ng of BSA was undetectable. Thus, NaCl was only useful to destain NG stained protein.

Most destaining alternatives to date have been tested on the Neuhoff cCBB. Although the composition of BioSafe (Bio-Rad) is based upon the Neuhoff cCBB formulation, modifications may explain why it does not behave in the same manner. Thus, we looked at the composition of the Neuhoff stain for the possibility that one of the ingredients could be suitable as an alternative destain. The only ingredient that appeared a likely candidate was phosphoric acid, H₃PO₄, since the use of ammonium sulphate (AS) resulted in an increase in background, and methanol was not considered due to the associated hazards of use, expense, and possible risk that evaporation might result in large inconsistencies when destaining. The final concentration of H₃PO₄ in the Neuhoff stain is 2%. A range of H₃PO₄ concentrations between 1-5% were tested (APPENDIX A: A-4) and it was shown that BSA at 390 ng was detectable over all concentrations; however, 10 ng of BSA stained with BioSafe was only detected when destained with 1% H₃PO₄. Although this low load of BSA was detected after destaining with H₃PO₄ over concentrations of 1-3% when stained with NG, there was no advantage over water.

Examination of BioSafe stained protein revealed that destaining using 1% H₃PO₄ produced similar results to water, with regard to protein intensity (fluorescent
volume) and IPV (Figure 2-8). Although improvements to selectivity were not a consistent trend for all loads of all proteins when using H₃PO₄ as an alternative destain, there were some statistical improvements for four proteins when destained with H₃PO₄, 5×15 min. Sensitivity for all proteins was similar between water (4×15 and 5×15 min) and H₃PO₄ (5×15 min), however, inter-gel reproducibility was greater with H₃PO₄, in particular for low MW proteins (Table 2-10). With regard to IMPS more than half of the protein standards tested had significantly higher values when destained with H₃PO₄ (5×15 min) highlighting the advantage of this destain solution and wash sequence for improved BioSafe sensitivity.

NG stained protein, however, showed significant improvements to protein intensity (fluorescent volumes), sensitivity, selectivity and IPV when destained with 0.5 M NaCl (Figure 2-9 and Table 2-11). Selectivity was consistently enhanced over all loads for all proteins in comparison to destaining with water (Figure 2-9: H-N). Although NG sensitivity between water and NaCl was comparable, three standard proteins had significantly better sensitivity when destained with NaCl for 5×15 min (BSA, TYRP, LYS; Table 2-11). There was also an obvious trend in which the LLD values for all low MW proteins (i.e. < 29 kDa) were improved greatly when destained with NaCl relative to water. For the majority of standard proteins, destaining with NaCl was shown to yield significantly improved IMPS, specifically with 5×15 min washes, relative to water. Based on this evidence BioSafe stained protein was destained with 1% H₃PO₄ (5×15 min) and 0.5 M NaCl (5×15 min) was used for NG stained gels.
2.3.2.4. Assessment of storage conditions

It has been suggested that storage of NG stained gels in 20-25% AS stabilises CBB and hence allows long-lasting visible detection of proteins in-gel\(^{[199-200]}\). However, no quantitative assessment was provided in those studies. Here the effectiveness of AS as a storage solution for BioSafe and NG stained gels was examined. Examination of BioSafe stained gels revealed that protein intensity, selectivity, and IPV were maintained for up to one month after storage in 1% \(\text{H}_3\text{PO}_4\) or AS (Figure 2-10). Sensitivity of protein detection following storage in \(\text{H}_3\text{PO}_4\), 5% AS, and 20% AS also did not change dramatically over the period of one month (Table 2-12). For the majority of proteins, LLD was improved with up to one day of storage in \(\text{H}_3\text{PO}_4\); however, storage beyond this time (i.e. up to one month) resulted in poorer LLD values, particularly for low MW proteins. In comparison to \(\text{H}_3\text{PO}_4\), AS LLD values were affected up to one day, however, the most obvious advantage to AS (5% and 20%) as a storage solution was a trend in improved LLD values for all protein standards after one month (Table 2-12). For five proteins, IMPS was significantly better after two days of storage in 20% AS. From these results, it could be recommended that BioSafe stained protein can be stored in \(\text{H}_3\text{PO}_4\) for a period of up to one month. For the best quantitative results one day storage is the maximum, but if long term storage is required AS is most suitable.

Signal intensity of NG stained protein was maintained for up to two days of storage in all solutions tested, however, after one month storage signal intensities for proteins stored in 5% AS and NaCl (0.5 M) were dramatically reduced (Figure 2-11). Interestingly, fluorescence intensity and IPV of NG stained proteins was maintained when stored in 20% AS for one month, and this was reflected in the retained selectivity for five of seven proteins (Figure 2-11). Detection sensitivity after storage
in NaCl and 20% AS did not change dramatically over the period of one month. For the majority of proteins LLD was improved up to one day after storage in NaCl; longer storage resulted in poorer LLD values, particularly after one month, and particularly for low MW proteins. In comparison to NaCl, the LLD values for some proteins did change over the month storage in AS, however, the most obvious advantage to AS (5% and 20%) as a storage solution was a trend in improved LLD values for all protein standards after one month, especially for low MW proteins after storage in 20% AS (Table 2-13). For NG stained protein stored in any solution, IMPS does not clearly distinguish a superior storage condition; however, the majority of proteins had improved IMPS after one month. Short term storage (up to one day) in NaCl was thus shown to be reasonable for maintaining the sensitivity of detection; however, to maintain the level of sensitivity for a long storage period, 20% AS is most advantageous.

2.3.3. Assessing LDR

Proteomes derived from numerous sources not only consist of a number of different proteins but these proteins are present at vastly different concentrations. It is thus important that a protein detection method used for quantitative proteomics not only stain proteins proportionally but that this occurs over a wide range of concentrations – the Dynamic Range. The range at which a detection method obeys a uniform linear relationship between protein quantity and signal intensity is otherwise known as the LDR. Here the LDR of the optimised cCBB staining protocols was determined and compared to SR for the same isolated proteins standards (Figure 2-12 and Table 2-14).
Fluorescent volumes for all proteins were similar for the entire data set when stained with BioSafe or NG but were greater for SR (Figure 2-12). For most proteins the relationship between protein quantity and signal intensity is quite linear when stained with BioSafe, NG or SR (*R² values all above 0.97). The widest LDR was determined statistically as the maximum range in which protein quantity and signal intensity were most linear (i.e. R² closest to 1), and denoted as **R² (Figure 2-12 and Table 2-14). Due to the nature of the analysis the detection threshold is defined as the lowest practical sensitivity (i.e. the lowest amount of protein that can be statistically distinguished from background for quantitative image analysis). In regards to each stain, LDR varies from protein to protein, for most, SR staining yielded relatively narrow LDR in comparison to cCBB and this was particularly evident after staining with the NG protocol (Figure 2-13: A).

It was also interesting to note that while SR could detect protein down to 0.38 ng (LPS of BSA), LPS values for four proteins when stained by SR are higher than those of NG (and in two instances BioSafe) (Figure 2-13: A). When assessing staining between proteins, the three lowest protein loads that were consistently detected by all stains for all proteins tested were 6.1, 24 and 98 ng and these were thus used to calculate IPV (Figure 2-13: B). Although there is no significant difference between the IPV of all stains, the trend was toward the largest for SR.

2.3.4. Mass spectrometry compatibility of optimised cCBB protocols

The optimised cCBB staining protocols for near IRF detection have been shown to have similar sensitivity to SR and for most proteins a wider LDR, in particular using the NG staining protocol. Although quantitative characteristics of a stain are important for the evaluation of proteomes, compatibility with mass
spectrometry is also essential. Proteomics not only strives to detect as many proteins as possible and their relative concentrations but also to enable simplified identification of the detected proteins. Since mass spectrometry, whether it be MALDI or ESI, is used for this purpose it is vital that the staining protocol used does not negatively impact this identification process.

Although the protocol modifications implemented here for BioSafe and NG would not be considered unfavourable, compatibility with mass spectrometry for positive identification needed to be assessed. Due to limited resources, analysis was restricted to one of the seven isolated protein standards. Beta-galactosidase was chosen for this analysis since LDR was the widest with this protein for all three stains. For BioSafe, NG, and SR, β-galactosidase was positively identified as the top protein hit for each independent replicate for ESI-MS (n=3; APPENDIX A: Table A-7). In addition, the source was positively identified as E. coli, which was stated by the supplier (Sigma). Interestingly, protein stained with both of the optimised cCBB protocols had higher MASCOT scores in comparison to SR (APPENDIX A: Table A-7). In addition, sequence coverage was also greater for both BioSafe and NG relative to SR, however, only BioSafe was found to be significantly different (Figure 2-14: A).

When the same samples were used for MALDI-MS, β-galactosidase was also positively identified, however, for NG and SR this was only true for two replicates (APPENDIX A: Table A-8). In the remaining replicate, despite the presence of a number of peptides, the ion scores were below 72 and thus could not be used by MASCOT for identification. In comparison to ESI, the MALDI MASCOT scores and sequence coverage were much lower (APPENDIX A: Table A-7 and Figure 2-
14: B). Thus, whether using either ESI- and/or MALDI-MS, both optimised cCBB staining protocols are compatible with mass spectrometry for successful protein IDs.
Figure 2-1. Filter and PMT combination for optimal sensitivity with BioSafe.

A dilution series of pure protein standards (seven in total) was resolved by 1DE and detected by BioSafe-IRF. Gels were stained overnight with BioSafe and destained with water, 4×15 min. Fluorescent volumes significantly discriminated from background, (A-G) 740 LP and (H-N) 750 LP. The capacity to discriminate protein from background, $S^2/N$ ratio [(O-S) 740 LP and (T-X) 750 LP]. (Y) Variability in staining between different proteins (i.e. IPV) 740 LP and 750 LP. All data obtained at PMT settings of 600, 700, 800, 900, 1000V. Error bars represent standard error of the mean (n = 3).
Figure 2-1
Figure 2-1
Figure 2-1
Figure 2-2. Filter and PMT combination for optimal sensitivity with NG.

A dilution series of pure protein standards (seven in total) was resolved by 1DE and detected by NG-IRF. Gels were stained overnight with NG and destained with water, 4×15 min. Fluorescent volumes significantly discriminated from background, (A-G) 740 LP and (H-N) 750 LP. The capacity to discriminate protein from background, $S^2/N$ ratio [(O-S) 740 LP and (T-X) 750 LP]. (Y) Variability in staining between different proteins (i.e. IPV) 740 LP and 750 LP. All data obtained at various PMT settings of 600, 700, 800, 900, 1000V. Error bars represent standard error of the mean (n = 3).
Figure 2-2
Figure 2-2
Figure 2-2
Figure 2-3. Comparison of SR and NG using the FLA-9000.

Mouse total membrane protein was resolved by 2DE and stained with SR or the method developed by Butt [296] (NG). Fluorescence was detected with the FLA-9000, using the 750 LP filter, and 600V PMT setting. This figure represents the quantitative image analysis of total spots detected for each stain. Error bars represent standard error of the mean (n=3).
Figure 2-3
Figure 2-4. Optimal stain time for BioSafe – 4 vs. 20 h

A dilution series of pure protein standards (seven in total) were resolved by 1DE and detected by BioSafe-IRF. (A-G) Quantitative image analysis identified fluorescent volumes significantly discriminated from background. (H-N) The capacity to discriminate protein from background, $S^2/N$ ratio. (O-U) Stain sensitivity in relation to LLD and LPS – IMPS. (V) Variability in staining between different proteins (i.e. IPV). Error bars represent standard error of the mean (n = 3).
Figure 2-4
Figure 2-4
Figure 2-5. Optimal stain time for NG – 4 vs. 20 h

A dilution series of pure protein standards (seven in total) were resolved by 1DE and detected by NG-IRF. (A-G) Quantitative image analysis identified fluorescent volumes significantly discriminated from background. (H-N) The capacity to discriminate protein from background, S^2/N ratio. (O-U) Stain sensitivity in relation to LLD and LPS – IMPS. (V) Variability in staining between different proteins (i.e. IPV). Error bars represent standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, p < 0.05) in comparison to a 4 h stain time are denoted by *. 
Figure 2-5
Figure 2-6. Optimal destain time for BioSafe – 4 vs. 20 h

A dilution series of pure protein standards (seven in total) were resolved by 1DE, stained with BioSafe and destained with water, 4×15 and 5×15 min. Quantitative image analysis identified fluorescent volumes significantly discriminated from background, (A-G) 4 h and (H-N) 20 h stain time. The capacity to discriminate protein from background, $S^2/N$ ratio, (O-U) 4 h and (V-AB) 20 h stain time. (AC-AI) Stain sensitivity in relation to LLD and LPS – IMPS. (AJ) Variability in staining between different proteins (i.e. IPV). Error bars represent standard error of the mean (n = 3). Statistically significant differences between 4×15 and 5×15 min washes denoted by * and differences between stain time (i.e. 4 vs. 20 h) denoted by † [Student’s t-test: * (†) p < 0.05, ** (††) p < 0.01].
Figure 2-6
Figure 2-6
Figure 2-6
Figure 2-6
Figure 2-7. Optimal destain time for NG - 4 vs. 20 h

A dilution series of pure protein standards (seven in total) were resolved by 1DE, stained with BioSafe and destained with water, 4×15 and 5×15 min. Quantitative image analysis identified fluorescent volumes significantly discriminated from background, (A-G) 4 h and (H-N) 20 h stain time. The capacity to discriminate protein from background, $S^2/N$ ratio, (O-U) 4 h and (V-AB) 20 h stain time. (AC-AI) Stain sensitivity in relation to LLD and LPS – IMPS. (AJ) Variability in staining between different proteins (i.e. IPV). Error bars represent standard error of the mean (n = 3). Statistically significant differences between 4×15 and 5×15 min washes denoted by * and differences between stain time (i.e. 4 vs. 20 h) denoted by † [Student’s t-test: * (†) p < 0.05, ** (††) p < 0.01].
Figure 2-7
Figure 2-7
Figure 2-7
Figure 2-7
Figure 2-8. Alternate destain for BioSafe.

A dilution series of pure protein standards (seven in total) were resolved by 1DE stained with BioSafe for 20 h and destained with water or H₃PO₄, 4×15 and 5×15 min washes. (A-G) Quantitative image analysis identified fluorescent volumes significantly discriminated from background. (H-N) The capacity to discriminate protein from background, S²/N ratio. (O-U) Stain sensitivity in relation to LLD and LPS – IMPS. (V) Variability in staining between different proteins (i.e. IPV). Error bars represent standard error of the mean (n = 3). Statistically significant differences between wash sequence (i.e. 4×15 vs. 5×15 min) for a specific destain condition, are denoted by *, differences between water and H₃PO₄ for the same wash sequence, denoted by † [Student’s t-test: * (†) p < 0.05, ** (††) p < 0.01, *** p < 0.001].
Figure 2-8
Figure 2-8
Figure 2-9. Alternate destain for NG.

A dilution series of pure protein standards (seven in total) were resolved by 1DE stained with NG for 20 h and destained with water or NaCl, 4×15 and 5×15 min washes. (A-G) Quantitative image analysis identified fluorescent volumes significantly discriminated from background. (H-N) The capacity to discriminate protein from background, $S^2/N$ ratio. (O-U) Stain sensitivity in relation to LLD and LPS – IMPS. (V) Variability in staining between different proteins (i.e. IPV). Error bars represent standard error of the mean ($n = 3$). Statistically significant differences between wash sequence (i.e. 4×15 vs. 5×15 min) for a specific destain condition, are denoted by *; differences between water and NaCl for the same wash sequence, denoted by †

[Student’s t-test: * (†) $p < 0.05$, ** (††) $p < 0.01$, ††† $p < 0.001$].
Figure 2-9
Figure 2-9
A dilution series of pure protein standards (seven in total) were resolved by 1DE, stained with BioSafe for 20 h and destained with 1% H$_3$PO$_4$, 5×15 min. After imaging gels were stored in 1% H$_3$PO$_4$, 5% or 20% AS and imaged after 1 h, 2 h, one day, two days and one month storage. (A-AI) Quantitative image analysis identified fluorescent volumes significantly discriminated from background. (AJ-BR) The capacity to discriminate protein from background, S$^2$/N ratio. (BS-DB) Stain sensitivity in relation to LLD and LPS – IMPS. (DC-DG) Variability in staining between different proteins (i.e. IPV). Error bars represent standard error of the mean (n = 3). Statistically significant differences in comparison to H$_3$PO$_4$ are denoted by * (Student’s t-test: * p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 2-10
Figure 2-10
Figure 2-10
Figure 2-10
Figure 2-10
Figure 2-10
Figure 2-11. Assessment of NG storage.

A dilution series of pure protein standards (seven in total) were resolved by 1DE, stained with BioSafe for 20 h and destained with 1% H$_3$PO$_4$, 5×15 min. After imaging gels were stored in 0.5 M NaCl, 5% or 20% AS and imaged after 1 h, 2 h, one day, two days and one month storage. (A-AI) Quantitative image analysis identified fluorescent volumes significantly discriminated from background. (AJ-BR) The capacity to discriminate protein from background, S$^2$/N ratio. (BS-DB) Stain sensitivity in relation to LLD and LPS – IMPS. (DC-DG) Variability in staining between different proteins (i.e. IPV). Error bars represent standard error of the mean (n = 3). Statistically significant differences in comparison to NaCl are denoted by * (Student’s t-test: * p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 2-11
Figure 2-11
Figure 2-11
Figure 2-11
Figure 2-11
Figure 2-12.  Linear dynamic range of BioSafe, NG and SR.

