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Toward the development of an analysis methodology for the elucidation of proteome
differentiation between two strains of *Saccharomyces cerevisiae*

Stephanie M. Prosa Carter

December 8, 2008

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in Clinical Chemistry

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Dedicated to my Husband, Daughter, Son, Mom, and Dad.

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Thank you to Dr. Kim's Proteomics Research Lab and to Dr. Evans for providing the strains.

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I also thank my Mom and Dad and my brothers and sister, Joey, Nicole, and Christopher and extended family for all of the love, support, and encouragement you've given me.

If it weren't for all of the support and encouragement that I've received from all of you, I would not have the confidence that I've acquired through the last two years.

Abbreviations

A

Asn (asparagine)

B

BSA (Bovine Serum Albumin)

BSE (Bovine Spongiform Encephalopathy)

C

°C (Degrees Celsius)

CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)

cm (centimeter)

C terminal (carboxyl terminal)

CV (Coefficient of Variation)

D

DTT (D,L-dithiothreitol)

E

EDTA (ethylenediaminetetraacetic acid)

ERF3 (Eukaryotic Release Factor 3)

G

Gln (glutamine)

g (gravity)

H

HCl (Hydrochloric Acid)

HSP104 (Heat Shock Protein 104)

Hsp70 (heat shock protein 70)

I

IEF (Isoelectric Focusing)

IPG (Immobilized pH Gradient)

K

kDa (kiloDalton)

L

λ (lambda)

M

M (molar)

M domain (Middle domain)

mA (milliamp)

mg (milligram)
mL (milliliter)
mM (millimolar)
M_r (molecular weight)
mRNA (messenger RNA)
MS (Mass Spectrometry)

N
nm (nanometer)
N terminal (amino terminal)

O
OD (optical density)

P
pI (Isoelectric point)
[PIN+] (Psi INducible)

S
S. cerevisiae (*Saccharomyces cerevisiae*)
SDS (sodium dodecyl sulfate)
SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

T
TE Buffer (tris-EDTA buffer)
TEMED (N,N,N',N'-tetramethylethylenediamine)
TCA (Trichloroacetic Acid)
TGS Buffer (Tris-Glycine-SDS Buffer)
2D (Two-dimensional)
2DE (Two-dimensional electrophoresis)
2D-PAGE (Two-dimensional polyacrylamide gel electrophoresis)

U
μA (microamp)
μg (microgram)
μL (microliter)

V
V-hr (volt hour)

W
W (watts)
w/v (weight by volume)

Y
YPD (yeast- extract/dextrose/peptone media)

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Equation 2:
$$\%T = \left[\frac{(\text{acrylamide}(g) + \text{bisacrylamide}(g))}{100mL} \right] * 100$$

Abstract

Through continued development, the two-dimensional PAGE gel technique developed by O'Farrell in 1975 has emerged as the hallmark technology utilized in virtually all protein expression analyses. The principle objective of this study was the development and validation of a proteome analysis methodology using this technique suitable for use in determining the quantifiable proteome differences between prion containing [PSI+] and wild type [psi-] strains of *Saccharomyces cerevisiae*. In addition to the development of an assay, which yields highly reproducible whole proteome isolates from both strains, was the elicitation of statistically significant proteome changes between the two strains. Thus, this study concludes that there is a quantifiable and reproducible effect on protein expression conveyed by the prion state in yeast, which warrants future studies to realize this implication within the context of epigenetics. Future studies will further benefit via the implementation of the methodology proven viable in this work in determining the specific nature of the observed proteome differential expression.

Introduction

Prions

According to the “protein-only” hypothesis, a prion is defined as an “infectious protein” that acts by heritable protein-based genetic elements that cause phenotypic changes without an underlying nucleic acid change (1). These phenotypic changes are passed on from parent to progeny. In order to be defined as a prion, the protein must encode itself, affect its own synthesis, or affect its own form or structure after synthesis (2). As described by Stanley Prusiner, “prions are unprecedented infectious pathogens that cause a group of invariably fatal neurodegenerative diseases mediated by an entirely novel mechanism. Prion diseases may present as genetic, infectious, or sporadic disorders, all of which involve modification of the prion protein (PrP), a constituent of normal mammalian cells” that are known to be the causal agents in numerous diseases including bovine spongiform encephalopathy (BSE) and amyloid disease in humans, such as Alzheimer’s, Creutzfeldt-Jakob disease, and Kuru (3,4). The prion structure propagates by a conformational change of the original protein creating an amyloid fold that aggregates as beta sheets which lends extreme stability to the substrates. In other organisms, such as certain fungi, it is proposed that prions serve as an evolutionary advantage through protein-based epigenetic inheritance (5).

[PSI+] and [psi-] S. cerevisiae

In 1965, Brian Cox discovered an unusual pattern of inheritance, [PSI+], in strains of *S. cerevisiae*, which was auxotrophic (nutrient dependent) for adenine, via the *ade2-1* gene, by a nonsense mutation (6). This *ade2-1* allele prevents growth on medium deficient in adenine and leads to the accumulation of pigmented metabolic intermediates. This pigmented color was red, while the strains containing the wild-type *ade2-1* allele were white. These white derivatives

contained a tRNA suppressor, SUQ5 (ochre suppressor- UAA). Cox was never able to identify the mutation responsible for the suppression, but proposed that the modification was due to a SUQ5 mediated cytoplasmic determinant (6). In 1987, Tuite et al. proposed that the protein determinant of [PSI+] is Sup35; a cytoplasmic protein encoded by a nuclear gene which functions as a component of the translation termination complex, eukaryotic release factor 3 (ERF3) (7). The prion form segregates to its progeny as a dominant gene during meiosis via a non-Mendelian fashion. When [PSI+] and [psi-] haploid strains fuse, the prion form converts proteins in the non-prion form through the cytoplasm. The inheritance of the [PSI+] state may be differentiated when grown on yeast- extract/dextrose/peptone (YPD) media (7). [PSI+] strains appear white to light pink, while [psi-] strains appear red as depicted in Figure 1.



Figure 1. Morphology of [PSI+] and [psi-] *S. cerevisiae* when grown on YPD media at 30°C. [PSI+] strains do not require adenine and have a white to light pink morphology (left), while the [psi-] strain require adenine and accumulate a red metabolite (right).

Later, in 1994, Reed Wickner presented evidence that [URE3] is the prion form of the ure2 protein in yeast that affects nitrogen catabolism (8). He simultaneously suggested that [PSI+] was a prion form of the Sup35 protein due to functional similarities between the two mechanisms of inheritance involving the self-propagation of alternate protein conformations (8). Sup35 is one of the two subunits of the translation termination factor, the other being Sup45. It

has been observed that the conformational change to the prion state may actually decrease protein activity and mimic loss-of-function mutations. The nonsense suppression of the [PSI+] phenotype results in a partial loss of Sup35 activity. The conversion from the active to the inactive form of Sup35 results in changes in Sup35 copy number and is the mechanism proposed by Wickner et. al. (7,8,9). The changes in Sup35 copy number implicate a protein-only model of inheritance for the prion. The amino-terminal (N) terminal region of the Sup35 protein is essential in causing the conversion and contains several imperfect oligopeptide repeats that are usually rich in Gln (glutamine) and Asn (asparagine) with few aliphatic amino acids (alanine and valine) that have been found to be essential in converting between the [PSI+] and [psi-] states (7, 8, 9). Refer to Figure 2 for a diagram of the Sup35 structural domain organization. According to the literature, an 8-9 amino acid repeat region comprised of Gln and Asn falls between amino acids 8 and 33 (9) with five complete and one partial copies of this particular repeat in the region (7). Thus, the removal of any of the repeats prevents the sustainment of the [PSI+] state, and as such, mutations of Gln and Asn residues and mutations of certain oligopeptide repeats do not propagate as the prion form (1).

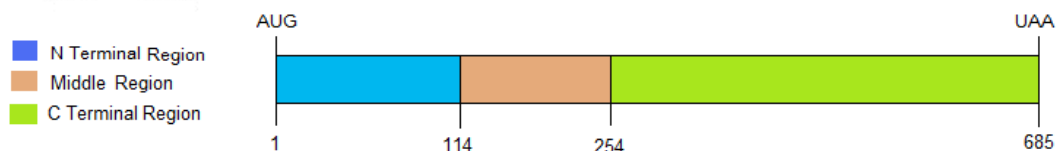


Figure 2. Structural domain of Sup35 protein organization where the numbers represent amino acid numbers: Depending on the strain, literature cites that the N terminal region consists of amino acids 1 to 114 or 1 to 123 and the C terminal region 254-685. AUG represents the start codon and UAA a stop codon. The N terminal region is essential for the conversion of [psi-] to [PSI+] (10).

Once the [PSI+] element is induced, the over-expression of Sup35 is no longer needed and is self-perpetuating. Normal, or active Sup35 protein is required for translation termination at stop

codons in messenger RNA (mRNA). Under normal circumstances, protein synthesis termination is signaled by one of the termination codons: UAA (ochre), UAG (amber), or UGA (opal) at the ribosomal A site (11). The prion form, or the inactive form, causes nonsense suppression and stop codon read-through. Sup35 has two binding sites for Sup45: one in the carboxyl-terminal (C) terminal domain and the other in the N terminal prion domain containing part of the M (middle) domain. The N and M domains allow Sup35 to acquire a stable prion conformation and allow the conversion between [PSI+] and [psi-] states. The C terminal region is responsible for the translation-termination activity and is essential for viability (12). When True and Lindquist removed the N and M regions of Sup35 in [psi-], there was no apparent effect. When N and M regions were deleted in [PSI+] cells, the prion was lost and “translational fidelity” was restored (12). Ter-Avanesyan et. al. (10,13) performed cytoduction experiments (“abortive matings that mix cytoplasmic factors without nuclear fusion and produce progeny with haploid parental genotypes (10)”) to study the effects of the C terminal region. The [PSI+] prion was transferred to strains in which Sup35p C region replaced the wild-type Sup35p. Fragments of Sup35p were expressed via a plasmid so that [PSI+] could propagate. [Psi-] strains were able to propagate [PSI+] without the presence of full length Sup35 and as a result, the C terminal region was not determined to be necessary for [PSI+] propagation (10). There has also been disagreement over the presence of Sup45 in Sup35 aggregates. It has been observed that Sup45 was present only in aggregates containing Sup35 containing binding sites for Sup45. One explanation, proposed by Wickner et. al (14), is that the component of the suppression by the prion is the sequestering of Sup45. Another group found no difference in sedimentation properties of Sup45 in prion containing strains. A third study concluded that Upf1 (regulator of nonsense transcripts), a

component of the nonsense mediated mRNA decay pathway, is present in [PSI⁺] aggregates (14).

In addition, the chaperone protein, Heat shock protein 104 (HSP104), is required for the induction of certain yeast prions, including [PSI⁺] by facilitating the folding of proteins (15). HSP104 is a member of the ClpB/Hsp100 family of molecular chaperones whose function is stress tolerance (16). HSP104 resolves thermally denaturing proteins and when overexpressed, can cure [PSI⁺] forms. Additionally, when HSP104 is not present, [PSI⁺] forms are cured. Therefore, [PSI⁺] propagation requires an intermediate level of HSP104. There are two models that support this conclusion as proposed by Uptain and Lindquist in 2000 (1). First, Sup35 conversion may proceed via an unstable state created by HSP104. The overexpression of Sup35 induces [PSI⁺] formation when it interacts with HSP104. When HSP104 is highly overexpressed, Sup35 prion disaggregates and the prion is cured. The second model suggests that HSP104 disaggregates Sup35 prion aggregates causing less aggregation to be passed on to daughter cells. Highly overexpressed HSP104 cures [PSI⁺] when all Sup35 prion aggregates are resolved. In contrast to the first model, the second model does not require HSP104 to convert Sup35 to the prion state. The overproduction of Ssalp, a member of the HSP70 family, partially blocks the curing of [PSI⁺] via the overproduction of HSP104 (14). Hsp70 is a universally conserved essential protein that acts in many cellular processes where proteins are incompletely folded, such as translation and membrane transport (17). The characterization of HSP70 by Jones et. al. (17) suggests that interactions of HSP70 with co-chaperones reveal that altered HSP70 function antagonizes amyloid propagation and may provide an explanation for effects seen in some earlier overexpression studies discussed here.

Although its function is unknown, the [PIN+] (Psi INducible) prion: the misfolded form of the Rnq1 protein (rich in asparagines and glutamine residues) is present in the [PSI+] form and induces [PSI+] when Sup35 is overexpressed (18). The prion model for [PIN+] is supported by the finding that the deletion of HSP104 leads to the loss of the [PIN+] prion. In contrast to [PSI+], the [PIN+] prion is not dependent on the N terminal region of Sup35, but may be dependent on the C terminal region, the region essential for growth, thus increasing the difficulty in studying this hypothesis in the C terminal region (10). The [PSI+]/[PIN+] relationship is the first evidence that the presence of one prion influences the appearance of another and provides further evidence that HSP104 is a necessary component of the prion mechanism; recall the previous discussion that discussed the role of HSP104 in the [PSI+] determinant. The true nature of this [PIN+] in [PSI+] mechanism is still under speculation (10).

Changes in genetic or epigenetic regulators, such as the prion models discussed previously, will affect the mechanisms of proper translation termination at stop codons and ultimately protein synthesis. If the frequency that ribosomes read through stop codons is great enough, the phenotype of the cell may be altered. Changes of this nature (proteome) may be quantitated by analyzing the entire proteome of both strains ([PSI+] and [psi-]) using a method such as two-dimensional gel electrophoresis.

Theory of the 2D Electrophoresis Method

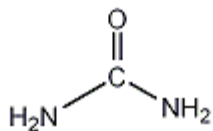
“Proteome analysis is a direct measurement of proteins in terms of their presence and relative abundance (19,20)”. Studying proteins entails separating all the proteins of an organism, called the proteome, and identifying them. As previously discussed, the “protein-only” hypothesis states that a prion acts by heritable protein-based genetic elements that cause phenotypic changes without an underlying nucleic acid change (1) suggesting that there may be

differential protein expression between [PSI+] and [psi-] strains. *S. cerevisiae* contain 5,773 (21) or 5,726 (22) protein coding genes.

In order to test this hypothesis, two-dimensional (2D) electrophoresis developed by O'Farrell in 1975 (23), may be a useful technique in comparing the differential proteome expression, if any, in [PSI+] and [psi-] *S. cerevisiae*. After sample preparation, proteins are separated according to their isoelectric point via isoelectric focusing in the first dimension, and according to molecular weight via sodium dodecyl sulfate (SDS) electrophoresis in the second dimension (23).