A dilution series of pure protein standards (seven in total) was resolved by 1DE, and detected with optimised BioSafe and NG protocols or SR. (A-Q) Quantitative image analysis identified fluorescent volumes significantly discriminated from background with respect to protein load. Multiple regression analyses indicate the maximum range over which fluorescent signals increase linearly with respect to protein load. * Indicates regression analysis of the entire data set from the LPS (*R^2). ** Indicates the regression analysis of the largest linear range from the LPS (**R^2). Error bars represent standard error of the mean (n = 3).
Figure 2-12
**Figure 2-13. Assesment criteria for BioSafe, NG and SR.**

(A) Graphical representation of the widest LDR for BioSafe, NG and SR (i.e. **R**). (B) Inter-protein variability of BioSafe, NG and SR. Error bars represent standard error of the mean (n = 3).
Figure 2-13

**A**

**B**

- **BioSafe**
- **NG**
- **SR**

**ng of protein**

0.38 1.5 6.1 24 98 390 1600 6300 25000

**% Inter-Protein Variability**

0 20 40 60 80 100

**Figure 2-13**
Figure 2-14. Mass spectrometry compatibility – BioSafe, NG and SR.

(A) Average ESI-MS sequence coverage values for BioSafe, NG and SR. (B) Average MALDI-MS sequence coverage values for BioSafe, NG and SR. Error bars represent standard error of the mean (n = 3). Statistically significant differences in comparison to SR are denoted by * (Student’s t-test: ** p < 0.01). NOTE: no statistical analysis could be performed on MALDI data since SR only had one replicate and was not sufficient to satisfy the analysis.
<table>
<thead>
<tr>
<th>Organic Protein Standard</th>
<th>Abbreviation</th>
<th>Nominal MW (kDa)</th>
<th>Sigma Product No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-galactosidase from <em>Escherichia coli</em></td>
<td>BGAL</td>
<td>116</td>
<td>G8511</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>BSA</td>
<td>66</td>
<td>A7517</td>
</tr>
<tr>
<td>Carbonic Anhydrase from Bovine Erythrocyte</td>
<td>CA</td>
<td>29</td>
<td>C2273</td>
</tr>
<tr>
<td>Lysozyme from Chicken Egg White</td>
<td>LYS</td>
<td>14.3</td>
<td>L4631</td>
</tr>
<tr>
<td>Phosphorylase b from Rabbit Muscle</td>
<td>PHOSB</td>
<td>97</td>
<td>P4649</td>
</tr>
<tr>
<td>Soybean Trypsin Inhibitor from <em>Glycine max</em></td>
<td>STI</td>
<td>20</td>
<td>T9767</td>
</tr>
<tr>
<td>Trypsinogen, PMSF treated from Bovine Pancreas</td>
<td>TRYP</td>
<td>24</td>
<td>T9011</td>
</tr>
</tbody>
</table>
Table 2-2. Extinction coefficients of isolated protein standards.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession Number</th>
<th>MW (kDa)</th>
<th>$E_{\text{Molar}}$ (M$^{-1}$ cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-galactosidase</td>
<td>P00722</td>
<td>11648</td>
<td>261 690</td>
</tr>
<tr>
<td>Glycogen phosphorylase, muscle form</td>
<td>P00489</td>
<td>97.29</td>
<td>120 140</td>
</tr>
<tr>
<td>Serum albumin precursor (BSA)</td>
<td>P02769</td>
<td>66.43</td>
<td>42 925</td>
</tr>
<tr>
<td>Carbonic anhydrase 1</td>
<td>Q1LZA1</td>
<td>28.82</td>
<td>43 430</td>
</tr>
<tr>
<td>Pancreatic anionic trypsinogen</td>
<td>Q547S4</td>
<td>26.29</td>
<td>46 130</td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>Q9LLX2</td>
<td>17.98</td>
<td>17 210</td>
</tr>
<tr>
<td>Lysozyme C precursor</td>
<td>P00698</td>
<td>14.31</td>
<td>37 970</td>
</tr>
<tr>
<td>Protein Standard</td>
<td>Nominal MW (kDa)</td>
<td>Protein Concentration (mg ml$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------</td>
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<td></td>
</tr>
<tr>
<td>BGAL</td>
<td>116</td>
<td>2.36</td>
<td></td>
</tr>
<tr>
<td>PHOSB</td>
<td>97</td>
<td>1.63</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>66</td>
<td>3.73</td>
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<tr>
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<tr>
<td>LYS</td>
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<td>3.45</td>
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Table 2-4. BioSafe filter and PMT for optimal sensitivity (LLD).

<table>
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<tr>
<th>Protein Standard</th>
<th>740 LP</th>
<th>750 LP</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>600V</td>
<td>700V</td>
</tr>
<tr>
<td>BGAL</td>
<td>10.4 ± 3.6</td>
<td>10.8 ± 4.0</td>
</tr>
<tr>
<td>PHOSB</td>
<td>8.9 ±2.0</td>
<td>9.8 ± 1.7</td>
</tr>
<tr>
<td>BSA</td>
<td>9.3 ± 1.0</td>
<td>10.0 ± 1.0</td>
</tr>
<tr>
<td>CA</td>
<td>7.2 ± 0.1</td>
<td>7.3 ± 0.8</td>
</tr>
<tr>
<td>TYRP</td>
<td>11.3 ± 0.9</td>
<td>10.5 ± 1.2</td>
</tr>
<tr>
<td>STI</td>
<td>19.5 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>LYS</td>
<td>13.6 ± 0.3</td>
<td>14.3 ± 1.6</td>
</tr>
</tbody>
</table>

Error represents standard error of the mean (n = 3).
Statistically significant differences (Student’s t-test, * p < 0.05, ** p < 0.01, *** p < 0.001) in comparison to 740 LP filter for the same PMT setting.
Table 2-5. NG filter and PMT for optimal sensitivity (LLD).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Standard</th>
<th>740 LP</th>
<th>750 LP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>600V</td>
<td>700V</td>
</tr>
<tr>
<td>BGAL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PHOSB</td>
<td>35.5 ± 7.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TYRP</td>
<td>40.3 ± 9.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STI</td>
<td>107.7 ± 47.6</td>
<td>154.2 ± 53.4</td>
<td>207.1 ± 68.9</td>
</tr>
<tr>
<td>LYS</td>
<td>-</td>
<td>-</td>
<td>85.9 ± 19.5</td>
</tr>
</tbody>
</table>

Error represents standard error of the mean (n = 3).
Statistically significant differences (Student’s t-test, * p < 0.05) in comparison to 740 LP filter for the same PMT setting.
Table 2-6. Stain time sensitivity (LLD) – BioSafe.

<table>
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<tr>
<th>Protein Standard</th>
<th>4 hrs</th>
<th>20 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGAL</td>
<td>5.0 ± 1.6</td>
<td>4.4 ± 1.3</td>
</tr>
<tr>
<td>PHOSB</td>
<td>5.7 ± 1.9</td>
<td>4.4 ± 1.5</td>
</tr>
<tr>
<td>BSA</td>
<td>9.9 ± 3.1</td>
<td>7.0 ± 2.1</td>
</tr>
<tr>
<td>CA</td>
<td>3.2 ± 1.3</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>TYRP</td>
<td>13.3 ± 6.6</td>
<td>13.2 ± 2.9</td>
</tr>
<tr>
<td>STI</td>
<td>14.7 ± 6.2</td>
<td>19.7 ± 4.4</td>
</tr>
<tr>
<td>LYS</td>
<td>8.8 ± 1.9</td>
<td>10.8 ± 2.0</td>
</tr>
<tr>
<td>Protein Standard</td>
<td>4 hrs</td>
<td>20 hrs</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>BGAL</td>
<td>5.7 ± 1.3</td>
<td>4.3 ± 1.4</td>
</tr>
<tr>
<td>PHOSB</td>
<td>7.0 ± 1.9</td>
<td>6.1 ± 1.8</td>
</tr>
<tr>
<td>BSA</td>
<td>10.7 ± 2.9</td>
<td>10.0 ± 2.3</td>
</tr>
<tr>
<td>CA</td>
<td>5.8 ± 1.1</td>
<td>4.1 ± 1.2</td>
</tr>
<tr>
<td>TYRP</td>
<td>18.4 ± 4.2</td>
<td>15.0 ± 3.6</td>
</tr>
<tr>
<td>STI</td>
<td>20.0 ± 4.1</td>
<td>15.5 ± 2.7</td>
</tr>
<tr>
<td>LYS</td>
<td>19.7 ± 3.7</td>
<td>10.8 ± 1.5</td>
</tr>
</tbody>
</table>
### Table 2-8. Water destain sensitivity (LLD) – BioSafe.

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>4 hrs</th>
<th>20 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4×15 min</td>
<td>5×15 min</td>
</tr>
<tr>
<td>BGAL</td>
<td>5.0 ± 1.6</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>PHOSB</td>
<td>5.7 ± 1.9</td>
<td>7.0 ± 1.3</td>
</tr>
<tr>
<td>BSA</td>
<td>9.9 ± 3.1</td>
<td>10.3 ± 3.3</td>
</tr>
<tr>
<td>CA</td>
<td>3.2 ± 1.3</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>TYRP</td>
<td>13.3 ± 6.6</td>
<td>15.8 ± 3.9</td>
</tr>
<tr>
<td>STI</td>
<td>14.7 ± 6.2</td>
<td>14.7 ± 2.3</td>
</tr>
<tr>
<td>LYS</td>
<td>8.8 ± 1.9</td>
<td>11.0 ± 1.9</td>
</tr>
</tbody>
</table>
Table 2-9. Water destain sensitivity (LLD) – NG.

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>4 hrs</th>
<th>20 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4×15 min</td>
<td>5×15 min</td>
</tr>
<tr>
<td>BGAL</td>
<td>5.7 ± 1.3</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>PHOSB</td>
<td>7.0 ± 1.9</td>
<td>6.4 ± 1.4</td>
</tr>
<tr>
<td>BSA</td>
<td>10.7 ± 2.9</td>
<td>10.7 ± 2.7</td>
</tr>
<tr>
<td>CA</td>
<td>5.8 ± 1.1</td>
<td>5.8 ± 1.4</td>
</tr>
<tr>
<td>TYRP</td>
<td>18.4 ± 4.2</td>
<td>16.7 ± 3.3</td>
</tr>
<tr>
<td>STI</td>
<td>20.2 ± 4.1</td>
<td>23.7 ± 4.7</td>
</tr>
<tr>
<td>LYS</td>
<td>19.7 ± 3.7</td>
<td>15.8 ± 3.1</td>
</tr>
</tbody>
</table>
Table 2-10.  BioSafe sensitivity (LLD) – phosphoric acid destain.

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>H$_2$O</th>
<th>H$_3$PO$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4×15 min</td>
<td>5×15 min</td>
</tr>
<tr>
<td>BGAL</td>
<td>1.6 ± 0.3</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>PHOSB</td>
<td>1.6 ± 0.5</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>BSA</td>
<td>2.8 ± 0.6</td>
<td>3.2 ± 1.4</td>
</tr>
<tr>
<td>CA</td>
<td>1.7 ± 0.7</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td>TYRP</td>
<td>3.4 ± 1.5</td>
<td>4.3 ± 1.5</td>
</tr>
<tr>
<td>STI</td>
<td>6.9 ± 2.1</td>
<td>8.3 ± 3.1</td>
</tr>
<tr>
<td>LYS</td>
<td>5.0 ± 1.4</td>
<td>7.5 ± 2.8</td>
</tr>
</tbody>
</table>

Statistical significance between 4×15 min and 5×15 min washes within one destain strategy (Student’s t-test, *p < 0.05, ** p < 0.01, *** p < 0.001)

Statistical significance between destain strategies (i.e. H$_2$O vs. H$_3$PO$_4$) for the same wash step (Student’s t-test, $^\dagger$ p < 0.05, $^{\ddagger\ddagger}$ p < 0.01, $^{\ddagger\ddagger\ddagger}$ p < 0.001)
Table 2-11.  NG sensitivity (LLD) – sodium chloride destain.

<table>
<thead>
<tr>
<th>Protein Standards</th>
<th>H₂O</th>
<th>NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4×15 min</td>
<td>5×15 min</td>
</tr>
<tr>
<td>BGAL</td>
<td>3.5 ± 0.5</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>PHOSB</td>
<td>4.4 ± 0.3</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>BSA</td>
<td>6.9 ± 0.5</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>CA</td>
<td>3.6 ± 0.3</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>TYRP</td>
<td>13.4 ± 1.2</td>
<td>14.2 ± 1.9</td>
</tr>
<tr>
<td>STI</td>
<td>12.7 ± 1.6</td>
<td>12.0 ± 1.6</td>
</tr>
<tr>
<td>LYS</td>
<td>12.5 ± 0.5</td>
<td>12.3 ± 1.8</td>
</tr>
</tbody>
</table>

Statistical significance between destain strategies (i.e. H₂O vs. NaCl) for the same wash step (Student’s t-test, ‡ p < 0.05, ‡‡ p < 0.01, ‡‡‡ p < 0.001)
Table 2-12. BioSafe storage sensitivity (LLD).

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>1% H$_3$PO$_4$</th>
<th>5% AS</th>
<th>20% AS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No storage</td>
<td>1 h storage</td>
<td>1 day storage</td>
</tr>
<tr>
<td>BGAL</td>
<td>3.3 ± 0.5</td>
<td>1.4 ± 0.2*</td>
<td>3.8 ± 1.3</td>
</tr>
<tr>
<td>PHOSB</td>
<td>4.4 ± 0.8</td>
<td>1.1 ± 0.2*</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>BSA</td>
<td>8.8 ± 1.9</td>
<td>4.3 ± 0.5</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>CA</td>
<td>3.8 ± 0.7</td>
<td>2.7 ± 0.3</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>TYRP</td>
<td>14.6 ± 1.2</td>
<td>12.8 ± 0.4</td>
<td>8.2 ± 2.7</td>
</tr>
<tr>
<td>STI</td>
<td>11.5 ± 1.2</td>
<td>11.3 ± 0.9</td>
<td>8.1 ± 2.9</td>
</tr>
<tr>
<td>LYS</td>
<td>10.1 ± 1.5</td>
<td>9.3 ± 0.6</td>
<td>6.9 ± 2.9</td>
</tr>
</tbody>
</table>

Statistically significant differences (Student’s t-test, * p < 0.05) in comparison to no storage within the same storage condition (i.e. difference in storage time), differences to H$_3$PO$_4$ for the same storage time is denoted as † p < 0.05, †† p < 0.001 (i.e. difference in storage condition).
Table 2-13.  NG storage sensitivity (LLD).

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>NaCl</th>
<th>5% AS</th>
<th>20% AS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>1 h</td>
<td>1 day</td>
</tr>
</tbody>
</table>
|                  | storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| 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storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage| storage|storage

Statistically significant differences (Student’s t-test, * p < 0.05, ** p < 0.01) in comparison to no storage within the same storage condition (i.e. difference in storage time), differences to H₃PO₄ for the same storage time is denoted as † p < 0.05, †† p < 0.01, ††† p < 0.001 (i.e. difference in storage condition).
## Table 2-14. Linear dynamic range.

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>Regression analysis of entire data set ((R^2))</th>
<th>Regression analysis of widest linear range from LPS ((\ddagger R^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BioSafe</td>
<td>NG</td>
</tr>
<tr>
<td>BGAL</td>
<td>25000 – 0.38 ng ( (R^2 = 0.9989) )</td>
<td>25000 – 0.38 ng</td>
</tr>
<tr>
<td>PHOSB</td>
<td>25000 – 6.1 ng ( (R^2 = 0.9889) )</td>
<td>25000 – 6.1 ng</td>
</tr>
<tr>
<td></td>
<td>( (R^2 = 0.9859) )</td>
<td>( (R^2 = 0.9855) )</td>
</tr>
<tr>
<td>BSA</td>
<td>25000 – 6.1 ng ( (R^2 = 0.9814) )</td>
<td>25000 – 1.5 ng</td>
</tr>
<tr>
<td></td>
<td>( (R^2 = 0.9896) )</td>
<td>( (R^2 = 0.9889) )</td>
</tr>
<tr>
<td>CA</td>
<td>25000 – 24 ng ( (R^2 = 0.9820) )</td>
<td>25000 – 6.1 ng</td>
</tr>
<tr>
<td></td>
<td>( (R^2 = 0.9812) )</td>
<td>( (R^2 = 0.9879) )</td>
</tr>
<tr>
<td>TYRP</td>
<td>25000 – 98 ng ( (R^2 = 0.9939) )</td>
<td>25000 – 1.5 ng</td>
</tr>
<tr>
<td></td>
<td>( (R^2 = 0.9901) )</td>
<td>( (R^2 = 0.9908) )</td>
</tr>
<tr>
<td>STI</td>
<td>25000 – 0.38 ng ( (R^2 = 0.9799) )</td>
<td>25000 – 0.38 ng</td>
</tr>
<tr>
<td></td>
<td>( (R^2 = 0.9771) )</td>
<td>( (R^2 = 0.9874) )</td>
</tr>
<tr>
<td>LYS</td>
<td>25000 – 6.1 ng ( (R^2 = 0.9802) )</td>
<td>25000 – 0.38 ng</td>
</tr>
<tr>
<td></td>
<td>( (R^2 = 0.9811) )</td>
<td>( (R^2 = 0.9787) )</td>
</tr>
</tbody>
</table>
2.4. Discussion

2.4.1. Optimisation of FLA-9000 for cCBB-IRF

The FLA-9000 imager is a versatile instrument as it can detect proteins stained with SR, Cy3 and Cy5 (i.e. reactive labels used for differential gel electrophoresis), Pro-Q Diamond (phosphoproteins) and Pro-Q Emerald 488 (glycoproteins). This means that a single gel can be sequentially stained by numerous approaches and imaged using the same instrument. Thus, here the imaging instrument can be eliminated as a contributing factor for discrepancies in detection.