Sample Preparation

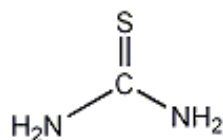
Sample preparation and quality are directly associated with the outcome of 2D electrophoresis results. The entire proteome must be adequately represented on a protein map, and as a result, the modification and/or loss of proteins must be minimal. The sample preparation methodology is sample dependent and should result in “complete solubilisation, disaggregation, denaturation, and reduction of the proteins in the sample (24)”, as well as free of other disturbing agents, such as salts, ionizing agents, detergents, etc. that may alter the native state of a protein (19). Optimal protein solubilisation is sample dependent and is achieved with the use of chaotropic agents, detergents, reducing agents, buffers, and ampholytes. The most common chaotropic agents are urea and thiourea, which disrupt hydrogen bonding, and thus, the protein's three-dimensional structure disrupting aggregation. Most commonly, a mixture of urea and thiourea (2M thiourea and 5-8M urea) are used (19).



urea

Chemical Formula: $\text{CH}_4\text{N}_2\text{O}$
Molecular Weight: 60.06

Figure 3. Urea ($\text{CH}_4\text{N}_2\text{O}$)



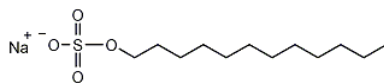
thiourea

Chemical Formula: $\text{CH}_4\text{N}_2\text{S}$
Exact Mass: 76.01

Figure 4. Thiourea ($\text{CH}_4\text{N}_2\text{S}$)

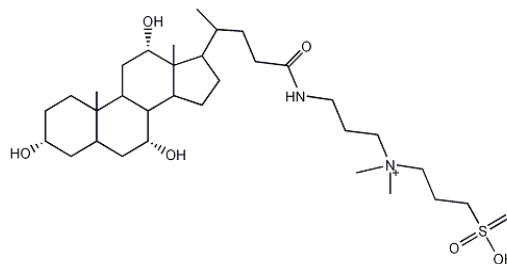
Detergents must be non-ionic so that proteins maintain their native charge and act by disrupting hydrophobic interactions, and as such, increasing the solubility of proteins according to their pI.

The most common detergents are sodium dodecyl sulfate (SDS) and CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), however, SDS is known to affect protein migration to its pI (it affects the protein's charge), thus concentrations of 1-2% of CHAPS (octylglucoside) are usually recommended (19).



sodium dodecyl sulfate
Chemical Formula: $\text{C}_{12}\text{H}_{25}\text{NaO}_4\text{S}$
Exact Mass: 288.14

Figure 5. Sodium dodecyl sulfate
($\text{C}_{12}\text{H}_{25}\text{NaO}_4\text{S}$)



Chemical Formula: $\text{C}_{32}\text{H}_{59}\text{N}_2\text{O}_7\text{S}^+$
Exact Mass: 615.4

Figure 6. CHAPS ($\text{C}_{32}\text{H}_{59}\text{N}_2\text{O}_7\text{S}^+$)

It is crucial that any solubilisation agents used be compatible with Isoelectric Focusing; i.e.: should not increase the solution's ionization strength, which would produce high currents during focusing and/or alter a protein's native state (19). It is imperative to note that when comparing two distinct protein mixtures, that optimal solubilisation may vary from sample to sample, and as

such, should be established concurrently. The metric of successful sample preparation will be the verified mass of protein extract via a modified Bradford Assay.

The sample preparation of *S. cerevisiae* poses great difficulty; yeast is surrounded by a thick, tough, and rigid cell wall, which makes protein extraction difficult. One protein extraction technique, enzymatic digestion, has been focused on known proteins and not complex protein mixtures. In addition, enzymatic digestion is not recommended for large sample volumes and is expensive. It also requires a second step: lysing spheroplasts (a cell from which the cell wall has been almost completely removed) that are formed during digestion (19). A second technique, by sonication, is a successful method of cell disruption; however, it can only be used on a single sample at a time and may leave some yeast cells intact due to the cells rigidity. The sonication method is the preferred method for lysing less rigid cellular organisms, such as bacterial cells (19). A more popular method of lysing yeast are with the use of glass beads, however, the use of glass beads can lead to the loss of protein by sticking to the beads during disruption (19). Cellular lysis with glass beads lends to be more convenient and is the least expensive methodology that will be employed during this study.

Once the cellular lysate is obtained, it may be necessary to perform a precipitation assay to remove any disturbing agents, so that a native protein sample may be obtained. Generally, a sample's proteins are precipitated and concentrated, while impurities, or disturbing agents are removed with the supernatant (19). Although there are various precipitation methodologies, including chloroform/methanol, ammonium sulfate, Trichloroacetic Acid (TCA), and acetone precipitations, with the most frequently used precipitation method being TCA followed by acetone washes, Lei Jiang, et. al. (25), have concluded that sample precipitation via TCA and acetone, followed by ultracentrifugation yielded the most optimal results. The mechanism in

which TCA works is still under speculation. It is proposed by Sivaraman, et al (26), that an acid-induced hydrophobic aggregation of proteins occurs, which is instigated by the three chloro groups on the molecule (represented in Figure 7) (26).

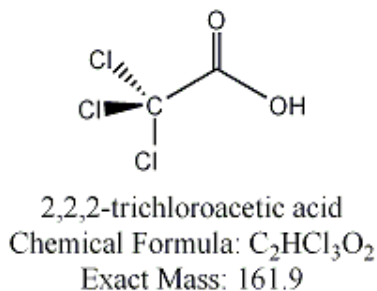


Figure 7. Trichloroacetic acid ($C_2HCl_3O_2$)

This work will comprise of a TCA precipitation followed by acetone washes and sonication to resolubilise the protein pellet in an IEF compatible buffer that can be introduced to isoelectric focusing (25).

Lastly, and most importantly, it is imperative that the sample preparation methodology is reproducible from sample to sample. To avoid variability in results, it is crucial that consistent sample processing conditions are maintained (27). The general schema is depicted in Figure 8 below.

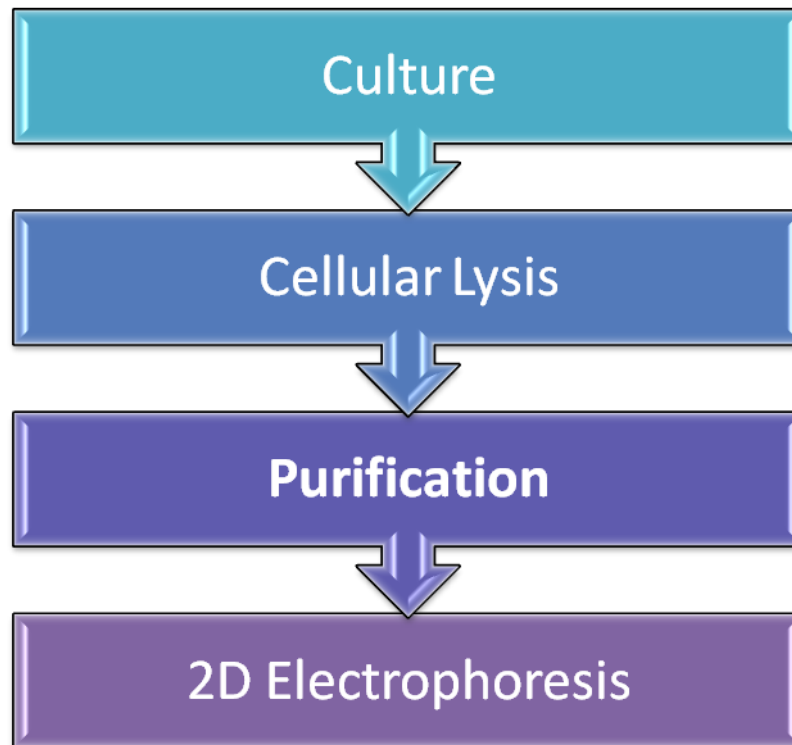


Figure 8. The general schema of sample preparation for Two-Dimensional Electrophoresis

First Dimension: Isoelectric Focusing (IEF)

The isoelectric point (pI) of a protein is the pH in which the protein will not migrate in an electrical field, and thus reaches equilibrium. Since proteins are amphoteric molecules, they carry a positive, negative, or neutral charge, that is dependent on the pH of the surrounding environment; i.e.: the ability to protonate or deprotonate functional groups present within the molecules side chains (19). The pH in which the net charge is zero is the protein's pI. The pI of a protein varies, with the majority between pH 4 and 7 (19). Isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01 (28). Figure 9 below illustrates proteins resolved to their pI on a pH 3-10 IPG (Immobilized pH gradient) gel before and after isoelectric focusing.

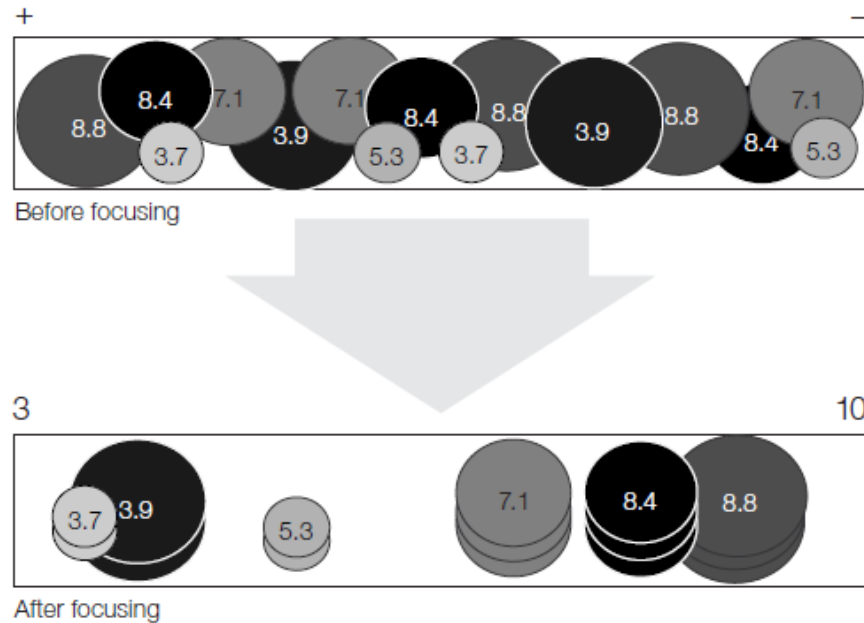


Figure 9. Proteins resolved on a pH 3-10 IPG strip to their isoelectric point (19)

The preferable method of performing the first dimension is via Immobilized pH gradients (IPGs)

because the technique offers a variety of benefits including:

- increased resolution
- stability of the pH gradient
- variation of the pH interval used
- increased loading ability
- high reproducibility
- commercially available
- reveals minimal distortion by salts present in the sample
- control of the pH, buffering capacity, and ionic strength
- easy separation of the sample by IEF (24)

Proteins are introduced into an immobilized pH gradient (IPG) gel that is created with derivatives of acrylamide containing reactive double bonds and buffering groups (acrylamido buffers) that are covalently bonded into polyacrylamide gels to form the desired pH gradient.

The general structure of the acrylamido buffer is $\text{CH}_2=\text{CH}-\text{CO}-\text{NH}-\text{R}$. Typically, R is either a carboxyl group $[-\text{COOH}]$ or a tertiary amino group, for example $-\text{N}(\text{CH}_3)_2$ (29). IPG strips are difficult to make and are conveniently available commercially. If using a commercially available

IPG strip (distributed dehydrated), the sample is allowed to rehydrate the strip to its original thickness before introducing it to IEF. Two methods for rehydrating IPG strips are active rehydration and passive rehydration. Active rehydration applies a low voltage current during rehydration, usually 50milliamps (mA) to allow proteins to enter the gel matrix via two mechanisms; absorption and current. The advantage is that larger proteins enter the strip via the electrical “pull”, with the disadvantage being that smaller proteins have a higher risk of being lost from the strip. With passive rehydration, proteins enter the gel matrix by absorption only (19). After rehydration, the IPG strip can then be isoelectrically focused via application of either a constant or stepping current according to the IPG methodology and separated by its isoelectric point as discussed previously (28). Once equilibration has been reached, i.e.: all of the proteins have reached their pI and are immobile in the pH gradient, the second dimension may be executed.

Second Dimension: Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels separate proteins according to their electrophoretic mobility, which is defined as a “function of length of a polypeptide chain or molecular weight as well as higher order protein folding, posttranslational modifications, and additional factors (30)”. Before IPG strips can be separated via SDS-PAGE, strips need to be equilibrated in a buffer containing SDS and a reducing agent to ensure that protein charge, mass, and shape are uniform. The first equilibration buffer, containing DTT (DL-Dithiothreitol) reduces sulfhydryl groups. The second equilibration buffer, containing Iodoacetamide alkylates sulfhydryl groups (19,30). The mechanism is illustrated in Figure 12.

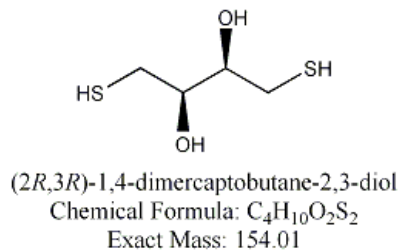


Figure 10. DTT (C₄H₁₀O₂S₂)



Figure 11. Iodoacetamide (C₂H₄INO)

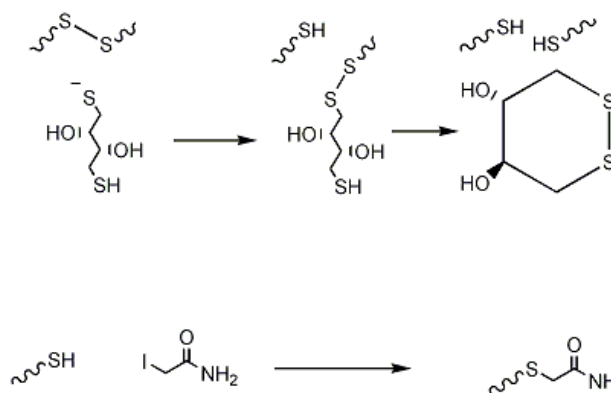


Figure 12. Mechanism of reduction (top) and alkylation (bottom) of disulfide bonds via action of DTT and Iodoacetamide respectively.

The equilibrated IPG strips can be transferred to an SDS-PAGE gel and overlaid with agarose containing a tracking dye; Bromphenol Blue. The SDS gel electrophoresis of samples ultimately leads to separation of samples by molecular weight (M_r); dodecyl sulfate coats proteins essentially in proportion to their mass. SDS denatures secondary and non-disulfide-linked tertiary structures and applies a negative charge, which is proportionate to the protein's mass. The ratio SDS binds varies from 1.1 – 2.2 g SDS/g protein (19) and generally conveys a uniform charge to all protein proportionate to their mass. A critical factor in the separation of proteins in this dimension is pore size, with the effective pore size of the gel being inversely related to the ratio of acrylamide (Figure 13) and bis crosslinker (Figure 14) monomers (31).

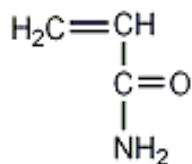


Figure 13. Acrylamide (C₃H₅NO)

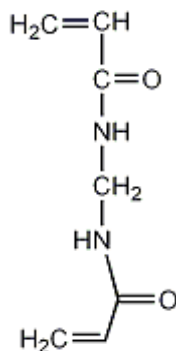


Figure 14. N,N'-Methylenebisacrylamide (C₇H₁₀N₂O₂)

There are two important proportionalities present in the gel system, which ultimately determine pore size, defined as %T (the acrylamide monomer concentration) and %C (the ratio of the crosslinker: acrylamide ratio) and are resolved as follows (31):

$$\text{Equation 1: } \%T = \left[\frac{(\text{acrylamide}(g) + \text{bisacrylamide}(g))}{100mL} \right] * 100$$

$$\text{Equation 2: } \%C = \left[\frac{\text{bisacrylamide}(g)}{(\text{acrylamide}(g) + \text{bisacrylamide}(g))} \right] * 100$$

In general, the increase in pore size (decrease in %T) will allow proteins to travel faster through the matrix proportionate to their mass, with the subsequent decrease in pore size being more restrictive and decrease the progression of proteins through the matrix (dependant on size) (31).

Refer to Table 1.

Table 1. Acrylamide and Crosslinker effective resolution with respect to peptide molecular weight (31).