Originally the detection of protein by CBB was carried out using densitometry. Recently, CBB has been shown to fluoresce in the near-infrared region of the spectrum and this has been shown to enable the sensitive detection of proteins in-gel \cite{230, 259, 296}. Since CBB-IRF detection is currently not widely employed for protein detection, the only other imager available for capturing CBB-IRF is the Odyssey (LiCor, Lincoln, NB), by dually exciting CBB at 685 and 785 nm and collecting emission using the 700 nm channel i.e. < 750 nm. The FLA-9000 is advertised as a multipurpose imaging instrument as it can accommodate up to four lasers simultaneously and a range of filter modules. Nonetheless, although the lasers to excite CBB were supplied with the instrument, the most suitable filter to capture CBB-IRF was not.

The spectral data for CBB-IRF has not been well established but work suggested the 685 and 785 nm as the lasers of choice \cite{296}. However, the FLA is not capable of dual excitation; the 685 nm laser was chosen since a stronger response upon excitation was produced (Coorssen Lab, unpublished observations). Based on known emission characteristics, a range of LP filters - 725, 733, 740, 750, 800 nm – were tested. Initial examination of gels demonstrated that the 725 and 733 nm LP filters were inadequate for collection of CBB-IRF since no protein bands were visible, possibly due to an overlap between the excitation and emission profiles of CBB. The remaining LP filters
(i.e. 740, 750, 800 nm) looked promising since the resulting images revealed a number of protein bands; however, as the 800 nm filter produced a grainy image, this was abandoned as unsuitable for supporting quantitative analyses. Thus, the 740 and 750 nm LP filters were pursued. It was clear that although the 740 LP filter could capture BioSafe-IRF it was extremely inefficient for collection of NG-IRF (Figure 2-2); in comparison to the 750 LP filter, detection using the 740 LP filter was weak. To date only one other study has imaged CBB-IRF using the FLA-9000, and used a PMT setting of 900V \[354\]. Although high precision for seven isolated protein standards was using a PMT setting of 900V, it has been demonstrated in this study that increasing the voltage on the PMT beyond 600V poses no advantage since reproducibility at 600V is high and favourable for the detection of low abundance protein.

It is essential that imaging equipment, whether it be a laser scanner or CCD-based camera, performs optimally. The Odyssey and the FLA-9000 similarly excite CBB using a 685 nm laser. The Odyssey collects CBB-IRF using the 700 nm channel (i.e. < 750 nm), whereas the FLA-9000 as established in this study collects CBB-IRF above 750 nm. Apart from this discrepancy both imagers can be used to detect CBB-IRF. It is interesting to note that while a 750 LP filter was applied throughout this study, a BPFR700 filter has been used to capture CBB-IRF with the FLA-9000, however, there was no data presented on the intensity of protein signal, IPV, \( S^2/N \) for this filter \[354\].

2.4.2. cCBB protocol optimisation

To ensure that the performance of the newly developed cCBB-IRF protocol \[296\] was maintained using a different imaging system, it was tested using the FLA-9000. The results presented here concur with previous results \[296\], revealing no significant difference in spot detection between SR and cCBB (Figure 2-3). However, the tendency for cCBB to detect slightly fewer spots was still seen. Overall spot detection in this
study for SR and CBB was lower than previously seen\textsuperscript{[296]}, but this may be due to the different mouse strain used, C57BL/10ScSn × mdx/mdx (dystrophic mice) in this study vs. 192-SV-EV. In addition, different spot detection software was used, Delta2D in this study vs. Progenesis Workstation v2005 in previous work\textsuperscript{[296]}; differences may also thus be dependent on the very different spot detection algorithms each software employs (APPENDIX A: Figure A-10). Considering that CBB-IRF detection (i.e. total spot number) was unaffected regardless of the fact that a different imager was used (FLA-9000 in this study in comparison to studies using the Odyssey\textsuperscript{[259, 296]}), this validated the use of the FLA-9000 for further optimisation of the cCBB protocol.

The optimisation of cCBB staining for increased sensitivity has remained of interest for proteomics mainly due to its low cost and simplicity. The majority of the CBB protocol changes in the past were implemented for improved densitometric detection\textsuperscript{[178-181, 183, 185-187, 189-196, 199-200, 303-304]}. A systematic study investigating 14 of the most common CBB formulations using a unified standard staining protocol revealed two CBB stains as considerable competitive fluorescent alternatives to SR\textsuperscript{[296]}. These stains were the colloidal Coomassie formulation developed by Neuhoff\textsuperscript{[199-200]} and the commercially available BioSafe Coomassie stain (Bio-Rad). Application of these stains using the unified protocol for the detection of native proteomes, presented slightly poorer detection of low abundance proteins in comparison to SR. It is possible that the unified standard protocol used resulted in sub-optimal performance of the BioSafe and NG stains. The unified protocol\textsuperscript{[296]} involved only a 4 h stain time and very rigorous water destain mode, with the overall protocol from fixing (i.e. after electrophoresis) to destaining requiring approximately 13.5 h to complete.

When optimising a staining protocol there are numerous aspects that can be altered including stain time, dye concentration/type, destain time, and type of destain solvent\textsuperscript{[180, 186-189, 191-193, 195, 197, 199-200, 304]}. The unified standard protocol used a 4 h stain
time; however, with CBB it is very common to stain overnight. Here, regardless of stain
time (i.e. 4 vs. 20 h), no significant differences were seen for sensitivity of protein
detection and this was also the case even with additional destain washes. Since it has
been shown that cCBB staining can completely saturate protein bands after 2 h\[200]\), this
may be one reason why no differences were seen with longer staining times. Since the
last option for optimising CBB staining for sensitive detection was the investigation of
alternate destaining reagents the 20 h stain time was chosen. The rationale here was
based on improved signal intensity, especially for NG; and reduced variability for some
protein standards, in particular low MW proteins – TYRP, STI, for BioSafe.

Since the development of cCBB, the primary destaining reagent has been water.
Throughout the years there have been a variety of solvents tested, however, the risks
involved with their use and/or poor analysis has limited their potential as alternate
destaining reagents\[188, 191-193]\). The only reagent that appeared to have some impact was
sodium chloride (NaCl), resulting in gels with clearer background and vibrant protein
bands\[191]\). Although this qualitative assessment suggested NaCl to be useful for
destaining CBB stained protein, only quantitative assessments can statistically confirm
this. When NG stained proteins were destained with NaCl, there was increased signal
intensity and selectivity (i.e. improved contrast – $S^2/N$) consistent with the findings of
Sreeramulu\[191]\). In addition, sensitivity and IPV were also improved. Destaining NG
stained protein with NaCl was remarkably improved in comparison to water and it has
been suggested to be due to the NaCl weakening the interaction between CBB and the
polyacrylamide matrix\[191]\). Although it may be interesting to investigate the effect of a
range of other salts, as some have been shown to be less effective than NaCl\[191]\).

Although NaCl was not useful for destaining BioSafe stained protein, an alternate
destain was pursued based on the CBB formulation developed in the late 1980s\[199-200]\).
The components comprising the cCBB stain are phosphoric acid, ammonium sulphate,
CBB, methanol and water. Methanol was not pursued due to cost, potential hazards and risk of high variability due to evaporation during destaining. Preliminary assessment using ammonium sulphate (AS) revealed that instead of reducing CBB from protein free regions of the polyacrylamide gel, the reverse occurred; CBB was actually retained in the gel. The sole function of destaining is to reduce background, so AS was not pursued further and the only remaining component, phosphoric acid, was examined. When using H$_3$PO$_4$ to destain BioSafe stained protein, fluorescence signal, IPV and S$^2$/N were very similar to water, however, inter-gel reproducibility for low MW proteins was improved with H$_3$PO$_4$. This trend was also seen for NG stained protein destained with NaCl. It is important that the destain approach, and staining, does not contribute to inter-gel variability. If variability between gels is large, then differences between control and disease samples might be observed only for high abundance proteins as they yield the strongest signals. This is of great concern since changes in expression of low abundance proteins will not be considered significant due to low signal with large error.

BioSafe and NG were previously shown to yield high sensitivity using a standard protocol$^{[296]}$, and it has been clearly demonstrated in this study that detection for each stain has been enhanced by destaining with specific reagents. Although it is interesting that even though BioSafe is based upon the preparation developed by Neuhoff$^{[199-200]}$, it is not surprising that a different destain improves detection since the proprietary modifications introduced to develop the stable commercial cCBB stain may influence the manner in which the stain binds. This highlights an important issue on how minor changes to stain composition can have an impact on the effectiveness of a destaining reagent.

One problem associated with cCBB stained protein is storage. For storage in water, it is typical to see the solution turn blue in colour over time and proteins ‘disappearing’. Storage of CBB stained gels in AS has been suggested to prevent this
from occurring \cite{199}. This stripping of CBB from protein is concerning for two reasons. First, if gels are left to sit on the bench while waiting to be imaged, the variation between replicates increases. Second, such an increase in gel variability can then have dramatic affects on comparisons between samples. Although it is desirable to image the gels immediately after destaining, and this can be achieved by using a staggered staining/destaining approach, in a busy laboratory with an imaging instrument in high demand, this is not always possible. Based on preliminary assessments of cCBB stained protein when exploring alternate reagents for destaining, AS was noted to promote the retention of CBB on protein as well as matrix \cite{199}. Since destained gels have reduced background, storage of these gels in AS to retain CBB on protein would be useful. Even so the impact of using of AS to store CBB stained protein and maintain sensitivity was not quantitatively examined until this study. For both BioSafe and NG there were no significant differences between any of the solutions (i.e. NaCl or H$_3$PO$_4$, 5% and 20% AS) with regards to short term storage. However, it was clear that long term storage using 20% AS was definitely beneficial since the quantitative assessment of these gels was somewhat affected after one month. Not only is storage important for quantitative proteomics but also for picking protein spots or bands for subsequent mass spectrometric analyses – there is no need for the gel to be restained and reimaged, thus saving time and labour. On saying this, storage over one month may result in evaporation causing the gels to shrink, so caution must be taken if storing beyond this time frame.

By logically testing each step of the cCBB staining protocol (i.e. staining, destaining and storage), sensitive detection of proteins could be achieved using CBB-IRF. Apart from sensitivity, this protocol is simple, economical, environmentally friendly, and reproducible. Although sensitivity, inter-protein variability and selectivity
are all important characteristics, the capacity of a stain for accurate quantitation is equally important for effective proteomic analyses.

2.4.3. Assessment of LDR

The optimised cCBB protocols in this study provide an alternate method to achieve sensitive detection by fluorescent measures. However, the protein standard sample used to test sensitivity, IPV, and selectivity were present at concentrations below 119 ng of protein. In a native sample, the range of protein abundances can be quite vast, so not only is it important for a stain to detect protein at low concentrations but also to produce a linear response regardless of concentration. In the literature, the linear dynamic range of densitometric cCBB can vary between users (spanning within) 30-1000 ng\(^{[217, 250, 355]}\), which is restricted to higher protein quantities, while the LDR for SR was reported to span between 1-1000 ng\(^{[217, 250, 355]}\). Only recently has the LDR for CBB-IRF been revealed to vary only slightly from protein to protein, but for four standard proteins the LDR of CBB exceeded that of SR.\(^{[296]}\). Here the LDR was also shown to vary between proteins; for four proteins the LDR of NG exceeded that of SR, whereas this was only true for two proteins with BioSafe. Even more interesting is the marked difference in LDR between NG and BioSafe, which may be a result of stain modifications. Unlike the previous study\(^{[296]}\), SR and CBB were both imaged using the FLA-9000 permitting a more comparable analysis and excluding imager type as a possible contributing variable to differences in detection.

In the literature SR is commonly noted to detect protein down to 1-2 ng. In this study the LDR practical sensitivity only shows this to be true for two proteins, BSA and PHOSB. On examining the data for all replicates it was revealed that even though SR could detect bands of lower loads, the variability among protein fluorescent volumes for the replicate gels was high; thus statistical analysis indicated that there could not in fact
be discriminated from background. Although this does not seem to be a problem for proteins at high abundance, the concern rises when examining those that are of lower abundance. More importantly, this level of variation when examining native proteomes could potentially conceal protein expression differences between conditions. Thus, it is important for a protein stain to detect low amounts of protein, and provide a large linear response with low variability between replicates.

As mentioned previously, the LDR for each isolated protein standard is different when stained with BioSafe, NG, or SR, but more interesting is that when all proteins are detected by the same stain there are still remarkable differences in LDR. The variability in LDR between the stains (i.e. SR vs. BioSafe vs. NG) may be attributed to the fact that CBB and SR have different target amino acid residues. The binding mechanism of CBB is suggested to be between the sulfonic group of CBB and the basic amino acids in addition to aromatic residues and hydrophobic interactions \[^{75, 89-91}\]. The binding mode of SR is unclear but is suggested to be through non-covalent, electrostatic and hydrophobic interactions \[^{254}\]. Although the dye interactions of CBB and SR are similar, the target residues for SR are unknown.

For SR, BioSafe and NG there is notable variation in LDR between the seven proteins tested. It is clear that the amino acid sequence of a protein has a significant impact on the way a stain binds, since no two proteins have the same amino acid sequence. It may also be possible that the amino acids surrounding the target amino acid influence dye binding. Hence, if the target residue is surrounded by amino acids that will enhance binding a large response may result, whereas those that will cause a steric hindrance will reduce the response. This may explain the reasons why two proteins relatively similar in MW differ greatly in LDR.
2.4.4. Mass spectrometry compatibility of optimised cCBB protocols

Another important feature for a protein stain is compatibility with protein characterisation methods. Since the introduction of mass spectrometry it has become the preferred method for the identification and sequencing of proteins [46, 123, 126, 356-357]. After proteins have been resolved and stained in-gel, those of interest can be excised and processed for analysis by mass spectrometry. However, it is important that the staining method applied does not introduce changes to the protein and/or have a negative impact on the mass spectrometric process itself. These problems are of great concern particularly when silver nitrate staining is applied, given that it has been shown to have the poorest compatibility with MALDI- and ESI-MS [223]. However, specific improvements to the silver staining method can enhance MS compatibility, albeit with a sacrifice in detection sensitivity [212, 214-215, 224-225]. Since the MS compatibility of the optimised cCBB protocols is unknown, it would be useful to determine whether identification by MS is compromised by the newly introduced changes.

MALDI and ESI are the most common ionisation methods used and compatibility for both optimised cCBB methods was investigated in parallel with SR. For MALDI MS, all three stains produced the same level of sequence coverage. This was also seen for ESI; however, sequence coverage was much greater in comparison to MALDI. Although the level of sequence coverage by MALDI is small, it is not unusual [223]. However, this may be due to the fact that peptides are separated via LC prior to ionisation, unlike MALDI in which the unfractionated digest is spotted onto the target platform prior to ionisation. It may also be possible that the method used for MALDI sample preparation may have discriminated against obtaining good spectra since a sample not taken to dryness will tend to cause losses in the ZipTip cleanup stage. Examination of the MALDI Mascot search results revealed that a large number of ion scores for a MS/MS match between the experimental data and database sequences were
considered as non-random events. Interestingly this was seen for all samples analysed by MALDI and since there were only a few matches used for positive hit selections, it is no surprise that a small percent of the sequence was covered. Since, beta-galactosidase was positively identified with adequate sequence coverage by both MS strategies (MALDI and ESI) regardless of which of the three stains was used, it is clear that the optimised cCBB methods are MS compatible.

2.5. Conclusion

Here optimised methods for sensitive cCBB-IRF have been quantitatively characterised for improved in-gel detection of proteins. It was shown that longer staining time coupled with specific destaining reagents could deliver sensitive protein detection, similar to that initially characterised for cCBB-IRF in 2008 [296]. For BioSafe and NG stained gels, destaining with H₃PO₄ and NaCl, respectively, proved beneficial for sensitive protein detection via CBB-IRF. Although little was known about the effects of storage on CBB stained protein, it was shown that storage in respective destain solutions was suitable for short periods of time, however, 20% AS was ideal for longer term storage (i.e. up to one month). In comparison to SR, NG performed similarly and in some instances better in terms of detection sensitivity and LDR; BioSafe was to some extent inferior in both aspects. The results suggest that there is great potential for the cCBB-IRF method to be utilised for quantitative proteomics. The main limitation of the optimisation strategy to this point was that the analysis involved only isolated protein standards. This was sufficient to determine the optimal variables for sensitive detection by cCBB staining from a purely chemical perspective. Nonetheless, proteomics involves the investigation of the native protein complement in tissues, organisms and other biological samples. This collection of proteins as a whole will behave much differently than isolated protein standards, and for this reason the
optimised cCBB staining protocol was next tested for native proteome analysis to ensure thorough characterisation of the new in-gel protein detection protocols.
CHAPTER 3: Sensitive fluorescence detection of native proteomes using an optimised CBB protocol

3.1. Introduction

To investigate biological proteomes, 2DE remains as one of the most widely used techniques, in some cases complemented by alternate approaches such as ‘shot-gun’ LC/MS and protein chips [126, 151, 358-361]. Using 2DE, proteins are resolved sequentially by two physio-chemical properties, isoelectric point (pI) and then molecular weight (MW). Since the likelihood that two unrelated proteins possess the identical pI and MW is small, each spot resolved by 2DE theoretically represents one protein. 2DE was initially believed to be unreliable for resolving large, hydrophobic, alkaline/acidic, low abundance and/or membrane proteins, however, modern methodological optimisations have minimised these potential limitations highlighting the power and versatility of such a well-characterised and mature technology [10, 13-14, 19, 21, 35, 103, 300, 302, 362-364]. In addition, 2DE is not limited by sample type since it has been widely applied to resolve a number of diverse proteomes derived from mammalian, plant, insect samples and other species [22, 24, 63, 365].