Acrylamide concentration		<i>M</i> , range of sample polypeptides
%T	%C	
Uniform acrylamide concentration gels		
5	2.6	25,000 to 300,000
10	2.6	15,000 to 100,000
10	3.0 ^a	1,000 to 100,000
15	2.6	12,000 to 50,000

The gel matrix relies exclusively on two critical compounds to form cross-linked acrylamide polymers. The generation of the co-polymer matrix is resultant of a vinyl polymerization system instantiated by the spontaneous generation of free radicals (32). Free radicals in the PAGE gel system are created via rapid degradation of Ammonium Persulfate (Figure 15), a process that is accelerated by TEMED (Figure 16). These persulfate free radicals react with acrylamide monomers to free radical forms, which then spontaneously reassemble into polymer chains of the molecule. These polymers are randomly cross-linked via bis-acrylamide radicals, forming the gel matrix (Figure 17). Additionally, SDS is added to the gel as well as a buffered pH solution to adjust the resolving gel's pH to approximately 8.8 (31).

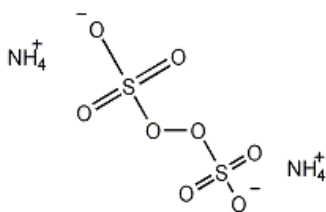


Figure 15. Ammonium Peroxydisulfate
(NH₄)₂S₂O₈

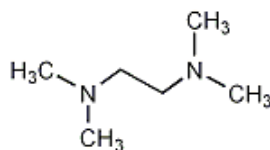


Figure 16. *N*¹,*N*¹,*N*²,*N*²-tetramethylethane-
1,2-diamine (C₆H₁₆N₂)

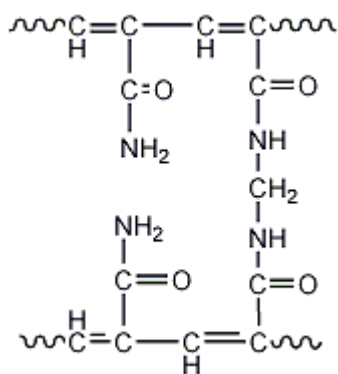


Figure 17. PAGE gel polymer excerpt exhibiting bis-mediated crosslinking

When performed in conjunction with IEF, the SDS gel is carried out perpendicularly to the first dimension and is stained so that proteins become visible (19, 30). Figure 18 below

illustrates the equilibration and SDS-PAGE separation of proteins by M_r in relation to the IEF run.

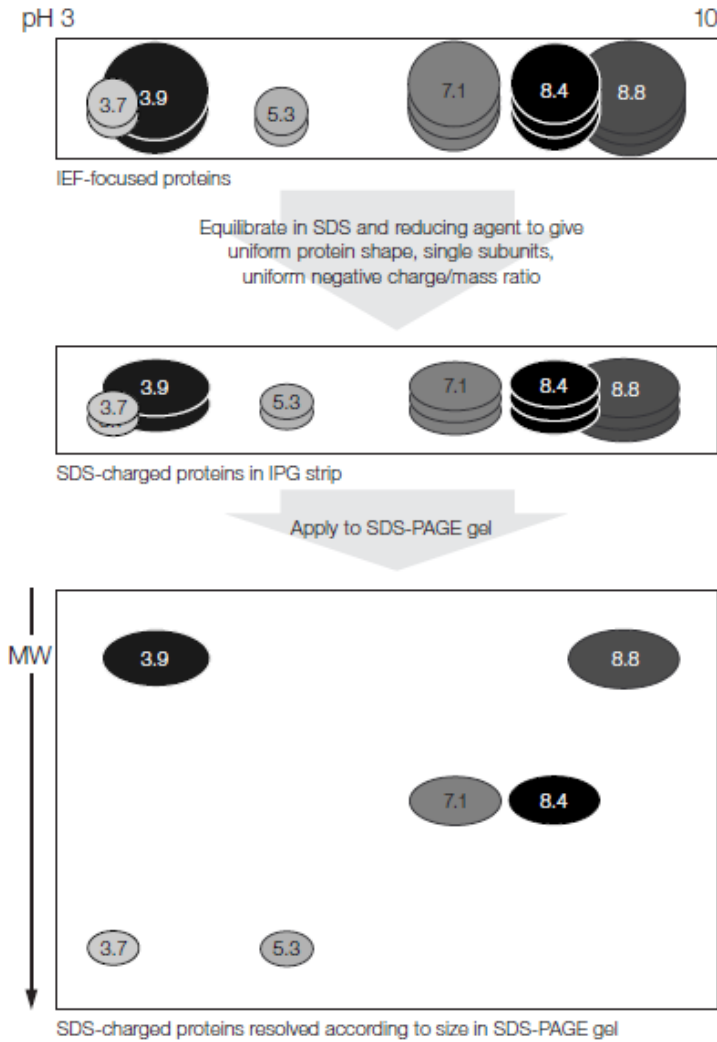


Figure 18. Flow diagram depicting IEF focused proteins, equilibration, and SDS-PAGE separation perpendicularly to IEF (19).

After separation via SDS-PAGE, the two-dimensional separation must be stained for protein spot visualization. Although this work will not be going into the details of specific staining methodologies, the more common stains used are Coomassie Brilliant Blue R-250, Silver Staining, and SYPRO Ruby Red. While other staining methods exist, which may yield more quantifiable results; they are not readily compatible with mass spectrometry and as a result

are not considered viable alternatives for this study in the event an MS analysis is performed (19). Coomassie Brilliant Blue R-250 is the most common staining method and is the least expensive method. Coomassie R-250 requires a protein density of $5\mu\text{g}/\text{mm}^2$ detection (19,33). Silver stain contains silver nitrate that binds to a protein's cysteine residues for protein detection. A second fixing step, with an acetic acid and alcohol solution, is required. The color intensity can be quantitated to the amount of protein present. Silver staining can be up to 100 times more sensitive to protein detection in comparison to Coomassie Brilliant Blue R-250. Silver staining can detect spots containing $50\text{ng}/\text{mm}^2$ of protein per spot (19,33), however, the technique is more time consuming than Coomassie Brilliant Blue R-250 and is typically more sensitive than most laboratories need. Lastly, SYPRO Ruby Red is a fluorescent stain sensitive to $25\text{ng}/\text{mm}^2$ of protein that is easily visualized with ultraviolet light at 635nm (19, 33). In concordance with silver staining, SYPRO Ruby Red stain is far more sensitive than needed in most qualitative experiments, as well as expensive (19). Each of these staining methods creates a two-dimensional map, where each spot characterizes an individual set of pI/Mr coordinates (19). These protein maps are powerful tools in proteome analysis since protein spots of interest may be excised from the 2D gel, digested into fragments by specific proteases, and identified using mass spectrometry (MS) (34).

Reproducibility of Results

Since 2D electrophoresis is a powerful methodology in studying complex protein mixtures, it is reasonable to assume that sample-to-sample variation, as well as naturally occurring variation between samples, i.e.: mutations, posttranslational modifications, etc. can affect the reproducibility of 2D gels, as well as technical and experimental variations.

Qualitative changes refer to the appearance or disappearance of spots on a gel with the number of

spots represented on a gel varying due to even slight technical and experimental deviations, however discouraged, between gels (35). Quantitative changes refer to intensity of spots and are affected by similar technical and experimental variation, as well as limitations of staining and imaging techniques (35). Challapalli et. al. (36) investigated the quantitative reproducibility of 2D-PAGE and proposed using triplicate gels to create an “average gel” via computerized software packages to yield higher correlation coefficients. In addition, Choe et. al. (35) observed that when maintaining consistent sample preparation, sample load, and image analysis between a single sample run on multiple gels, 95% of spots present on three replicate gels exhibit less than a 0.52 coefficient of variation (standard deviation/mean) in stain intensity (% volume). When increasing the number of replicate gels to four, the coefficient of variation increased to 0.57. This result is consistent with one of the major drawbacks to this analysis type and is directly attributable to the presence or absence of a data point in the individual gel data set. The absence of a spot on one gel significantly increases the associated variability in the data set, with the likelihood of this occurrence increasing as the sample size increases (35,36). Thus, this effect is the principal motivator for running triplicate gel runs of each strain for the analysis and is consistent with established practices.

Statement of Intent

The principle objective of this study is two-fold, with the first being to demonstrate a protein extracting procedure for [PSI+] and [psi-] *S. cerevisiae* that meets the expectations of reliable and reproducible sample preparation methodology as discussed previously. Secondly, to determine if previous studies (previously reviewed) can be correlated with differential protein expression, if any, between the two strains, via the use of two-dimensional electrophoresis. Triplicate gels of both [PSI+] and [psi-] *S. cerevisiae* will be used to create “master” gels of each

strain with the use of the PDQuest software package to undergo a comparative analysis in an effort to describe the effectivity of the protocol used as well as to quantitate differences in the proteome (if any).

Materials and Methods

Chemicals, reagents, and media used in this study were obtained from Ambion, Amresco Inc, Biorad Inc, Fischer Scientific, Sigma Chemical Company, and VWR International Inc.

Cell Cultures

The strains used in this study were L1751 *Mata ade1-14 leu2-3,112 ura3-52 trp1-289 his3-200 [psi-]* and L1763 *Mata ade1-14 leu2-3,112 ura3-52 trp1-289 hs3-200 [PSI+]* and were obtained from Dr. Susan Liebman at the University of Illinois at Chicago. The protocols for “GCAT growing yeast” were followed: The strains were streaked on YPD agar media and incubated at 30±1°C until the colonies grew to be approximately 1.5mm in diameter. Using the aseptic technique, single isolate colonies were inoculated in side arm flasks containing between 50 to 100mL YPD broth and incubated in a shaking incubator at 30±1°C until the cell concentration was approximately 1x10⁷ cells/mL, which was verified using both a direct hemocytometer count of each culture and by reaching an optical density (OD) between 0.80 and 0.95 at 595nm in a Genesys 10vis spectrophotometer.

Sample Preparation

In order to determine the most appropriate (with specific respect to protein yield and purity) sample preparation technique, two distinct sample preparation procedures were attempted. After sample preparation, the isolates were either used immediately for a TCA precipitation, Modified Bradford Assay, or were frozen at -80°C until needed.

Lysis Buffer + 10% SDS

The cell cultures were transferred to 50mL centrifuge tubes and were centrifuged for two minutes at 12,000x g (Sorvall Instruments GLC-4 General Laboratory Centrifuge). After removing the supernatant from the pellet, 480µL Lysis Buffer and 48µL 10% SDS was added to the pellet. The mixture was gently vortexed for 5 seconds and was transferred to a prepared 1.5mL screw cap tube with ice-cold zirconia beads (height ~2.5 cm). The screw cap tube was vortexed for ten minutes and was proceeded by centrifugation at 13.2g and 4°C for 60 minutes (eppendorf Centrifuge 5415R).

Sterile TE Buffer

The cell cultures were transferred to 50mL centrifuge tubes and were centrifuged for two minutes at 12,000x g (Sorvall Instruments GLC-4 General Laboratory Centrifuge). After removing the supernatant from the pellet, 1000µL¹ sterile TE Buffer (10mM Tris, 1mM EDTA, pH 8.0) was added to the pellet. The mixture was gently vortexed for 5 seconds and was transferred to a prepared 1.5mL screw cap tube with ice-cold zirconia beads (height ~2.5 cm). The screw cap tube was vortexed for twenty minutes and was proceeded by centrifugation at 13.2 g and 4°C for 60 minutes (eppendorf Centrifuge 5415R).

TCA Precipitation

Volume of supernatant was determined and 100% Trichloroacetic acid was added to yield a final TCA concentration of 10%. The sample was incubated at -20°C for 10 minutes and then centrifuged at 13.2g (eppendorf Centrifuge 5415R) for 5 minutes and 4°C. The supernatant was discarded. Two acetone washes were performed: For the first wash, 500µL 90% acetone was

¹ A 600µl aliquot was attempted and subsequently abandoned due to low protein concentration yield.

added to the pellet. The acetone/pellet mixture was incubated at room temperature for 5 minutes, and then centrifuged at 13.2g for 5 minutes and 19°C; discarding the supernatant. An additional acetone wash of 800µL 90% acetone was added to the pellet with the acetone/pellet mixture being incubated at room temperature for 5 minutes, and then centrifuged at 13.2g for 5 minutes and 19°C. The supernatant was discarded and the length of the remaining pellet was measured in order to calibrate the volume of Rehydration Buffer needed. 30µL of 0.2M NaOH was added to the pellet; the mixture was gently vortexed for 3 seconds and incubated at room temperature for 2 minutes. 20µL of Bio-Lyte 3/10 Ampholytes was added to 1000µL Rehydration Buffer (8M urea, 2% CHAPS, 50mM DTT, 0.001% Bromphenol Blue).² 300 to 600µL of the Rehydration Buffer/Ampholyte mixture was added to the pellet and incubated at room temperature for 10 minutes. The pellet/Rehydration Buffer/Ampholyte mixture was sonicated gently at 8W for 10 seconds, followed by a 30 second rest period on ice, and was then centrifuged at 13.2g for 5 minutes and 19°C; with the supernatant being collected as the protein extract.

Modified Bradford Assay

Bovine Serum Albumin (BSA) standards were prepared at specific concentration intervals via addition of 5.0mg/mL stock solution to 10λ 0.1M HCl to attain final concentrations of [0.0 (blank), 5, 10, 15, 20, 25, 30, 35, and 40µg/mL]. The standards were allowed to incubate at room temperature for one to two minutes before 80λ of distilled water was added. After a five-minute incubation period at room temperature, 4mL Bradford dye was added to each tube and was allowed to incubate for five minutes at room temperature. The B tube was prepared similar to the standard tubes, without the addition of BSA stock solution. In addition to the standards, two test tubes were prepared for each sample by adding 10λ 0.1M HCl and the lysate

² 300µL was added to pellets <4mm in length, 600µL for pellets >4mm in length

to the tubes. In one test tube, 5 λ of the lysate was added; the other tube contained 10 λ . This procedure was repeated with remaining samples and was allowed to incubate at room temperature for one to two minutes. To these sample tubes, 80 λ of Milli-Q water was added and the tubes were allowed to incubate for five minutes at room temperature before adding 4mL of the Bradford Dye. In order to measure the absorbance of each of the standards and the samples, they were transferred to cuvettes. The Genesys 10vis Spectrophotometer was set to 595nm to determine the absorbance for each standard and sample. The absorbencies of the standards were used to create a Bradford Standard Absorbance Curve with a desired minimum R² value of at least 0.98³. Linear regression analysis was then used to estimate the protein concentration of the unknown concentration of the samples.

2D Electrophoresis

Isoelectric focusing was performed using the BioRad Protean IEF Cell and Bio-Rad Ready-Strip IPG strips (7cm and 17cm). The SDS-PAGE was performed using either the Biorad Mini-Protean 3 cell and the Biorad Power Pac 300 power supply (7cm) or the Biorad Protean II XL and the Thermo electron Corporation EC570-90 power supply (17cm). 7cm strips were used to determine proof of principle, while 17cm strips were used to obtain increased resolution for triplicate analyses.