The complexity of the proteome can be simplified using pre-/sub- and/or post-fractionation [53, 69, 97]. Focus can also be directed to the analysis of sub-proteomes such as the phospho- or glycoproteomes [44, 48]. Even though the sample density can be reduced by any of these measures, it is common for some proteins to be present in hyperabundance (i.e. at the extreme high end of native protein expression levels). This can be a problem for 2DE and other protein resolution techniques since other proteins localised in the same region may be ‘hidden’, so removal of these hyperabundant proteins is often implemented. However, the downfall of this approach is that not only
are the offending hyperabundant proteins removed but so are any/all the proteins associated with it\textsuperscript{[55]}.

It is clear that problems once associated with protein resolution are no longer a primary issue for 2DE. Rather effective detection of the proteome seems to be the more critical issue\textsuperscript{[239,258]}. Densitometric detection of proteomes resolved by 2DE is inferior to fluorescent alternatives\textsuperscript{[259]} due to high background staining. A number of fluorescent protein stains are currently available including Deep purple\textsuperscript{[256-257]}, C-16 fluorescein\textsuperscript{[366]} and the reactive Cy dyes\textsuperscript{[276,367]} (used primarily for differential gel electrophoresis). However, sensitive protein detection by Sypro Ruby (SR) makes it the generally preferred fluorescent stain\textsuperscript{[217]}.

Densitometry of CBB stained protein cannot compete with SR in terms of detection sensitivity. However, detection of soluble and membrane proteomes by CBB near-infrared fluorescence (IRF) was recently shown to be potentially competitive with SR; and remarkably superior to densitometric CBB\textsuperscript{[259]}. In addition, signal to noise (S/N) was also shown to be enhanced by CBB-IRF relative to SR\textsuperscript{[259]}. Systematic characterisation of 14 CBB stain formulations via IRF revealed that Neuhoff\textsuperscript{[200]} and BioSafe Coomassie (BioRad) exhibited similar sensitivity to SR\textsuperscript{[296]}. However, the unified staining protocol used in that study resulted in slightly poorer detection of native proteomes by CBB relative to SR. In this study, optimisation of the cCBB staining protocol was shown to retain a similar level of sensitivity as seen previously and delivered a reasonable linear dynamic range as assessed using seven isolated protein standards (Chapter 2)\textsuperscript{[296]}.

Information on sensitivity and linear dynamic range is essential to quantitatively assess the performance of a stain to be used for in-gel protein detection. However, the analysis only explored isolated protein standards which do not reflect the inherent complexities associated with assessing a native proteome. Thus, it is imperative that
newly developed protein staining protocols are also tested on native proteomes. The work presented here will thus focus on the performance of the optimised cCBB staining protocols when used for native proteome detection relative to SR.
3.2. **Materials and Methods**

3.2.1. Chemicals

All consumables were of electrophoresis grade or higher quality. Electrophoresis consumables including acrylamide, tris-glycine SDS buffer, Coomassie Brilliant Blue G-250 dye were purchase from Ameresco Inc. (Solon, OH). A commercially available, ready-made CBB formulation, Bio-Safe™ Coomassie Stain (BioSafe), SYPRO Ruby protein gel stain, Bio-lyte broad range carrier ampholytes (pH 3-10) and each of four Bio-lyte narrow range ampholytes (pH 3-5, 6-8, 7-9 and 8-10) were purchased from Bio-Rad Laboratories (Hercules, CA).

3.2.2. Native protein sample preparation

3.2.2.1. Tissue homogenisation

The method used here was essentially the same as that mentioned in Section 2.2.2.1 (Chapter 2) for mouse brains. Arabidopsis thaliana Wt (Columbia) seeds were germinated in standard potting soil mix at 25 °C in a seedling tray after imbibing the seeds at 4 °C for 48 h. Once rosette leaves emerged, plants were transferred to 0.5 L pots of soil. Soluble nutrients were added regularly (Aquasol). Plants were grown in a greenhouse at 25 °C and harvested just after bolting. Arabidopsis thaliana leaves were harvested fresh and left intact prior to snap freezing in liquid nitrogen and subsequent automated frozen disruption.

3.2.2.2. Pre-fractionation of powdered protein sample

Powdered sample (Section 3.2.2.1) was processed according to Section 2.2.2.2., with the following changes implemented for A. Thaliana. For A. thaliana homogenates, a solution of 40 mM HEPES (pH 7.4), 660 mM NaCl, 2× PIC was used in place of 2× PBS to restore isotonicity and 20m M HEPES (pH 7.4), 330 mM NaCl, 1× PIC was
used in place of 1× PBS/1× PIC when suspending the membrane pellet prior to the second ultracentrifugation step.

3.2.2.3. Identification of A. thaliana insoluble material

Prior to ultracentrifugation, large pellets of insoluble material remained after cell lysis. This was subjected to solubilisation using 2% SDS and quantitation via the protein concentration assay (Section 3.2.3) of this sample revealed minimal residual protein (0.93% of total protein extracted), supporting the efficiency of the automated frozen disruption and lysis method used for pre-fractionation and the efficiency of the subsequent protein extraction. Since A. thaliana belongs to the plant kingdom, it was hypothesised that the insoluble material remaining after protein solubilisation may have been cellulose since it is the primary component of a plant cell wall and is a polysaccharide; thus it will not be effectively extracted in a protein solubilisation buffer. To determine whether the insoluble material was indeed cellulose a modified method based on those of Updegraff and Gama and Carder was developed.

Briefly, the method is as follows: approximately 10-15 mg of cellulose (Sigma-Aldrich) or 10–15 µL of insoluble A. thaliana material were added to separate tubes. To each, 4 mL of 13.5 M sulphuric acid or water (control) was added and incubated for one h with regular agitation before diluting with water to 50 mL. A 1.5% (w/v) agarose in 0.1 M sodium phosphate buffer, pH 6 (4.5 ml) was prepared and dissolved at 121 °C for five min. When cool to touch, 0.5 mL of sample mixture was added to the liquid agarose solution, vortexed to mix, and 3 mL of each reaction mixture was added to one well of a 6-well microtitre plate (Greiner) and left to solidify.

Each well was stained with an equal volume of 0.1% (w/v) Congo red in distilled water for 30 min with constant agitation (400 rpm) using a microtitre plate vortex. After staining, wells were rinsed with H₂O for 10 s and then twice with 1 M NaCl (5
Wells were then soaked in 5% HAc for 5 min. Any sample stained by Congo red will become purple. The last step can be optional since wells that contain intact cellulose will be stained red, and addition of HAc causes a color change, whereas reactions with hydrolysed cellulose remain clear after both steps. As can be seen in the image below the insoluble material was determined to be cellulose when tested in parallel with the control.

NOTE: Although the method presented here used cellulose as the polysaccharide control, as this method is applicable to any polysaccharide, the results may indicate that the insoluble material was indeed polysaccharide in nature but not necessarily only cellulose.

3.2.3. Protein concentration assay

Carried out as described in Section 2.2.2.3 (Chapter 2).

3.2.4. Two-dimensional gel electrophoresis (2DE)

2DE using the mini-SDS PAGE format was carried out as described in Section 2.2.3. (Chapter 2). For the Tall-SDS PAGE format the following changes were implemented: 300 μg total protein load, and passive rehydration using 17-cm (tall) non-linear pH = 3-10 IPG strips (Bio-Rad Laboratories, Hercules, California). Linear ramping to 10 000V over 2 h and 10 000V constant voltage for 75000 volt-hours. Tall
2DE gels were either stained using SR or the optimised NG protocol (Section 2.2.3 and 2.2.6, respectively).

3.2.5. Detection

All gels stained with either BioSafe, NG and SR, were imaged with the FLA-9000 (FUJIFILM Corporation, Tokyo, Japan) as mentioned previously in section 2.2.7.7. Access to the Odyssey Imager (LiCor, Lincoln, NB) was granted by Associate Professor Mark Molloy at Macquarie University. Gels were excited simultaneously using the dual lasers at 685 and 785 nm, and emission was collected in the 700 channel i.e. < 750 nm. Images were acquired at 84 µm pixel resolution and medium quality. The Odyssey creates 16-bit tiff images by design.

3.2.6. 2DE image analysis

Analysis of 2DE gel images was carried out using Delta2D 4.0 (DECODON Gmbh, Greifswald, Germany) according to company instructions, with the following changes. Briefly, gel images (16-bit tiff) before being imported into the Delta2D software were cropped in Multigauge v3 (FUJIFILM Corporation, Tokyo Japan) to remove marker, dye front and non-gel components (APPENDIX B: Figure B-1). Once imported into Delta2D, total spot numbers were determined using raw images. Then each group of images were warped and fused (Average) to produce a representative image, and spots were detected on this fused image. By selecting the average fusion option, the spot pattern obtained was only of the spots consistently present within all three replicate gels (i.e. 100% reproducible spots). The spot pattern from the representative image was then transferred to all raw images. To ensure minimal user bias, images were cropped to similar dimensions using Multigauge prior to import into Delta2D; and spots detected using Delat2D were not filtered as suggested by DECODON.
3.3. Results

3.3.1. Total proteome detection

When a protein stain is developed for protein detection, the quantitative data presented is generally established using protein standards. This can provide an insight into the quantitative capacity of a stain, but the composition of a native sample is extremely different to isolated protein standards. Thus, when investigating the potential of a protein stain for quantitative proteomics, the staining protocol must also be tested with regard to the detection of native proteomes.

To demonstrate the versatility of the optimised cCBB staining protocols, detection of two diverse tissue types, mammalian and plant were evaluated. Analyses were also carried out in parallel with SR staining. The proteomes from each tissue were also pre-fractionated into membrane and soluble samples, and total detected spot numbers of each sample were obtained for BioSafe, NG and SR (Figure 3-1). In all instances, BioSafe yielded similar total spot detection relative to SR. However, total spot detection of membrane (mouse and plant) and soluble (plant) samples by NG was shown to be significantly greater than SR. Interestingly, comparison between the optimised cCBB staining protocols revealed NG as the superior cCBB stain for total spot detection. Overall, NG displayed the best performance for total spot detection, regardless of tissue and sample type.

Using Delta2D, the spot patterns on representative images for SR, BioSafe and NG could be superimposed and a deeper look into the comparative distribution of the total protein spots detected could be examined (Figure 3-2). As expected, the majority of total protein spots detected were common to SR, BioSafe and NG. Interestingly, the percentage of additional spots unique to BioSafe or SR was minor, between 2-9%; NG was the only stain to consistently detect a greater number of stain specific spots for all samples, >18%. Since cCBB protocol optimisation was carried out using isolated
protein standards it was not clear whether the level of sensitivity seen would also apply to native proteomes, however, the data presented here strongly supports the optimisation data, indicating that the optimised cCBB protocol is a promising fluorescent alternative for sensitive detection of proteins in-gel (isolated and native protein).

3.3.2. Proteome detection – mini vs. tall-gel format

The proteome analyses thus far have been completed using the mini-gel format, but it is common to resolve proteomes using the tall-gel format. To ensure that the results of the optimised cCBB staining protocols, in particular for NG, are not limited to the mini-gel format (mouse brain total soluble spot detection: NG-809, SR-609), the optimised NG staining protocol was also applied to the tall-gel format. For effective comparison with the mini-gel format the same mouse brain sample preparations for total membrane and soluble protein were used. When using the tall gel format, NG also detected a significantly greater number of total spots relative to SR for soluble protein (964 vs. 786, respectively) (Figure 3-3). Although the number of spots detected were slightly greater for tall-gels relative to mini-gels, this is not unusual since tall-gels have a greater resolving area.

During scanning of membrane proteins resolved by tall 2DE, a shading effect at the pH 10 region occurred for NG stained gels (APPENDIX B: Figure B-2). This shading had dramatic impacts on total spot detection when analysed by Delta2D resulting in massive and unrealistic increases in total spot number. Since this shading was not seen before a second triplicate set of membrane resolved tall gels was run and the shading effect appeared once again. This shading effect was thought to be a consequence of reduced protein spot number in combination with reduced fluorescence intensity of stained proteins. Therefore a concentrated molecular marker sample was placed at the pH 10 end of the IPG to increase protein fluorescence intensity in the basic
area to eliminate shading and hence permit the visualisation of native sample proteins resolved in this region. Even with this attempt to improve image quality, only one replicate resulted in a similar spot number to SR. It was obvious that this method for membrane samples, in particular, requires improvement.

3.3.3. Comparison of IRF imagers for native proteome detection

Currently the Odyssey manufactured by LiCor is the one imager that can be used to capture CBB-IRF [259, 296, 354]. In this study the FLA-9000 was also shown to be valuable for cCBB-IRF detection of both isolated protein standards and native proteomes and it would be most interesting to also assess the performance of the optimised cCBB protocols for BioSafe and NG using the Odyssey.

Mouse brain membrane proteins resolved by 2DE were stained with either optimised NG or BioSafe. Due to limited access and the location of the Odyssey, NG and BioSafe stained gels, after imaging with the FLA-9000, were stored in 0.5 M NaCl and 1% H₃PO₄, respectively, at 4 °C prior to imaging the next day (i.e. at Macquarie University). These storage solutions were shown to preserve detection sensitivity (Chapter 2: Section 2.3.2.4), thus any differences in total spot number would be attributable to the imager. For NG stained protein, the FLA-9000 yielded significantly greater total spot detection relative to the Odyssey, however, this was not so for BioSafe (Figure 3-4). Furthermore, the ratio of spots detected (FLA/Odyssey) for both NG and BioSafe (1.21 ± 0.01 and 1.09 ± 0.03, respectively) does support the FLA imager as the imaging system to yield improved detection even with BioSafe. Although two completely different imaging systems were used, NG still detected a greater number of total protein spots relative to BioSafe. Thus, using the FLA-9000 imager with the optimised NG staining protocol is the most favourable arrangement for enhanced detection of naive proteomes.
Figure 3-1. Total spot detection of animal and plant proteomes

Animal (Mouse brain; A & B) and plant proteomes (A. thaliana leaf; C & D), membrane and soluble, were resolved by 2DE, mini-format. After staining (optimised BioSafe, NG or SR), gels were imaged using the FLA-9000. Error bars represent standard error of the mean (n = 3). Statistically significant differences in comparison to SR are denoted * and between BioSafe and NG † (Student’s t-test: * (†) p < 0.05, *** (†††) p < 0.0001). NOTE: Due to the limited amount of protein material obtained for A. thaliana membrane and sub-optimal performance of BioSafe, only SR and NG staining protocols were tested.
Figure 3-1
**Figure 3-2. Distribution of spots detected**

BioSafe, NG and SR gel images were warped to a representative gel image, spot patterns were compared using Delta2D and represented with Venn diagrams to reveal common and unique spots between the three stains. (A) Mouse brain membrane protein. (B) Mouse brain soluble protein. (C) A. thaliana membrane protein. (D) A. thaliana soluble protein. Total detected spots are bold in brackets, total stain unique/common spots detected are non-bold and below is the % of unique spots as a fraction of the largest total spot count (given by NG in all conditions) is represented in brackets.

NOTE: Due to the limited amount of protein material obtained for A. thaliana membrane and sub-optimal performance of BioSafe, only SR and NG staining protocols were tested.
Mouse brain

A. thaliana leaf

Membrane

Soluble

Figure 3-2
Figure 3-3. Total spot detection using tall format gels

Mouse brain soluble protein was resolved by 2DE, tall format. After staining, optimised NG and SR, gels were imaged using the FLA-9000. Error bars represent standard error of the mean (n = 3). Statistically significant differences relative to SR is denoted * (Student’s t-test: * p < 0.05).
Figure 3-3
Figure 3-4.  Spot detection of cCBB stained gels: FLA-9000 vs. Odyssey

Mouse brain membrane protein was resolved by 2DE, mini format. After staining, optimised BioSafe and optimised NG, gels were imaged using the FLA-9000. The same gels were stored in appropriate storage solutions and then imaged the following day using the Odyssey (LiCor). Error bars represent standard error of the mean (n = 3). Statistically significant differences between imagers are denoted as * and between BioSafe and NG for the same imager, † (Student’s t-test: †† p < 0.01, *** (†††) p < 0.0001).
Figure 3-4

![Graph showing total spot number for FLA-9000, Odyssey, BioSafe, and NG across different imagers.](image-url)
3.4. Discussion

3.4.1. Native proteome detection

One of the most widely used methods for resolving complex proteomes is 2D). It is a versatile technique by which numerous sample types can be assessed \[22, 24, 63, 371\]. Although problems associated with resolving particular protein species has been an issue for 2DE decade or more ago, but the main issue now is one of detection \[239, 258\]. Detection of the proteome is commonly achieved using post-electrophoretic protein stains (i.e. staining after electrophoresis). Since the protein stain is the limiting factor for detection, it is important that it can enable assessment of as much of the proteome as possible.

There is a vast array of protein stains available including those used for densitometry and fluorescence \[75, 217, 228, 253, 257, 259\]. Since SYPRO Ruby (SR) is currently the most sensitive protein stain available \[217\], it is the stain of choice; but its use is accompanied with a substantial financial cost. Originally used for densitometric, cCBB, protein detection, was shown to be a potential competitive fluorescent alternative \[259, 296\]. However, detection of native proteomes using a unified CBB staining protocol was slightly poorer relative to SR \[296\]. Optimised cCBB staining protocols, for BioSafe and NG, yielded high sensitivity when using isolated protein standards (Chapter 2). Although the data provided strong evidence to support high sensitivity protein detection using these staining protocols, the question was whether sensitivity would also be improved for native proteome detection. Here the optimised protocols for both BioSafe and NG were compared to SR, and in all instances NG was superior for total spot detection regardless of sample type (i.e. mammalian vs. plant) or fraction (membrane vs. soluble), highlighting the versatility of the NG staining protocol. Not only was total spot detection improved with NG, but also the number of stain specific spots was greater in comparison to BioSafe and SR. It must be noted, that even though a greater
number of stain specific spots were seen with NG in comparison to SR and BioSafe, this result was heavily relied on the detection software. In some instances one spot determined by the software for SR stained protein was indeed recognised as two and sometimes even three spots when using NG. It may be possible that the fluorescence intensity of SR is so strong that the software cannot distinguish between two or more highly abundant spots in close proximity, whereas this may not be the case with NG since the spot number was definitely increased. However, although the number may be smaller than seen here, there were a few spots present in NG that were completely absent from SR stained gels.

Resolving proteomes is generally achieved using the mini (6 cm) and/or the tall (20 cm) format gels. Up to now cCBB staining protocol optimisation and 2DE analyses were all performed using the mini format and it may be possible that performance may differ when applying this protocol to the tall gel format. Since NG performance was superior to BioSafe, it was tested in parallel to SR using the tall gel format. It was clearly shown that NG total spot detection of mouse brain total soluble protein was greater relative to SR. However, detection of mouse brain total membrane protein was difficult due to the presence of shading in the basic region on the resulting images (APPENDIX B: Figure B-2). It is not clear why this happened predominantly for membrane protein, but may be due to the fact that membrane samples have a reduced protein density; combined with the increased resolving area, and perhaps more CBB dye molecules are retained as background.

Membrane samples, in particular in the basic region, have a lower protein density than the neutral region; in addition these proteins are low abundance. To overcome the shading effect, high concentrations of molecular marker was placed at the pH 10 end of the IEF strip, to increase protein fluorescence in this region, and potentially reduce the effect of shading possibly promoted by the CBB dye molecules retained within the
polyacrylamide matrix. However, shading was only minimised for only one of the three gel replicates. Although total spot detection for this replicate was similar to SR, imaging across the membrane replicates was inconsistent and may be due to ineffective destaining among replicate gels. It was noticed after imaging and cleaning of the scanning plate, that the cleaning tissue retained a strong blue colour, whereas this was minimal with the mini gel format. This could mean that the volume of destaining solution was insufficient to remove the CBB dye from the gel. However, the volume of destaining solution was kept constant relative to the mini format (i.e. 10-fold the gel volume, 50 mL for a 5 mL mini gel and 300 mL for a 30 mL tall gel); in addition the speed for agitation was also kept constant (60 rpm). The only other factor that may have caused this effect could have been the container size, which in turn restricted motion and hence the rate at which the destaining solution could in fact remove the CBB dye from the matrix and yielding inconsistent results. This is likely since the containers used for the mini-gel format have sufficient room for ample gel movement, whereas with the tall gels, the containers restrict agitation of the gel in all directions. Due to the size of the large gels this was the most suitable sized container available at the time. It was clear, however, that adjustments to the protocol would be required to permit reproducible imaging of NG stained membrane samples resolved using tall gels.

3.4.2. Imager dependent proteome detection

There are a variety of imagers available that can be used for detecting proteomes resolved in-gel, whether it be for densitometry and/or fluorescence. Currently one imager used to capture CBB-IRF is the Odyssey (LiCor, Lincoln, NB) and it has been shown to be applicable for the sensitive detection of proteins in-gel [259, 296]. Here, the FLA-9000 (FUJIFILM, Tokyo, Japan) was optimised to capture CBB-IRF and was also shown to be advantageous for sensitive in-gel protein detection (Chapter 2). It has been
noted that stain sensitivity can differ between imagers. Thus the sensitivity of the optimised cCBB protocols were assessed using both the FLA-9000 and the Odyssey. Interestingly, detection by BioSafe was similar between the imagers, whereas NG displayed poorer detection when using the Odyssey. It is unclear why NG detection suffers greater losses when using the Odyssey but it may be due to differences in stain formulation and/or instrument design. Although the FLA-9000 and Odyssey imagers are both effective for cCBB-IRF detection, it is clear that NG detection is superior on the FLA-9000, whereas BioSafe detection is unaffected regardless by the imager used.

3.5. Conclusion

Here it was shown that the optimised NG protocol for native proteome detection was superior relative to both BioSafe and SR when using either the FLA-9000 or Odyssey imager. Not only did cCBB-IRF detection yield sensitive in-gel protein detection but is also cost effective, environmentally safe, and doesn’t require protection from light. As a result cCBB staining is identified as a competitive alternative to SR for sensitive fluorescent in-gel protein detection.
FUTURE DIRECTIONS

The optimised cCBB protocols presented here yield high sensitivity using the IRF detection approach. However, the analyses concentrated primarily on fluorescence. It would be interesting if the optimised cCBB protocols could also improve densitometric detection beyond the commonly achieved 4-8 ng of protein. Sensitive protein detection was achieved after 20 h of staining and specific destaining; but it may be of interest to observe the outcome of destaining with NaCl and H₃PO₄ after 4 h staining. Although the changes implemented here concentrated on stain time, destain time and destain solution it may be interesting to investigate the effect of temperature, adjusting NaCl concentration and/or alternate salt solutions for destaining. It is interesting to note that while AS is also a salt, AS seems to stabilise the CBB-protein interaction to prevent any loss of CBB, which is similar to NaCl which has a different purpose (i.e. destaining). It is intriguing how two different salts can have such a positive effect and it would definitely be of great interest to understand the chemistry of how these salts promote their effect with CBB and CBB-protein.

The formulation of cCBB developed by Neuhoff was used here without alteration, perhaps by decreasing the concentration of dye (i.e. CBB, it may be possible that background staining could be reduced without affecting protein intensity and sensitivity, particularly with the 20 h staining protocol. Throughout the literature there are also a number of dyes that have similar densitometric properties to CBB (i.e. Fast Green and Trypan Blue); if these fluoresce within the imaging parameters defined for CBB-IRF (i.e. 685 nm excitation and > 750 nm LP emission), they may also be suitable for use as additives to potentially improve detection by either enhancing the interaction of CBB to protein and/or improve protein detection by binding to alternate sites on the protein.
Preliminary observations suggest that this indeed could be the case (Coorssen, personal communication). Other future work could involve assessment of other protein sample types (i.e. marine, bacterial, fungal) could further highlight the versatility (i.e. broad applicability) of CBB as a sensitive fluorescent stain. In the data presented here it was noted that LDR varied between proteins for a given staining protocol and it would be interesting to distinguish whether this is dependent on the amino acid sequence of an individual protein and/or amino acid cluster(s) within that sequence to better understand the interaction of CBB to proteins. Here MS compatibility was also carried out on one protein at 100 ng. To highlight the effectiveness of the cCBB staining protocols for positive MS IDs other proteins and a range of concentrations should be tested.

Here, BioSafe, NG, and SR stained gels were imaged using the same instrument, however, it may be possible that the FLA-9000 may not be the most suitable imager for SR since detection has been shown to be affected by the imaging instrument utilised. This highlights the necessity to test imagers for optimal detection, which was shown for both NG and BioSafe when using the FLA-9000 or Odyssey for detection. It was noted that membrane samples stained with NG yielded imaging disturbances ultimately affecting total spot detection in tall format gels. To overcome this, image quality for membrane samples using the tall gel format may be improved by increasing the time interval for each wash of the optimised protocol and/or, increase the number of destain washes to assist in further removal of CCB dye molecules and hence reducing background. Alternatively, since the FLA-900 was not manufactured to capture CBB-IRF, the instrument design may have contributed to this shading affecting image quality.
For gel-based proteomics, detection of protein was initially accomplished using densitometry; however, due to background issues fluorescent protein stains became more popular. Not only are the issues associated with background reduced but sensitivity is greater with the use of fluorescent stains. Currently the most widespread fluorescent stain used for sensitive in-gel protein detection is SYPRO Ruby; however, its use is accompanied with high cost, and increased variability, and poor total spot detection as highlighted in this thesis. Here, the FLA-9000 was optimised to detect CBB fluorescence and with specific modifications to the cCBB staining protocols, for BioSafe and NG, detection sensitivity was improved relative to water and reasonably similar LDR relative to SR. Application to native proteomes, both animal and plant, also displayed enhanced detection relative to SR (NG ≥ SR = BioSafe). In summary, cCBB has been re-visited as a fluorescent protein stain for sensitive detection of proteins in-gel. The data presented here shows that cCBB is equally effective as SR yet is less expensive to use while still being compatible with downstream mass spectrometry for high quality protein IDs. As such, cCBB represents a sensitive and economically viable alternative to SR.
CHAPTER 4: Zinc starvation induces a stress response in Saccharomyces cerevisiae that is mediated by the Msn2p and Msn4p transcriptional activators

This Chapter was previously published:


The author was responsible for conducting the experimental work, analysis of data and preparation of manuscript. A/Prof. Higgins was responsible for the experimental design and contributions to experimental procedures, analysis of data and preparation of manuscript. Professor Rogers contributed to the experimental design. Professor Dawes contributed to the experimental design and preparation of manuscript. Dr. Beckhouse contributed to the analysis of data and preparation of manuscript. Victoria Lyons gave aid in some experiments and A/Prof. Beh assisted in the analysis of data.
### ABBREVIATIONS USED IN THIS CHAPTER

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
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<tr>
<td>LZM</td>
<td>Limiting zinc medium</td>
</tr>
<tr>
<td>LZM+Zn</td>
<td>LZM with zinc</td>
</tr>
<tr>
<td>Msn2/4p</td>
<td>Msn2p and Msn4p</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>RSAT</td>
<td>Regulatory sequence analysis tools</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SSC</td>
<td>Saline sodium citrate</td>
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<tr>
<td>STRE</td>
<td>Stress-response element</td>
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<tr>
<td>STRE-lacZ</td>
<td>Reporter construct consisting of the STRE-containing promoter of the CTT1 gene fused in-frame with the bacterial lacZ gene</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>ZRE</td>
<td>Zinc-responsive element</td>
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4.1. Abstract

During the production of wine and beer, the yeast Saccharomyces cerevisiae can encounter an environment that is deficient in zinc, resulting in a 'sluggish' or a 'stuck' ferment. It has been shown that the Zap1p-transcription factor induces the expression of a regulon in response to zinc deficiency; however, it was evident that a separate regulon was also activated during zinc deficiency in a Zap1p-independent manner. This study discovered the Msn2p and Msn4p (Msn2/4p) transcriptional activator proteins to be an additional control mechanism inducing the stress response during zinc deficiency. Promoter sequence analysis identified the stress-response element (STRE) motif, recognized by Msn2/4p, and was significantly enriched in the promoters of genes induced by zinc deficiency. An investigation using genome-wide analyses revealed a distinct regulon consisting of STRE-containing genes whose zinc-responsive expression was abolished in an msn2 msn4 double mutant. An STRE-driven lacZ reporter construct confirmed that expression of the genes within this regulon was perturbed by the deletion of MSN2 and MSN4 and also implicated Hog1p as a contributing factor. This research provides a better understanding of the molecular mechanisms involved in the yeast response to zinc deficiency during fermentation.
4.2. Introduction

Zinc is an essential micronutrient for cellular function. It is required by many proteins for structural stability \textsuperscript{372}, as a catalytic or co-catalytic factor \textsuperscript{373-375}, and has an effect on cell mechanisms \textsuperscript{376-377}. Zinc also plays a vital role in beer fermentation, stimulating early ethanol production, amino utilization and the creation of aroma compounds \textsuperscript{378}. However, the crop quality \textsuperscript{379} and the processing involved in wort production \textsuperscript{380} have a major influence on the zinc content available for fermentation. Therefore, it is important that suitable levels of zinc are maintained to avoid ‘sluggish’ and incomplete fermentations \textsuperscript{381-383}.

In Saccharomyces cerevisiae (S. cerevisiae), expression of the zinc transporters ZRT1, ZRT2 and ZRT3 is induced during zinc deficiency and mediated by the transcriptional activator, Zap1p \textsuperscript{384-385}. Using these genes, a conserved 11-bp consensus sequence, 5’-ACCYYNAAGGT-3’, known as the zinc-responsive element (ZRE) was identified and shown to be necessary for zinc-responsive transcriptional regulation via Zap1p \textsuperscript{385}. An elegant study by Lyons et al. (2000) \textsuperscript{386} used genome-wide expression analysis to determine an extended set of genes that form part of the zinc regulon in yeast controlled by Zap1p. A high proportion of the genes, 458, were induced during zinc deficiency with 24% of these containing the ZRE or ZRE-like binding motifs \textsuperscript{386}. A relatively small proportion, 46, of the induced genes was shown to be regulated by Zap1p, indicating that the remaining genes induced during zinc deficiency may be controlled by unknown transcriptional regulators. Recently the zinc-responsive regulon for Zap1p has been further characterized by examining the differential regulation of Zap1p targets, but no other regulatory proteins were identified \textsuperscript{387-388}.

Expression analysis in an industrial strain of S. cerevisiae under zinc-deficient growth conditions with maltose as the main carbon source \textsuperscript{389} identified a significant overlap with genes induced in the Lyons et al. (2000) \textsuperscript{386} study, but the presence of
genes that were known to be regulated by the homologous Msn2p and Msn4p (Msn2/4p) transcription factors was observed. Msn2/4p induces the expression of genes under conditions of stress via interaction with the consensus sequence, 5′-AGGGG-3′, known as the stress-response element (STRE) \[^{390-391}\]. Under stress conditions, it has been shown that a number of factors such as Hog1p, Whi2p/Psr1p and Glc7p/Bud14p activate Msn2/4p, which results in increased expression of its target genes \[^{392-394}\]. Under non stress conditions, Msn2/4p activity is down regulated by the Ras-cAMP-PKA pathway \[^{395-396}\]. To determine whether zinc deficiency includes a stress response mediated by Msn2/4p, interrogation of the promoter regions of zinc-responsive genes, microarray analysis of mutant strains and functional protein assays were carried out in this study.

4.3. Materials and Methods

4.2.1. Yeast strains, media and culture conditions

The yeast strains used were W303-1A (MATa ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11,15 can1-100) and Δmsn2,4 (W303-1A msn2-3::HIS3 msn4-1::TRP1). W303-1A-STRE-lacZ (W303-1A URA3::STRE-lacZ), Δmsn2,4-STRE-lacZ (Δmsn2,msn4 URA3::STRE-lacZ), Δbud14 (W303-1A-STRE-lacZ bud14::kanMX4), Δras2 (W303-1A-STRE-lacZ ras2::kanMX4) and Δwhi2 (W303-1A-STRE-lacZ whi2::kanMX4) were created in this study. Δhog1 (W303-1A hog1::TRP1) was obtained from Schüller et al. (1994) \[^{392}\]. Limiting Zinc Medium (LZM) was prepared in the same manner as the limiting Iron medium prepared by Eide and Guarente (1992) \[^{397}\], except that zinc (not iron) was the metal omitted from the metal stock. Under non-zinc limiting conditions, zinc was added to a final concentration of 12 mg L\(^{-1}\). Strains were grown overnight to mid-log phase (OD\(_{600}\) = 0.5) at 30 °C, cells harvested at 6000 g for 5 min and washed.
three times with distilled water. Cells were then transferred to LZM with zinc (LZM+Zn) and LZM with an initial OD<sub>600</sub> = 0.1, grown to mid-log phase and harvested before RNA isolation or β-galactosidase (β-gal) assays were performed.

4.2.2. DNA Manipulations

Plasmid CTT1-18 (CTT1-18/7x-lacZ), described previously by Marchler et al. (1993) [390], was digested with NcoI (Promega) and transformed into W303-1A [wild type (WT)], Δmsn2,4 and Δhog1, yielding in strains W303-1A-STRE-lacZ, Δmsn2,4-STRE-lacZ and Δhog1-STRE-lacZ, respectively. Transformants were selected for uracil prototrophy. In strain W303-1A-STRE-lacZ, deletions for BUD14, RAS2 and WHI2 were achieved using the primers: 5′-CGCAAGAGTCAGACTGACTCG-3′, 5′-ACTACCTC-CTCAACCCCAGTT-3′; 5′-TGACATT-TAGGACGGTGGAAGC-3′, 5′-TACGTTTCTC-TTCTGTGAGGCG-3′; 5′-TTTCTTTTCTCCCCCCAAAG-3′, 5′-TGTACGACTTTA-TTATGCGGG-3′; respectively, to amplify the specific gene deletion in BY4743 mutants (EUROSCARF, Frankfurt, Germany). The PCR fragments generated were transformed into W303-1A-STRE-lacZ and selected for geneticin resistance. Mutants were then confirmed by PCR using the primers: 5′-TTTCCAAGCAGATCCGGTGAT-3′, 5′-TGTTGGGATTCCATTGTTGA-3′; 5′-TCTTGAGT-GACGA-TCGTTTGT-3′, 5′-AAATAGCTCTCGGGCGAATA-3′; 5′-ATAGTGCAGAGAAGGAAA-G-3′, 5′-ATCGAATGCATACAGGCCTA-3′ for BUD14, RAS2 and WHI2, respectively, and NcoI (Promega) digestion.

4.2.3. Regulatory sequence analysis

Gene data sets from De Nicola et al. (2007) [387], Higgins et al. (2003), Lyons et al. (2000) [386] and Wu et al. (2008) [388] and this study were analysed for significant regulatory motifs using Regulatory Sequence Analysis Tools (RSAT) [398]. When
searching for motifs, five to eight oligonucleotide sizes were chosen for analysis. The P-value represents the probability that the motif could occur in the set of genes by chance.