First Dimension: Rehydration and IEF

7cm IPG strips

For each sample, the amount of the protein extract needed for a 100 μ g protein load was calculated. The total rehydration volume should be 125 μ L, so the amount of protein extract plus 5.0 μ L was subtracted and the resulting number was subtracted from the total volume. The resulting number is the amount of Rehydration Buffer needed. The appropriate amounts of

³One Proof of Principle Bradford Curve yielded an R² value of 0.91.

protein sample, Rehydration Buffer (8M urea, 2% CHAPS, 50mM DTT, 0.001% Bromphenol Blue), and 5.0 μ L of Bio-Lyte 3/10 Ampholytes were combined and added between the electrodes of the rehydration/equilibrium tray with the IPG strips gel side down onto the sample and overlaid with mineral oil. The sample was allowed to rehydrate either by active rehydration or passive rehydration overnight after which the Biorad PROTEAN IEF was programmed with the following conditions: a starting voltage of 0V with an ending voltage of 4,000V, 20,000 V-hr, with a focusing temperature of 20°C, and a maximum current of 50 μ A/IPG strip. After electrophoresis, the strips were removed from the focusing tray and transferred to a new rehydration/equilibrium tray. Prior to running the second dimension, the IPG strips were equilibrated in an SDS-containing buffer. 2.5mL SDS-PAGE Equilibrium Buffer I with DTT (6M urea, 0.375M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT) was added to the equilibrium/rehydration tray with the blotted IPG strips gel side up and was placed on a slow speed orbital shaker for ten minutes. After the incubation period, the buffer was decanted and 2.5mL SDS-PAGE Equilibrium Buffer II with Iodoacetamide (6M urea, 0.375M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) Iodoacetamide) was added to the IPG strip and was returned to the tray on the orbital shaker for ten minutes. Following incubation and decanting of excess buffer, the strips were ready to implement separation via the second dimension.

17cm IPG strips

For each sample, the amount of the protein extract needed for either a 250 μ g or 300 μ g protein load was calculated. The total rehydration volume should be 300 μ L, so the amount of protein extract plus 13.5 μ L was subtracted and the resulting number was subtracted from the total volume. The resulting number is the amount of Rehydration Buffer needed. The appropriate amounts of protein sample, Rehydration Buffer (8M urea, 2% CHAPS, 50mM DTT),

and 13.5 μ L of Bio-Lyte 3/10 Ampholytes were combined and added to either an IEF tray (if performing active rehydration) or a rehydration tray (if performing passive rehydration) with the IPG strips gel side down onto the sample and overlaid with mineral oil. Active rehydration of the sample proceeded at 20°C, with a maximum current of 50 μ A/IPG strip for at least 12 hours, proceeded with the following PROTEAN IEF conditions: a starting voltage of 0V with an ending voltage of 10,000V, 60,000 V-hr, with a focusing temperature of 20°C, and a maximum current of 50 μ A/IPG strip. If the sample was rehydrated passively, the sample was allowed to rehydrate (passive) at least 12 hours and no more than 19 hours on a slow speed orbital shaker in the rehydration tray. The passively rehydrated IPG strips were transferred between the wick-containing electrodes of the IEF tray with the IPG strips gel side down onto the sample and overlaid with mineral oil with the following parameters programmed for the IEF run: starting voltage of 0V with an ending voltage of 10,000V, 60,000 V-hr, with a focusing temperature of 20°C, and a maximum current of 50 μ A/IPG strip. After electrophoresis, the strips were removed from the focusing tray and transferred to a new rehydration/equilibrium tray. Prior to running the second dimension, the IPG strips were equilibrated in an SDS-containing buffer. 6mL SDS-PAGE Equilibrium Buffer I with DTT (6M urea, 0.375M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT) was added to the equilibrium/rehydration tray with the blotted IPG strips gel side up and was placed on a slow speed orbital shaker for ten minutes. After the incubation period, the buffer was decanted and 6mL SDS-PAGE Equilibrium Buffer II with Iodoacetamide (6M urea, 0.375M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) Iodoacetamide) was added to the IPG strip and was returned to the tray on the orbital shaker for ten minutes. Following incubation and decanting of excess buffer, the strips were ready to implement separation via the second dimension.

Second Dimension: SDS-PAGE

An overlay of bromophenol blue in agarose gel (0.5% low melt agarose in 1x Tris-Glycine-SDS (TGS) Buffer and 0.001% bromophenol blue) was prepared and the 10% or 12%⁴ SDS gel⁵ was placed in an AnyGel stand. The IPG strips were removed from the rehydration/equilibrium tray and dipped into 1x TGS Buffer (25mM Tris, 192mM glycine, 0.1% SDS) running buffer. The strip was placed onto the back plate of the SDS-PAGE gel above the IPG well and the overlay agarose was added so that the progress of the electrophoresis could be monitored. After polymerization, the gels were mounted into the electrophoresis cell and were placed into the reservoirs filled with cooled 1x TGS running buffer. For the 7cm IPG strips, the gels were run at a 200V constant (235mA). The 17cm IPG strips were run at 10 or 16mA/gel, 500V maximum for 30 minutes. After 30 minutes, the parameters were increased to 24, 26, or 28mA/gel and a 500V (maximum) until the dye front reached completion.

Coomassie Staining/Destaining

Gels were removed from plates and were incubated overnight on a slow speed orbital shaker in Coomassie stain (0.1% Coomassie Brilliant Blue R-250, 45% methanol, 10% acetic acid). Coomassie stain was decanted off and the gel was immersed in a large volume of high destain/fixing solution (50% 18 Milli-Q water, 40% methanol, 10% acetic acid) and Kim Wipes for one hour. The high destain was decanted off and low destain (84% 18 Milli-Q water, 10% methanol, 6% acetic acid) was added. Gels were destained until the background was visibly clear and were placed in 18 Milli-Q water. After 15 minutes, gels were imaged using the HP Scan Jet 5370C and saved (Dell Dimension E520 and removable flash drive).

⁴ Data not shown.

⁵ Gels were cast no more than 5 days prior and stored immersed in 0.5x TGS buffer at 4°C.

PDQuest Analysis

The scanned images (in triplicate), for the [psi-] strain, were imported into the PDQuest software to automatically select an image to be used for a master gel. The automated detection and matching tools were selected in order to select the faintest spot and the largest spot cluster. Smoothing parameters were increased to a 5x5 kernel size. Next, landmark spots were chosen and the default parameters were used for matching the spots between gels. The spots were shown on each gel to make sure all the spots were identified. Any missing spots were added to the analysis manually and any erroneous spots were removed; creating the master gel image. This process was repeated for the triplicate analysis of the [PSI+] strain to create the master gel image. Once the two master gel images were created, both the [psi-] and [PSI+] master gel images were imported into the PDQuest software package to do a comparative analysis of differentiation of protein expression. Landmark spots were set and master gel images were manually scanned for alignment.

Results

Proof of Principle Experimentation

A fifty-three hour growth curve of both [PSI+] and [psi-] *S. cerevisiae* grown in YPD media were used to observe the pattern of growth in each strain in order to determine when log-phase occurred. Log phase, or the period of exponential growth, is the optimal growing condition for cellular lysis as well as allowing for consistent sample preparation procedures (i.e.: consistent initial cell concentration) in which ample protein is being expressed. As a culture reaches stationary phase, nutrient depletion occurs, as well as the accumulation of toxins causing differential protein expression as a result of stressors induced on the organism, which is not desired. The initial growth curve is depicted in Figure 19. Log phase was determined to be

between approximately 15 and 25 hours for the [psi-] strain and between 10 and 20 hours for [PSI+] strain, with an optimal cellular concentration of approximately 1×10^7 cells/mL.