4.2.4. Microarray description, RNA labelling, and hybridization

Saccharomyces cerevisiae microarray slides were obtained from the Ramaciotti Centre for Gene Function Analysis (Sydney, NSW, Australia). Slides were Schott Nexterion® Slide A+ with an amino-link coating (Schott, Mainz, Germany) and spotted with 40-mer oligonucleotide probes for 6250 yeast ORFs (Version MWGSc6K; MWG Biotech, Ebersburg, Germany) in duplicate. Slides were preprocessed by baking at 120 °C and blocking with 5% (v/v) ethanol as per the manufacturer's instructions (Ramaciotti Centre, Sydney, NSW, Australia).

Total RNA was isolated using TRizol™ Reagent (Invitrogen, Carlsbad, CA) as outlined previously [399]. The integrity of the RNA was analysed using an RNA 6000 Nano LabChip® on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). RNA (20 µg) was reverse transcribed, labelled and hybridized as outlined previously [399] for the slides labelled with cyanine dyes, except that yeast tRNA was used instead of Escherichia coli tRNA. After hybridization, the slides were washed in 2 × saline sodium citrate (SSC), 0.2% sodium dodecyl sulphate (SDS) for 10 min, 2 × SSC for 10 min and 0.2 × SSC for 10 min before drying the slides by centrifugation. Biological duplicates were analysed in technical duplicate using a dye swap and slides were scanned using an Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA).

4.2.5. Data acquisition

Image analysis of the microarray slides was performed using GenePix Pro 6.0 (Molecular Devices). A signal for each gene was determined to be ‘present’ if there
were no artefacts associated with the spot and the program could identify the spot intensity above background intensity. Normalization was performed on the data using the LOWESS method in the GeneSpring GX 7.3.1 (Agilent Technologies) analysis software package. The genes whose expression ratio (WT/double mutant) was significantly different from unity were identified based on Welch's analysis of one-way ANOVA, where the variances were not assumed to be equal and a level of significance of 0.05 was set. The complete raw microarray data set is available at Gene Expression Omnibus (GEO) database (Accession number: GSE11878).

4.2.6. β-Gal assays

β-Gal activity was measured as described [400-401], and activity units were calculated as follows: 
\[ \frac{A_{420\text{nm}} \times \text{total reaction volume (ml)}}{0.0045 \times \text{incubation time (min)} \times \text{extract volume (ml)} \times \text{protein conc. (mg ml}^{-1}\text{)}} \] . The concentration of protein was determined using the Bradford method [402].

Fold induction for each strain tested was determined by dividing LZM β-gal activity by LZM+Zn β-gal activity. Statistical analysis on these fold induction levels for each strain was performed using a one-way ANOVA with Tukey’s comparison method and was used to highlight where the fold induction levels differ if they do [403]. The ANOVA determines whether the mean fold induction levels of at least one pair of WT or mutants were different. Using Tukey’s comparison method, we were able to determine for each pair of fold induction levels whether their differences were statistically and significantly different from zero. Such a test was carried out using a 0.05 level of significance. All statistical computation was carried out using MINITAB (version 15) (Minitab Inc., PA).
4.4. Results

4.3.1. Promoter analysis reveals an Msn2/4p consensus sequence in genes responsive to zinc deficiency

The analysis of the promoter regions of genes induced during the exposure of yeast to zinc-deficient culture conditions was performed using promoter analysis software, RSAT [398]. Two core consensus sequences for known transcriptional activator proteins were identified from this analysis (Table 4-1). The sequence 5′-AGGGG-3′ was significantly overrepresented in the promoters of induced genes during zinc-deficient conditions in industrial [389] and laboratory [386, 388] yeast strains, but was not identified from the results presented by De Nicola et al. (2007) [387]. Identification of the STRE strongly indicated that the Msn2/4p transcriptional activators may play a role in regulating gene expression under zinc-deficient conditions. Additionally, the promoter analysis identified a number of other promising motifs to which no known transcriptional activator binds; however, it seems likely that these result from variations of the STRE motif. As expected, the Zap1p consensus core sequence, 5′-ACCYYNAAGGT-3′, was also overrepresented in upregulated genes.

4.3.2. Microarray analysis of the msn2 msn4 double mutant during zinc deficiency

To identify whether Msn2/4p are active inducers of gene expression during zinc deficiency, we performed a genome-wide expression analysis on zinc-deficient WT cells in comparison to zinc-deficient cells of the msn2,4 double mutant. During zinc deficiency, 141 genes were differentially expressed between the WT and msn2,4 double mutant strains. Of these genes, 72 were significantly reduced in the msn2,4 double mutant compared with the WT (Table 4-2 and Table 4-3). It was then of interest to determine whether these 72 differentially expressed genes could be regulated by
Msn2/4p. This was performed by analyzing the promoter regions of the gene set using RSAT\(^{[398]}\). The analysis identified that the STRE motif is a highly significant sequence enriched in this regulon with a significance index twofold higher than the entire gene set (141 genes). This greater value of significance indicates that the STRE is likely to be a regulatory element. Of the 72 genes, 72% contained the consensus sequence for the STRE within their promoter region (Table 4-2). Further RSAT analysis of these genes revealed that they contained no consensus sequence for Zap1p and that many of the possible significant sequences derived contained the STRE motif.

Of the remaining genes that did not contain the consensus STRE sequence, 62% contained STRE-like sequences and 38% contained no STRE or STRE-like sequences, and yet their expression was considerably affected by the absence of Msn2/4p (Table 4-3). Although these 16 genes show no direct link to Msn2/4p due to the absence of the consensus STRE sequence, it may be possible that Msn2/4p recognizes a highly similar sequence for gene induction or plays a role with other factors which affects the expression of these genes.

These results revealed a general trend for the induction of genes containing STREs within their promoters. Genes that have a fold increase > 10 contain, on average, four STRE motifs and genes with lower induction levels have on average two to three STRE motifs (Table 4-2). However, as with most motifs, there were exceptions. Some highly expressed genes such as RTC3 and others were found to contain relatively few STREs whereas HSP12, which contains seven STREs, was found to have a fold change lower than expected. The results produced from genome-wide and promoter analyses show strong evidence to indicate that the Msn2/4p complex plays a role in regulating gene expression under zinc-deficient growth conditions.
4.3.3. Msn2/4p-dependent regulation of gene expression during zinc deficiency

A strain harboring mutations in both the MSN2 and the MSN4 genes in this study was shown to impair the expression of STRE-regulated genes during zinc deficiency. To provide further evidence of Msn2/4p-dependent regulation, the level of protein activity was examined using a reporter construct consisting of the STRE-containing promoter of the CTT1 gene fused in-frame with the bacterial lacZ gene (STRE-lacZ)\[390\]. WT and the msn2,4 double mutant strains were transformed with the STRE-lacZ reporter construct and were grown to logarithmic phase. Cells were then transferred to zinc-replete (LZM+Zn) and zinc-deficient (LZM) media and the level of β-gal activity was assayed (Figure 4-1: A). The WT strain showed increased β-gal activity during zinc deficiency, further validating the zinc-dependent transcription of STRE-containing genes identified in the microarray studies. In contrast, β-gal activity in the msn2,4 double mutant was severely reduced in the absence of zinc, confirming that gene induction during zinc deficiency was Msn2/4p-dependent.

Numerous upstream factors, such as Bud14p, Hog1p, Whi2p and Ras2p, have previously been associated with Msn2/4p activation of transcription \[392-394\]. Therefore, we chose to examine the effect of the deletions of each of the upstream factors on Msn2/4p activity during zinc deficiency (Figure 4-1: A). In the strain lacking HOG1, β-gal activity was reduced approximately fivefold during zinc-deficient conditions when compared with the WT strain, which resulted in a decrease in overall extent of induction to about half that of the WT (Figure 4-1: B). Using Tukey’s comparison method \[403\], the mean fold induction level for Δhog1 was significantly dissimilar to WT, but significantly similar to the msn2,4 double mutant (95% confidence), indicating that Hog1p may play a role in positively regulating Msn2/4p function. Under zinc-replete and zinc-deficient conditions, it was observed that the deletion of RAS2 induced large
amounts of β-gal activity in comparison with the WT. Although β-gal activity was increased dramatically, the degree of induction was statistically similar to that of the WT as was the response in the bud14 and whi2 mutant strains (Figure 4-1: B). The whi2 mutation exhibited low levels of Msn2/4p activation according to the reporter assay in comparison with the WT (Figure 4-1: A), indicating that Msn2/4p activity may be affected by its absence. However, the extent of change reported for Δwhi2 in replete and deficient conditions (Figure 4-1: B) was similar, as indicated by Tukey’s comparison, to that of the WT, indicating that the reduced reporter activity was due to other mechanisms not involved with zinc metabolism.
Figure 4-1. Zinc responsiveness of the STRE driven-lacZ reporter construct.

(A) Yeast strains, W303 WT, Δmsn2,4, Δbud14, Δhog1, Δras2 and Δwhi2 were grown and shifted to conditions with normal zinc (LZM+Zn) and limited zinc (LZM) until samples in the logarithmic phase (≈ OD 0.5) were collected and β-gal activity was measured. (B) The fold induction for each strain represents the ratio of induction in zinc limitation to normal zinc. The asterisk above Δhog1 denotes Tukey’s significance. Error bars in both figures represents SD from mean.
<table>
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<tr>
<th>Gene cluster</th>
<th>Promoter element</th>
<th>Putative-binding protein</th>
<th>P-value</th>
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<td><strong>All up-regulated genes in an</strong> &lt;br&gt;industrial strain of <em>S. cerevisiae</em></td>
<td>AGGGG</td>
<td>CCCCT</td>
<td>Msn2p/4p</td>
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<td><em>(Higgins et al., 2003)</em></td>
<td>ATGGG</td>
<td>CCCAT</td>
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<td>AAGGG</td>
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<td></td>
<td>AGGGAG</td>
<td>CTCCC</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CCCTTA</td>
<td>TAAGGGG</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CCCTAAAG^{†}</td>
<td>CTTTAGGG</td>
<td>Zap1p</td>
</tr>
<tr>
<td><strong>All up-regulated genes in a</strong> &lt;br&gt;laboratory strain of <em>S. cerevisiae</em></td>
<td>AGGGG</td>
<td>CCCCT</td>
<td>Msn2p/4p</td>
</tr>
<tr>
<td><em>(Lyons et al., 2000)</em></td>
<td>AAGGG</td>
<td>CCCCC</td>
<td>-</td>
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<tr>
<td></td>
<td>ACCCC</td>
<td>GGGGT</td>
<td>-</td>
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<tr>
<td></td>
<td>CCCCC</td>
<td>CCCCC</td>
<td>-</td>
</tr>
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<td></td>
<td>GGGGA</td>
<td>TCCCC</td>
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<td></td>
<td>CCCTTA</td>
<td>TAAAGGG</td>
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<tr>
<td></td>
<td>AGGGAG</td>
<td>CTCCC</td>
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<td></td>
<td>AAGGGG</td>
<td>CCCCC</td>
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<tr>
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<td>CCCCTA</td>
<td>TAGGGG</td>
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<td></td>
<td>CTTGGAG^{†}</td>
<td>CTCCAAGGG</td>
<td>Zap1p</td>
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<tr>
<td></td>
<td>CCCTTGGAA^{†}</td>
<td>TTCAAGGG</td>
<td>Zap1p</td>
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</table>

The sequences are shown significantly overrepresented as possible promoter motifs in industrial and laboratory strains of *Saccharomyces cerevisiae*.

^{†} Based on the consensus ZRE sequence, 5’-ACCYYNAAGGT-3’ (Lyons et al., 2000).

^{‡}Sequence derived by RSAT analysis of putative Zap1p-regulated genes in an industrial strain of *S. cerevisiae* (Higgins et al., 2003).
# Table 4-2. Potential Msn2/Msn4p target genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>No. of STREs</th>
<th>Significance Index</th>
<th>Expression Fold Change</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes with consensus STREs (AGGGG)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>YNR034W-A</td>
<td>Protein of unknown function</td>
<td>5</td>
<td>30.97</td>
<td>240.4</td>
<td>5.18 x 10^-3</td>
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<tr>
<td>DDR2</td>
<td>DNA damage responsive 2 protein</td>
<td>4</td>
<td>30.97</td>
<td>147.3</td>
<td>3.18 x 10^-3</td>
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<tr>
<td>HXX1</td>
<td>Hexokinase isoenzyme I</td>
<td>5</td>
<td>30.97</td>
<td>138.7</td>
<td>1.26 x 10^-3</td>
</tr>
<tr>
<td>GPH1</td>
<td>Glycogen phosphorylase</td>
<td>3</td>
<td>30.97</td>
<td>61.3</td>
<td>1.27 x 10^-4</td>
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<tr>
<td>YER067W</td>
<td>Protein of unknown function, high similarity to uncharacterised <em>S. cerevisiae</em> Yil057p</td>
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<td>54.9</td>
<td>5.58 x 10^-4</td>
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<td><strong>HSP12</strong></td>
<td>Heat shock protein of 12 kDa</td>
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<td>1.01 x 10^-2</td>
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<tr>
<td></td>
<td>Protein of unknown function involved in RNA</td>
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<td></td>
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<tr>
<td>RTC3</td>
<td>metabolism</td>
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<td>44.8</td>
<td>1.78 x 10^-2</td>
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<td>STF2</td>
<td>Protein of unknown function, high similarity to uncharacterised <em>C. glabrata</em> Cgl008745gp</td>
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<td>Phosphoglucomutase 2</td>
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<td>30.97</td>
<td>38.6</td>
<td>1.39 x 10^-3</td>
</tr>
<tr>
<td>HXT7</td>
<td>High-affinity glucose transporter nearly identical to Hxt6p</td>
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<td>27.9</td>
<td>1.56 x 10^-3</td>
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<tr>
<td></td>
<td>High-affinity glucose transporter nearly identical Hxt6p</td>
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<tr>
<td>HXT6</td>
<td>to Hxt7p</td>
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<tr>
<td>GLC3</td>
<td>Glycogen branching enzyme</td>
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<td>19.7</td>
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<tr>
<td><strong>CTT1</strong></td>
<td>Catalase T 1</td>
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<tr>
<td><strong>HSP26</strong></td>
<td>Heat shock protein of 26 kDa</td>
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<td><strong>MSC1</strong></td>
<td>Protein that affects meiotic homologous chromatid recombination</td>
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<td>11.1</td>
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<tr>
<td><strong>ARG1</strong></td>
<td>Argininosuccinate synthetase</td>
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<td>8.2</td>
<td>4.10 x 10^-4</td>
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<tr>
<td>ARG1</td>
<td>Argininosuccinate synthetase</td>
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<td>30.97</td>
<td>8.2</td>
<td>4.10 x 10^-4</td>
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<tr>
<td>DCS2</td>
<td>Non-essential, stress induced regulatory protein</td>
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<td>7.8</td>
<td>4.24 x 10^-3</td>
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<tr>
<td>HOR7</td>
<td>Protein of unknown function</td>
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<td>7.4</td>
<td>6.03 x 10^-3</td>
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<tr>
<td>YIL169C</td>
<td>Protein of unknown function</td>
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<td>6.8</td>
<td>8.45 x 10^-3</td>
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<tr>
<td></td>
<td>Phospholipid hydroperoxide glutathione peroxidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GPX1</td>
<td>Putative transcription factor containing a C_{2}H_{2}</td>
<td>2</td>
<td>30.97</td>
<td>6.8</td>
<td>2.20 x 10^-2</td>
</tr>
<tr>
<td>USV1</td>
<td>Zinc finger</td>
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<td>30.97</td>
<td>6.8</td>
<td>1.36 x 10^-2</td>
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<tr>
<td><strong>SPI1</strong></td>
<td>Stationary phase induced 1 protein</td>
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<td>30.97</td>
<td>6.7</td>
<td>5.65 x 10^-3</td>
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<tr>
<td>URA10</td>
<td>Orotate phosphoribosyltransferase 2</td>
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<td>30.97</td>
<td>6.1</td>
<td>9.59 x 10^-3</td>
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<td>YGP1</td>
<td>Cell wall-related secretory glycoprotein</td>
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<td>30.97</td>
<td>5.2</td>
<td>7.31 x 10^-3</td>
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<tr>
<td>NCE102</td>
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<td>5.1</td>
<td>1.98 x 10^-3</td>
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<td>HSP31</td>
<td>Heat shock protein 31</td>
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<td>4.5</td>
<td>7.00 x 10^-3</td>
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<td>CRS5</td>
<td>Copper-binding metallothionein</td>
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<td>30.97</td>
<td>3.9</td>
<td>2.64 x 10^-3</td>
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<td>TFS1</td>
<td>Carboxypeptidase Y inhibitor</td>
<td>2</td>
<td>30.97</td>
<td>3.7</td>
<td>9.89 x 10^-3</td>
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<td>COX5B</td>
<td>Subunit Vb of cytochrome c oxidase</td>
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<td>30.97</td>
<td>3.4</td>
<td>1.51 x 10^-3</td>
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<tr>
<td></td>
<td>Glycosylphosphatidylinositol-dependent cell wall protein</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YNL300W</td>
<td>Protein of unknown function that associates with ribosomes</td>
<td>1</td>
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<td>3.4</td>
<td>3.04 x 10^-4</td>
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<td>TMA10</td>
<td>Mitochondrial aldehyde dehydrogenase</td>
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<td>30.97</td>
<td>3.4</td>
<td>8.25 x 10^-3</td>
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<tr>
<td>PBI2</td>
<td>Cytosolic inhibitor of vacuolar proteinase B</td>
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<td>30.97</td>
<td>3.3</td>
<td>2.79 x 10^-3</td>
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<td>ALD4</td>
<td>Mitochondrial aldehyde dehydrogenase</td>
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Table 4-2.  Continued

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>No. of STREs</th>
<th>Significance Index*</th>
<th>Expression Fold Change</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>YJR096W</td>
<td>Putative xylose and arabinose reductase</td>
<td>1</td>
<td>30.97</td>
<td>3.1</td>
<td>1.69 x 10^-3</td>
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<td>MCR1</td>
<td>Mitochondrial NADH-cytochrome b5 reductase, involved in ergosterol biosynthesis</td>
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<td>30.97</td>
<td>3.0</td>
<td>1.86 x 10^-2</td>
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<td>SOD2</td>
<td>Mitochondrial superoxide dismutase</td>
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<td>30.97</td>
<td>3.0</td>
<td>9.00 x 10^-3</td>
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<tr>
<td>YJR008W</td>
<td>Putative protein of unknown function</td>
<td>2</td>
<td>30.97</td>
<td>3.0</td>
<td>3.36 x 10^-3</td>
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<td>OMC1</td>
<td>Protein of unknown function</td>
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<td>30.97</td>
<td>2.9</td>
<td>2.91 x 10^-2</td>
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<td>QM45</td>
<td>Protein of unknown function, major constituent of the mitochondrial outer membrane</td>
<td>3</td>
<td>30.97</td>
<td>2.9</td>
<td>3.70 x 10^-2</td>
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<tr>
<td>DMR48</td>
<td>Stress protein induced by heat shock, DNA damage, or osmotic stress</td>
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<td>30.97</td>
<td>2.9</td>
<td>2.08 x 10^-2</td>
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<tr>
<td>TDH1</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase, isozyme 1</td>
<td>1</td>
<td>30.97</td>
<td>2.8</td>
<td>6.27 x 10^-3</td>
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<tr>
<td>TPS2</td>
<td>Phosphatase subunit of the trehalose-6-phosphate synthase/phosphatase complex</td>
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<td>30.97</td>
<td>2.8</td>
<td>2.03 x 10^-3</td>
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<tr>
<td>MMF1</td>
<td>Mitochondrial protein involved in maintenance of the mitochondrial genome</td>
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<td>2.7</td>
<td>5.26 x 10^-3</td>
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<td>FMP16</td>
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<td>FMP46</td>
<td>Putative redox protein</td>
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<td>2.6</td>
<td>2.82 x 10^-3</td>
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<td>YER053C-A</td>
<td>Putative protein of unknown function</td>
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<td>30.97</td>
<td>2.6</td>
<td>6.07 x 10^-3</td>
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<td>YMR291W</td>
<td>Putative kinase of unknown function</td>
<td>3</td>
<td>30.97</td>
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<td>5.05 x 10^-3</td>
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<td>MDH1</td>
<td>Mitochondrial malate dehydrogenase</td>
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<td>GSP2</td>
<td>GTP binding protein</td>
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<td>30.97</td>
<td>2.3</td>
<td>2.03 x 10^-2</td>
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<td>COS8</td>
<td>Nuclear membrane protein</td>
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<td>PRB1</td>
<td>Vacuolar protease B</td>
<td>1</td>
<td>30.97</td>
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<td>2.64 x 10^-2</td>
</tr>
<tr>
<td>RIB5</td>
<td>Riboflavin synthase</td>
<td>1</td>
<td>30.97</td>
<td>2.1</td>
<td>4.95 x 10^-2</td>
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<td>FMP10</td>
<td>Protein of unknown function</td>
<td>2</td>
<td>30.97</td>
<td>2.1</td>
<td>5.79 x 10^-4</td>
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</tbody>
</table>

The genes listed showed Msn2/Msn4p-dependent regulation pattern whose promoters contain sequences that match the previously published STRE consensus sequence, 5′-AGGGG-3′. STRE, Stress Response Element. Msn2/Msn4p-dependent regulation of genes in bold has been confirmed independently either in this study or in other reports by lacZ reporter fusions and/or northern blotting.

*Calculated for each sequence by RSA-Tools program. The significance index is the minus log transform of the E-value.
†Probability that the genes by chance alone are regulated by Msn2/Msn4p. The p-values obtained from the raw microarray data and normalised using the LOWESS method and Welch’s parametric ANOVA. Each value is the average of three biological arrays with dye swaps.
Table 4-3.  Other Potential Msn2/Msn4p target genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>STRE like sequence</th>
<th>Significanc e Index*</th>
<th>Expression Fold Change</th>
<th>P-value†</th>
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<tbody>
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<td><strong>Genes with STRE-like sequences</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SNZ1</strong></td>
<td>Protein involved in vitamin B6 biosynthesis</td>
<td>AAGGG</td>
<td>8.59</td>
<td>5.7</td>
<td>$5.08 \times 10^{-4}$</td>
</tr>
<tr>
<td><strong>TMT1</strong></td>
<td>Trans-aconitate methyltransferase</td>
<td>AAGGG</td>
<td>8.59</td>
<td>4.3</td>
<td>$1.23 \times 10^{-2}$</td>
</tr>
<tr>
<td><strong>HSP30</strong></td>
<td>Heat shock protein of 30 kDa</td>
<td>ACCCC</td>
<td>18.57</td>
<td>3.8</td>
<td>$2.66 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCCC</td>
<td>9.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAGGG</td>
<td>8.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGGA</td>
<td>5.68</td>
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<tr>
<td><strong>YER079W</strong></td>
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<td>AAGGG</td>
<td>8.59</td>
<td>2.9</td>
<td>$2.14 \times 10^{-3}$</td>
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<tr>
<td><strong>SHM2</strong></td>
<td>Cytosolic serine hydroxymethyltransferase</td>
<td>ACCCC</td>
<td>18.57</td>
<td>2.8</td>
<td>$4.41 \times 10^{-2}$</td>
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<td><strong>QCR8</strong></td>
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<td>18.57</td>
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<td>5.68</td>
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<tr>
<td><strong>SEC9</strong></td>
<td>t-SNARE protein important for fusion of secretory vesicles with the plasma membrane</td>
<td>ACCCC</td>
<td>18.57</td>
<td>2.3</td>
<td>$2.63 \times 10^{-2}$</td>
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<tr>
<td><strong>ATP7</strong></td>
<td>Subunit d of the stator stalk of mitochondrial F1F0 ATP synthase</td>
<td>CCCCC</td>
<td>9.30</td>
<td>2.1</td>
<td>$2.67 \times 10^{-2}$</td>
</tr>
<tr>
<td><strong>CYB5</strong></td>
<td>Cytochrome b5</td>
<td>AAGGG</td>
<td>8.59</td>
<td>2.0</td>
<td>$2.30 \times 10^{-2}$</td>
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<tr>
<td><strong>Genes with no STREs or STRE-like sequences</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>UGP1</strong></td>
<td>UDP-glucose pyrophosphorylase</td>
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<td>-</td>
<td>11.3</td>
<td>$2.22 \times 10^{-2}$</td>
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<tr>
<td><strong>YMR173W-A</strong></td>
<td>Dubious open reading frame unlikely to encode a protein; overlaps verified gene</td>
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<td>-</td>
<td>4.5</td>
<td>$3.99 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>DDR48/YML173W*</td>
<td></td>
<td></td>
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<tr>
<td><strong>URA3</strong></td>
<td>Orotidine-5'-phosphate (OMP) decarboxylase</td>
<td>-</td>
<td>-</td>
<td>3.9</td>
<td>$4.05 \times 10^{-2}$</td>
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<tr>
<td><strong>RAS2</strong></td>
<td>GTP-binding protein</td>
<td>-</td>
<td>-</td>
<td>2.8</td>
<td>$1.14 \times 10^{-2}$</td>
</tr>
<tr>
<td><strong>BNA1</strong></td>
<td>3-hydroxyanthranilic acid dioxygenase</td>
<td>-</td>
<td>-</td>
<td>2.6</td>
<td>$1.57 \times 10^{-2}$</td>
</tr>
<tr>
<td><strong>YHR122W</strong></td>
<td>Protein of unknown function required for establishment of sister chromatid cohesion</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>$7.62 \times 10^{-3}$</td>
</tr>
<tr>
<td><strong>MCM10</strong></td>
<td>Essential chromatin-associated protein involved in the initiation of DNA replication</td>
<td>-</td>
<td>-</td>
<td>2.1</td>
<td>$4.61 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

*Calculated for each sequence by RSA-Tools program. The significance index is the minus log transform of the E-value.†Probability that the genes by chance alone are regulated by Msn2/Msn4p. The p-values obtained from the raw microarray data and normalised using the LOWESS method and Welch's parametric ANOVA. Each value is the average of three biological arrays with dye swaps.‡RSA-Tools analysis disregarded this gene from analysis.

The genes listed showed Msn2/Msn4p-dependent regulation pattern whose promoters contain sequences that match the previously published STRE consensus sequence, 5′-AGGGG-3′. STRE, Stress Response Element.

Msn2/Msn4p-dependent regulation of genes in bold has been confirmed independently either in this study or in other reports by lacZ reporter fusions and/or northern blotting.

**NOTE in manuscript this is supplementary data**
4.5. Discussion

Environmental niches introduce cells to dynamic conditions under which nutrients can be limiting or abundant. Cells must adjust their genomic expression program rapidly to adapt to these new conditions. Industrial fermentations can present the yeast with conditions requiring such an adaptation. Zinc availability is of major concern during the fermentation process because the deficiency results in sluggish and incomplete fermentation. When yeast cells are exposed to zinc-deficient conditions, Zap1p induces the expression of genes involved in increasing cellular zinc levels [384, 386]. Interestingly, a large subset of genes that were regulated in a Zap1p-independent manner was also induced under zinc-deficient growth conditions. The presence of an overrepresented STRE motif in these genes supported the hypothesis proposed by Higgins et al. (2003) [389] that Msn2/4p may play a role in inducing the expression of genes during zinc deficiency in addition to the induction of STRE-regulated genes in a variety of other stresses such as osmotic, oxidative, heat, pressure and nitrogen starvation [391, 404-408]. These upregulated genes harboring the STRE motif were also identified in zinc limitation studies [386, 388-389], adding further weight to the findings that Msn2/4p plays a role in the stress of zinc deficiency. Interestingly, the STRE motif was not identified in the upregulated gene set from data presented by De Nicola et al. (2007) [387]. The De Nicola et al. (2007) [387] study used continuously fed chemostat cultures instead of batch cultures and added enough zinc to the medium to sustain yeast growth. This is in contrast to this and other zinc-deficiency studies where zinc availability was decreased to a level that does not support long-term yeast growth, and thus better reflects the conditions found in industrial ‘stuck’ fermentations where yeast growth and fermentation cease. Additionally, this also suggests that the induction of the Msn2/4p regulon is a stress response as a result of zinc starvation rather than a cellular mechanism to overcome zinc deficiency.
A number of motifs with high homology to the STRE, but not identical, were also identified in the promoters of genes affected by the msn2,4 mutation. This suggests that they may function as activating sequences for Msn2/4p in a manner similar to the results of Lyons et al. (2000)\[386\], where ZRE-like sequences were present in genes that have been confirmed to be regulated by Zap1p. An example of this is the HSP30 gene, which was highly induced during zinc deficiency, but does not harbor the consensus STRE or ZRE motif. However, it does contain an STRE-like motif, 5’-AAGGG-3’, which may be functional. Mutational analysis of the core element of the consensus STRE \[391\] did not indicate that this predicted STRE-like motif would not be functional, and since that report, it has been shown that Msn2/4p does affect HSP30 induction \[409-410\]. Therefore, it may be possible that Msn2/4p can induce gene expression through this STRE-like element.

The transcriptional activation of the reporter gene contained within the CTT1-18/7x plasmid is regulated by STREs derived from the CTT1 gene \[390\]. The STRE-lacZ reporter showed that STRE-dependent expression in the msn2,4 double mutant was markedly affected and basal activity for the reporter gene was undetected. Induction was completely abolished when zinc was absent, providing compelling evidence to support Msn2/4p as an additional inducer of gene expression during this stress. Since we had provided evidence at the molecular level that Msn2/4p directs the expression of a specific regulon during zinc starvation, we sought to identify the molecular mechanism accountable for Msn2/4p activation for this condition. Screening of a number of mutants involved in controlling Msn2/4p activity revealed that its activation due to zinc starvation may be linked to HOG1. This mitogen-activated protein kinase has also been shown to regulate the expression of STRE-driven genes during osmotic stress \[392, 411\]. Recent research has shown that the role of Hog1p extends more widely than just in response to osmotic stress to include other stress conditions such as
exposure to heat, oxidants, citric acid, cesium chloride and arsenite. Screening of the RAS2 mutant showed that Ras2p and hence the Protein Kinase A (PKA) pathway negatively regulate Msn2/4p activity during zinc deficiency. The PKA signal transduction pathway regulates the nucleocytoplasmic shuttling of this transcription factor complex, and hence gene transcription, under conditions of stress. It is widely known that the PKA signal-transduction pathway negatively regulates Msn2/4p activity, resulting in its retention in the cytoplasm to prevent the activation of STRE-responsive genes under conditions of no stress, and it is therefore very interesting that this pathway also affects Msn2/4p activity during zinc starvation.

These results characterize a larger proportion of yeast genes that are upregulated in response to zinc deficiency and confirm that they are part of the Msn2/4p regulon. The molecular mechanisms controlling this regulon are similar to those for other stress conditions, which suggest this is the cells’ defence against zinc starvation. This research provides fermentation scientists with an improved understanding of the yeast response to zinc limitation and starvation, which may provide opportunities to overcome this challenge.

4.6. Acknowledgments

The support by the Australian Research Council's Linkage Projects funding scheme (project number LP0775238), Foster's Group Limited and The University of Western Sydney is acknowledged. Thanks to Kellie McNamara for all the technical support of. Thanks given to G. Marcheler for providing the CTT1-18/7x plasmid and C. Schüller for providing the HOG1 mutant strain.
**FUTURE DIRECTIONS**

It was shown that the expression of numerous STRE regulated genes were reduced in the double mutant, msn2,4 during zinc deficient conditions. Numerous pathways have been shown to regulate Msn2,4p activity, however, the specific pathway involved during zinc deficient conditions has not been revealed. Investigation revealed that Hog1p has some involvement in regulating Msn2,4p activity during zinc deficiency, however, to an extent Msn2,4p activity was still present.

Unlike the BY4743 yeast strain, the haploid W303 yeast strain does not have a deletion library i.e. a yeast mutant for each gene. Although only a small number of pathways mutants involved in Msn2,4p regulation, Hog1p was shown to be involved. However, since Msn2,4p was not completely reduced in the HOG1 mutant, it is clear that the Hog1p target is activated by an alternate protein and/or pathway. Thus looking into the Hog1p targets and examining these during zinc deficiency could identify which protein is involved downstream of Hog1p to activate Msn2,4p activity. It may also be of interest to identify the upstream players involved in activating Hog1p during zinc deficiency since Hog1p has been shown to be activated by two pathways, the phosphorelay mechanism involving Sln1p-Ypd1p-Ssk1p or the transmembrane osmosensor Sho1p.
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APPENDIX A: Optimisation of Coomassie Brilliant Blue staining as a competitive fluorescent alternative for sensitive in-gel protein detection - SUPPLEMENTARY DATA FOR CHAPTER TWO

Practical Examples of Quantitative Characteristics – LLD, S^2/N and LDR

**Lowest Limit of Detection:** LLD is calculated using the slope and background error.

<table>
<thead>
<tr>
<th>Protein Load (ng)</th>
<th>Protein Fluorescent Volumes</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
<td>Rep 3</td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>785454</td>
<td>791702</td>
<td>803983</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>374336</td>
<td>366595</td>
<td>380341</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>175895</td>
<td>165520</td>
<td>162264</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>70541</td>
<td>73898</td>
<td>59286</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>29616</td>
<td>40505</td>
<td>48805</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>17591</td>
<td>14311</td>
<td>12155</td>
<td></td>
</tr>
</tbody>
</table>

*statistically determined to be different to background, Students T-test (p < 0.05)

For each Replicate (Rep) the slope was determined using Fluorescent volumes and Protein Load values.

<table>
<thead>
<tr>
<th>SLOPE</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep 1</td>
<td>6512</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep 2</td>
<td>6537</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep 3</td>
<td>6681</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each Rep the respective background values are used to calculate 3 × BG SEM

<table>
<thead>
<tr>
<th>Background Fluorescent Volumes</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep 1</td>
<td>722468</td>
<td>725765</td>
<td>721807</td>
</tr>
<tr>
<td>Rep 2</td>
<td>709180</td>
<td>726536</td>
<td>728237</td>
</tr>
<tr>
<td>Rep 3</td>
<td>689796</td>
<td>736680</td>
<td>728471</td>
</tr>
<tr>
<td>678513</td>
<td>729228</td>
<td>730462</td>
<td></td>
</tr>
<tr>
<td>678398</td>
<td>702997</td>
<td>698657</td>
<td></td>
</tr>
<tr>
<td>680250</td>
<td>694579</td>
<td>697938</td>
<td></td>
</tr>
<tr>
<td>675642</td>
<td>697755</td>
<td>700614</td>
<td></td>
</tr>
<tr>
<td>671878</td>
<td>695681</td>
<td>701457</td>
<td></td>
</tr>
<tr>
<td>688269</td>
<td>709116</td>
<td>703990</td>
<td></td>
</tr>
<tr>
<td>3 × BG SEM</td>
<td>16934</td>
<td>16419</td>
<td>14366</td>
</tr>
</tbody>
</table>
For each Rep, the $3 \times$ BG SEM value is divided by the SLOPE of the same Rep.

<table>
<thead>
<tr>
<th>LLD</th>
<th>Rep 1</th>
<th>Rep 2</th>
<th>Rep 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.6</td>
<td>2.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

The LLD Rep values are then averaged and a SEM calculated.

**Signal Squared to Noise Ratio:** $S^2/N$ is used to highlight contrast between protein signal and background signal.

<table>
<thead>
<tr>
<th>Protein Load (ng)</th>
<th>Protein Fluorescent Volumes</th>
<th>Protein Fluorescent Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
</tr>
<tr>
<td>119</td>
<td>785454</td>
<td>791702</td>
</tr>
<tr>
<td>48</td>
<td>374336</td>
<td>366595</td>
</tr>
<tr>
<td>19</td>
<td>175895</td>
<td>165520</td>
</tr>
<tr>
<td>8</td>
<td>70541</td>
<td>73898</td>
</tr>
<tr>
<td>3</td>
<td>29616</td>
<td>40505</td>
</tr>
<tr>
<td>1.2</td>
<td>17591</td>
<td>14311</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Background Fluorescent Volumes</th>
<th>Rep 1</th>
<th>Rep 2</th>
<th>Rep 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>119</td>
<td>722468</td>
<td>725765</td>
<td>721807</td>
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<td>48</td>
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</tr>
<tr>
<td></td>
<td>688269</td>
<td>709116</td>
<td>703990</td>
</tr>
</tbody>
</table>

BG STDEV 16934 16419 14366

Each protein fluorescent volume is squared and divided by the BG STDEV for that Rep, resulting in $S^2/N$ ratio for each protein load of each Rep.

<table>
<thead>
<tr>
<th>Protein Load (ng)</th>
<th>$S^2/N$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep 1</td>
</tr>
<tr>
<td>119</td>
<td>36431008</td>
</tr>
<tr>
<td>48</td>
<td>8274745</td>
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<tr>
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<td>8</td>
<td>293846</td>
</tr>
<tr>
<td>3</td>
<td>51797</td>
</tr>
<tr>
<td>1.2</td>
<td>18273</td>
</tr>
</tbody>
</table>

The $S^2/N$ Rep values are then averaged and a SEM calculated for each protein at each protein load.
**Linear Dynamic Range:** Here the LDR was defined based on all protein loads (*R^2*), significant from background, and the LDR with the highest linear regression, widest LDR (**R^2**).

<table>
<thead>
<tr>
<th>Protein Load (ng)</th>
<th>Protein Fluorescent Volumes</th>
<th>Linear Regression (R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
</tr>
<tr>
<td>25000</td>
<td>4091755</td>
<td>3437946</td>
</tr>
<tr>
<td>6300</td>
<td>1392716</td>
<td>1256394</td>
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<tr>
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<td>503696</td>
<td>480077</td>
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<tr>
<td>24</td>
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</tr>
<tr>
<td>6.1</td>
<td>11113</td>
<td>18577</td>
</tr>
<tr>
<td>1.5</td>
<td>7539</td>
<td>12603</td>
</tr>
</tbody>
</table>

The protein dilution series (9 samples in total) used for LDR could provide up to 8 data sets, each set having the top load sequentially eliminated and with its own R^2 value. The number of R^2 values depends on how many protein fluorescent volumes were statistically distinguished from background.

Since each of the data sets have 3 Reps, there will always be 3 R^2 values comprising each of the average R^2 values (R^21-R^27). Then each R^2 values was compared to R^21 to determine whether the Regression for the smaller data sets was more linear, Students T-test (p < 0.05), this was then denoted as **R^2**, the widest LDR.
Figure A-1. Imaging of cCBB gels
Background fluorescence was determined from the matrix (i.e. no protein zones) of 1DE gels stained with NG with various concentrations of dye, 0.1% (normal), 0.05, 0.01, 0.005 and 0.001%. (A) Two images taken grouped as follows, 1st - 0.1 & 0.05%, 2nd - 0.01, 0.005 & 0.001%. (B) Each gel was imaged individually. On the right of each graph is a representative gel image. Error bars represent standard error of the mean (n = 3). Statistically significant differences to incubation 0.1% background are denoted by * (Student’s t-test, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). NOTE: data in Figure B obtained the next day for the same gels.
Figure A-2. Sensitivity to light.

1DE separation of seven protein standards (BGAL, PHOSB, BSA, CA, TYRP, STI, LYS) at 119 and 48 ng was stained with NG. Stained gels were destained with distilled water (4×15 min). Since CBB was to be treated as a fluorophore we wished to examine its performance in the presence or absence of light (standard lab conditions). Quantitative image analysis displayed those fluorescent volumes that are significantly discriminated from background (A, B). The stains capacity to discriminate protein from background is displayed by $S^2/N$ ratios for all proteins (C, D). Error bars represent standard error of the mean (n = 2). Statistically significant differences (Student’s t-test, p < 0.05) in comparison to incubation in the presence of light are denoted by *.

![Figure A-2](image-url)
Figure A-3. **Alternate destain - 0.5 M NaCl.**

1DE separation of pure BSA at 390 and 10 ng was stained with either BioSafe or NG. Stained gels were destained with either water or 0.5 M NaCl (4×15 min). Quantitative image analysis displayed those fluorescent volumes that are significantly discriminated from background (A, B, E, F). The stains capacity to discriminate protein from background is displayed by $S^2/N$ ratios (C, D, G, H). Error bars represent standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, p < 0.05) in comparison to water is denoted by * for a specific destain time; comparison between destain time for water or NaCl is denoted by †.
Figure A-3
Figure A-4. Alternate destain - H$_3$PO$_4$.

1DE separation of pure BSA at 390 and 10 ng was stained with either BioSafe or NG. Stained gels were destained with either water or H$_3$PO$_4$ (4×15 min) ranging from 1-5%. Quantitative image analysis displayed those fluorescent volumes that are significantly discriminated from background (A, B, E, F). The stain's capacity to discriminate protein from background is displayed by S'/N ratios for (C, D, G, H). Error bars represent standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, p < 0.05) in comparison to water are denoted by *.
Figure A-4
Figure A-5. Sensitivity to Photobleaching by increasing PMT.

1DE separation of seven protein standards (BGAL, PHOSB, BSA, CA, TYRP, STI, LYS) at 119 and 48 ng was stained with NG. Stained gels were destained with distilled water (4×15 min) (standard lab conditions). Since images at every time point were to be taken subsequently at 600V, 700V and 800V, we also examined taking images in the reverse sequence (800V, 700V, 600V) to determine whether taking images in this manner would affect the results obtained from these images. Quantitative image analysis displayed those fluorescent volumes that are significantly discriminated from background (A-C, G-I). The stains capacity to discriminate protein from background is displayed by \( S/N \) ratios for all standard proteins (D-F, J-L). Error bars represent standard error of the mean (n = 2). Statistically significant differences (Student’s t-test, \( p < 0.05 \)) in comparison to forward PMT voltage are denoted by *.
Figure A-6.  Water destain for optimal sensitivity with BioSafe (4 h).

1DE quantitative images analysis of pure protein standards (seven in total) were detected by BioSafe infrared fluorescence. Quantitative image analysis displayed those fluorescent volumes that are significantly discriminated from background; fluorescent volumes for all protein standards that underwent 4×15 min water washes and four consecutive 15 min water washes, at PMT settings of 600V, 700V and 800V and stain duration - 4 h, are displayed (A-U). The stains capacity to discriminate protein from background throughout these water destain steps is displayed by $S^2/N$ ratios for all standards (V-AP). The variation in staining for different proteins (i.e. IPV) is shown in Figures AQ, AR and AS. Error bars represent standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, p < 0.05) in comparison to water wash sequence, 4×15 min, are denoted by *.
Figure A-6
Figure A-6
Figure A-7. Water destain for optimal sensitivity with BioSafe (20 h).

1DE quantitative images analysis of pure protein standards (seven in total) were detected by BioSafe infrared fluorescence. Quantitative image analysis displayed those fluorescent volumes that are significantly discriminated from background; fluorescent volumes for all protein standards that underwent 4×15 min water washes and four consecutive 15 min water washes, at PMT settings of 600V, 700V and 800V and stain duration - 20 h, are displayed (A-U). The stains capacity to discriminate protein from background throughout these water destain steps is displayed by $S^2/N$ ratios for all standards (V-AP). The variation in staining for different proteins (i.e. IPV) is shown in Figures AQ, AR and AS. Error bars represent standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, $p < 0.05$) in comparison to water wash sequence, 4×15 min, are denoted by *. 

Figure A-7
Figure A-7
Figure A-8. Water destain for optimal sensitivity with NG (4 h).

1DE quantitative images analysis of pure protein standards (seven in total) were detected by NG infrared fluorescence. Quantitative image analysis displayed those fluorescent volumes that are significantly discriminated from background; fluorescent volumes for all protein standards that underwent 4×15 min water washes and four consecutive 15 min water washes, at PMT settings of 600V, 700V and 800V and stain duration - 4 h, are displayed (A-U). The stains capacity to discriminate protein from background throughout these water destain steps is displayed by $S^2/N$ ratios for all standards (V-AP). The variation in staining for different proteins (i.e. IPV) is shown in Figures AQ, AR and AS. Error bars represent standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, p < 0.05) in comparison to water wash sequence, 4×15 min, are denoted by *.
Figure A-8
Figure A-8
Figure A-8
Figure A-9. Water destain for optimal sensitivity with NG (20 h).  
1DE quantitative images analysis of pure protein standards (seven in total) were detected by NG infrared fluorescence. Quantitative image analysis displayed those fluorescent volumes that are significantly discriminated from background; fluorescent volumes for all protein standards that underwent 4×15 min water washes and four consecutive 15 min water washes, at PMT settings of 600V, 700V and 800V and stain duration - 20 h, are displayed (A-U). The stains capacity to discriminate protein from background throughout these water destain steps is displayed by S²N ratios for all standards (V-AP). The variation in staining for different proteins (i.e. IPV) is shown in Figures AQ, AR and AS. Error bars represent standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, p < 0.05) in comparison to water wash sequence, 4×15 min, are denoted by *.
**Figure A-9**

Fluorescent Volume (Au)

/S/N Ratio (Log 10)

LYS Load (ng per band)

PHOSB Load (ng per band)

CA Load (ng per band)

STI Load (ng per band)

TYRP Load (ng per band)

BGAL Load (ng per band)

BSA Load (ng per band)

600V

700V

800V

261
Figure A-9
Figure A-9
Figure A-10.  Total spot detection: Progenesis vs. Delta2D

2DE quantitative images analysis of mouse brain membrane (MBM) and A. thaliana soluble protein (ATSP) were detected by BR (BioSafe), NG and SR. Quantitative image analysis using Delta2D as described in this thesis was performed and the same image were processed by Progenesis as previously described [296]. The output is reported as total spot number. Error bars represent standard error of the mean (n = 3). Statistically significant differences to Progenesis are denoted as * (Student’s t-test, ** p < 0.01, *** p < 0.001, **** p < 0.0001). NOTE: the preparation of brain membrane (MBM) and A. thaliana soluble protein was performed as stated in Chapter 3 – methods section.

![Figure A-10](image-url)
Table A-1. **LLD of water destain for optimal sensitivity with BioSafe (4 h).**

1DE quantitative images analysis of pure protein standards (seven in total) were detected by BioSafe infrared fluorescence, after 4 h stain duration and destaining with water (4×15 min and then four consecutive 15 min washes). The following LLD values were derived from quantitative image analysis where fluorescent volumes were determined to be significantly discriminated from background. Images were taken using the 750 LP filter at PMT settings; 600V, 700V and 800V. Range of error represents standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, p < 0.05) for LLD compared to LLD of 600V for a single wash step is denoted by *.

<table>
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<th>Protein Standard</th>
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<th>7×15 min</th>
<th>8×15 min</th>
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<td>800V</td>
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<tr>
<td>BGAL</td>
<td>5.0 ± 1.6</td>
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<td>5.4 ± 0.5</td>
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<tr>
<td>PHOSB</td>
<td>5.7 ± 1.9</td>
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<tr>
<td>BSA</td>
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Table A-2. LLD of water destain for optimal sensitivity with BioSafe (20 h).

1DE quantitative images analysis of pure protein standards (seven in total) were detected by BioSafe infrared fluorescence, after 20 h stain duration and destaining with water (4×15 min and then four consecutive 15 min washes). The following LLD values were derived from quantitative image analysis where fluorescent volumes were determined to be significantly discriminated from background. Images were taken using the 750 LP filter at PMT settings; 600V, 700V and 800V. Range of error represents standard error of the mean (n = 3).

<table>
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<tr>
<th>Protein Standard</th>
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<td>700V</td>
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<td>4.6 ± 0.8</td>
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<td>4.4 ± 1.5</td>
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<td>4.9 ± 0.7</td>
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<td>3.7 ± 0.9</td>
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<tr>
<td>TYRP</td>
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<td>13.8 ± 2.2</td>
<td>14.2 ± 2.4</td>
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<tr>
<td>LYS</td>
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<td>10.4 ± 1.1</td>
<td>10.4 ± 1.2</td>
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Table A-3. LLD of water destain for optimal sensitivity with NG (4 h).

1DE quantitative images analysis of pure protein standards (seven in total) were detected by NG infrared fluorescence, after 4 h stain duration and destaining with water (4×15 min and then four consecutive 15 min washes). The following LLD values were derived from quantitative image analysis where fluorescent volumes were determined to be significantly discriminated from background. Images were taken using the 750 LP filter at PMT settings; 600V, 700V and 800V. Range of error represents standard error of the mean (n = 3). Statistically significant differences (Student’s t-test, p < 0.05) for LLD compared to LLD of 4×15 min wash of the same PMT setting is denoted by †.

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<th>6×15 min</th>
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<td>700V</td>
<td>800V</td>
<td>600V</td>
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<tr>
<td>BGAL</td>
<td>5.7 ± 1.3</td>
<td>7.4 ± 2.2</td>
<td>6.9 ± 2.5</td>
<td>6.1 ± 0.5</td>
<td>5.1 ± 0.9</td>
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<tr>
<td>PHOSB</td>
<td>7.0 ± 1.9</td>
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<td>7.0 ± 22.2</td>
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<td>10.7 ± 2.7</td>
<td>10.6 ± 0.5</td>
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<tr>
<td>CA</td>
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Table A-4. LLD of water destain for optimal sensitivity with NG (20 h).

1DE quantitative images analysis of pure protein standards (seven in total) were detected by NG infrared fluorescence, after 20 h stain duration and destaining with water (4×15 min and then four consecutive 15 min washes). The following LLD values were derived from quantitative image analysis where fluorescent volumes were determined to be significantly discriminated from background. Images were taken using the 750 LP filter at PMT settings; 600V, 700V and 800V. Range of error represents standard error of the mean (n = 3).

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>4×15 min</th>
<th>5×15 min</th>
<th>6×15 min</th>
<th>7×15 min</th>
<th>8×15 min</th>
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<td>600V</td>
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<td>700V</td>
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<tr>
<td>BGAL</td>
<td>4.3 ± 1.4</td>
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<td>6.3 ± 1.1</td>
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<td>4.5 ± 0.9</td>
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<tr>
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<td>6.1 ± 1.8</td>
<td>6.8 ± 1.2</td>
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<td>4.9 ± 0.9</td>
<td>5.1 ± 1.1</td>
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<td>9.5 ± 2.2</td>
<td>7.3 ± 2.6</td>
<td>7.9 ± 2.0</td>
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<td>4.2 ± 1.0</td>
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<tr>
<td>TYRP</td>
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<td>16.3 ± 4.8</td>
<td>15.2 ± 4.8</td>
<td>13.5 ± 1.6</td>
<td>14.3 ± 2.2</td>
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<td>STI</td>
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<td>16.7 ± 4.7</td>
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<td>16.0 ± 3.8</td>
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<tr>
<td>LYS</td>
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<td>14.2 ± 2.7</td>
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Table A-5. Standard mixture components and final concentrations

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<th>Standard Mixture</th>
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<th>µg per vial</th>
<th>Final Standard Concentrations (when mixed with matrix and diluents)</th>
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<td>1.0 pmol/µL</td>
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<tr>
<td>Calibration Mixture</td>
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<td>2.0 pmol/µL</td>
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<td></td>
<td>Glu&lt;sup&gt;1&lt;/sup&gt;-Fibrinopeptide B</td>
<td>5.1</td>
<td>1.3 pmol/µL</td>
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<td>ACTH (1-17 clip)</td>
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<td>ACTH (18-39 clip)</td>
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<td>1.5 pmol/µL</td>
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<td>ACTH (7-38 clip)</td>
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<td>Component</td>
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<td>(M+nH)n+ Average (Da)</td>
<td>(M+nH)n+ Monoisotopic (Da)</td>
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### Table A-7. Mass spectrometry compatibility – ESI

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<th>Mass (Da)</th>
<th>MASCOT Score</th>
<th>Sequence Coverage (%)</th>
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Table A-8. Mass spectrometry compatibility – MALDI

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</table>

*Since D616F5 had the same score as the top protein hit, it was also listed
APPENDIX B: Sensitive fluorescence detection of native proteomes using an optimised CBB protocol -
SUPPLEMENTARY DATA FOR CHAPTER THREE

Figure B-1. 2DE raw images and cropped images
(A) A typical representative raw image of a 2DE gel taken with the FLA-9000. The entire gel is present in the imaging window. The red outline box indicates the area selected for cropping. Due to interferences with image analysis, the cropped area contains everything but the protein marker (left) and dye front (bottom). The right and top margins were taken as close as possible to the pH 10 and top areas of the gel, respectively. (B) A representative cropped image. To ensure there is no discrepancies between cropped images the dimensions of the cropped area were as similar as possible between replicates.
Figure B-2. Shading images in Delta 2D
(A) Cropped mouse brain membrane 2DE FLA-9000 images were imported into Delta2D software. In the basic region of the gel (right) there is a degree of shading on the images. (B) Cropped mouse brain membrane with pH 10 molecular marker 2DE FLA-9000 images were imported into Delta2D software. In the basic region of the gel (right) there is a degree of shading for two of the replicate gel images, with shading reduced for one replicate (MBM_A_m_1).