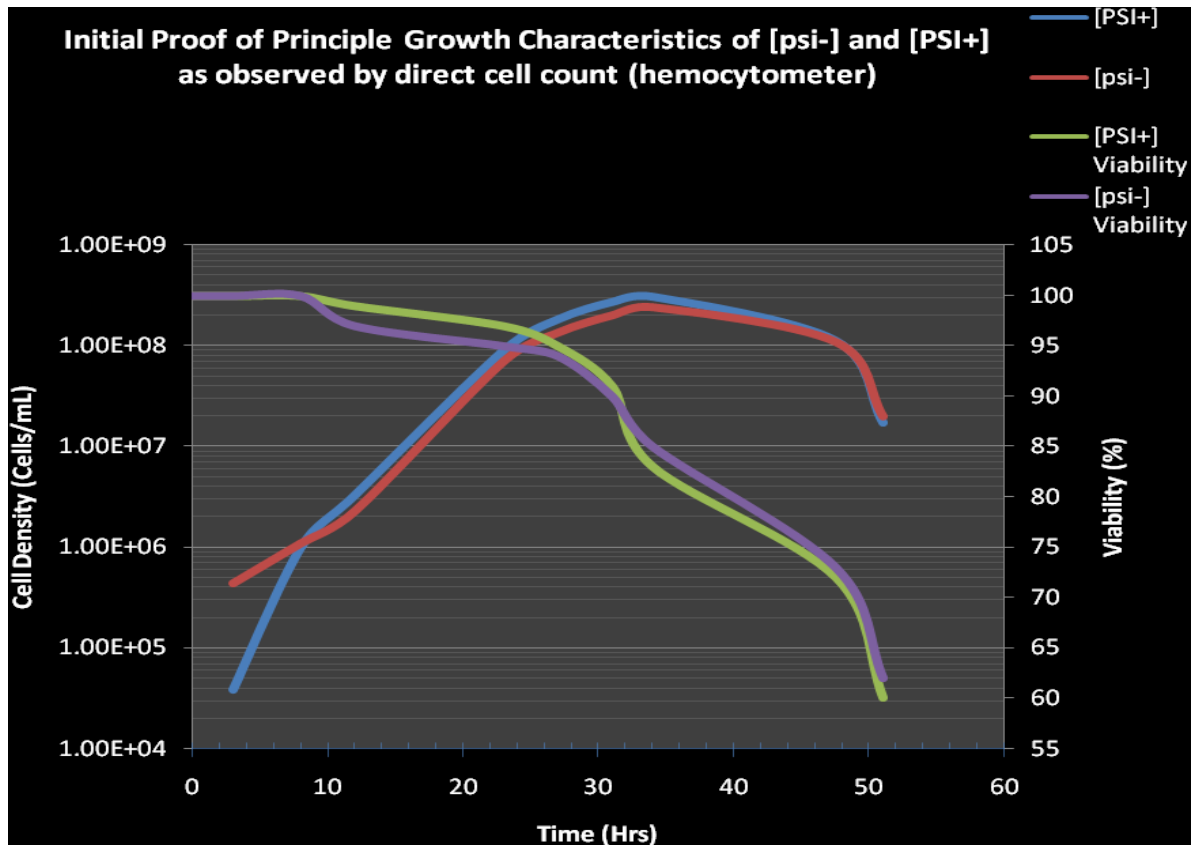


Figure 19. Initial Proof of Principle Growth Characteristics of [psi-] and [PSI+] as observed by direct cell count (hemocytometer) with viability determined by trypan blue dye exclusion.

Initial proof of principle experimentation illustrated potential differences in proteome expression between strains. Both [PSI+] and [psi-] protein extract samples were isolated via Lysis buffer and 10% SDS, with the protein concentrations depicted in Table 2 determined by using the equation of the line ($y = 0.0078x - 0.0001$, where the absorbance is y) determined from the Modified Bradford Assay (Figure 20):

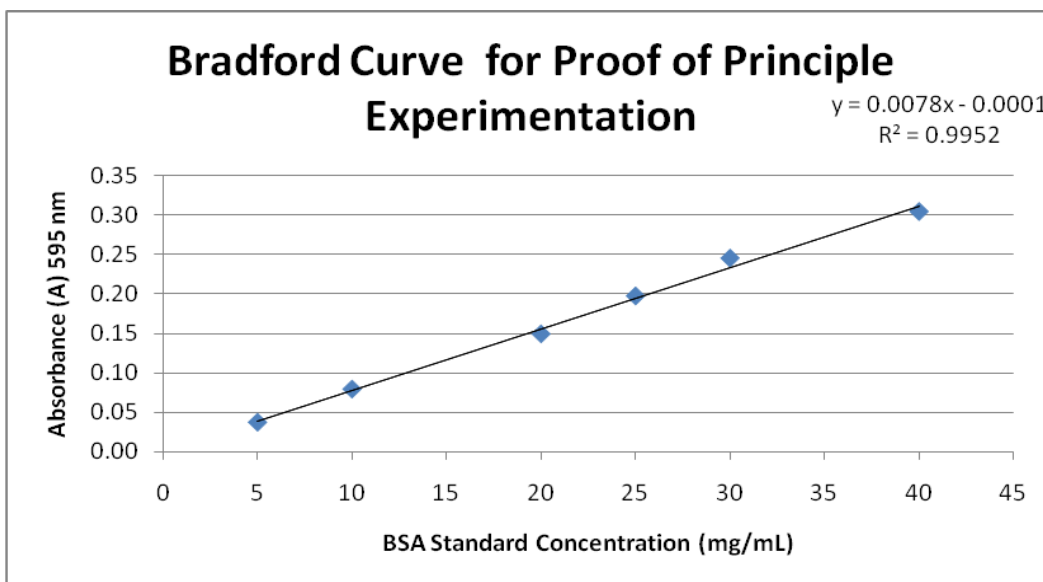


Figure 20. Bradford Curve for Proof of Principle Experimentation

Table 2. Proof of Principle Protein Extract Concentration Determination

Sample	Protein concentration
[PSI+]	3.0µg/µL
[psi-]	2.5µg/µL

The volume of sample to achieve a 100µg protein load was calculated and samples were isoelectrically focused via the use of 7cm pH 3-10 IPG strips. The use of a broad range IPG strip allows for a more accurate representation of the entire proteome, in general, the majority of proteins have a pI between 4 and 7 (20), and as such, the pH 3-10 strip will bracket that interval. Strips were focused according to the following parameters (Table 3):

Table 3. IEF Parameters for [psi-] and [PSI+] Proof of Principle Samples

IPG strip size	7 cm
IPG strip range	PH 3-10
Rehydration	Active
Maximum Voltage	4,000V
Volt-hours	20,000 V-hr
Focusing Temperature	20°C

Isoelectrically focused IPG strips were implemented into the second dimension with the use of 10% polyacrylamide gels in order to resolve proteins in the range between 30 to 150 kD (19) at a 200V constant (235mA), thus, allowing for a large interval for protein detection. Images of the

initial gel run are depicted in Figure 1.1 in Appendix 1. The resultant images depicted variation between samples; however, the number of spots was minimal, as well as streaky. Samples were centrifuged for 60 minutes, with the protein concentration verified by a Modified Bradford Assay (data not shown), with the resultant protein concentrations given in Table 4.

Table 4. Proof of Principle Protein Extract Determination of Centrifuged Samples

Sample	Protein concentration
[PSI+]	1.0 μ g/ μ L
[psi-]	1.5 μ g/ μ L

The protein concentration of the centrifuged samples seemed to result in a lower protein extract concentration compared to the samples that were not centrifuged for 60 minutes. The centrifuged samples were focused according to the same parameters as discussed previously, with the resultant images depicted in Figure 1.2 Appendix 1. Centrifuged samples resulted in “cleaner” gels; ie: gels with minimal streaking and the presence of more spots. The relative location of spots on non-centrifuged versus centrifuged samples were found to be fairly consistent, with greater resolution resulting from centrifuged samples, thus, was the justification for centrifuging samples prior to IEF constantly.

Increased Resolution

A pooled (average of three samples per strain) growth curve of both [PSI+] and [psi-] *S. cerevisiae* grown in YPD media were used to observe the pattern of growth in each strain in order to determine when log-phase occurred. The growth curve is depicted in Figure 21. Log phase was determined to be between approximately 7 and 25 hours for the [psi-] strain and between 5 and 22 hours for [PSI+] strain, with an optimal cellular concentration of

approximately 1×10^7 cells/mL. The individual growth curves, in triplicate, are depicted in Appendix 2⁶.

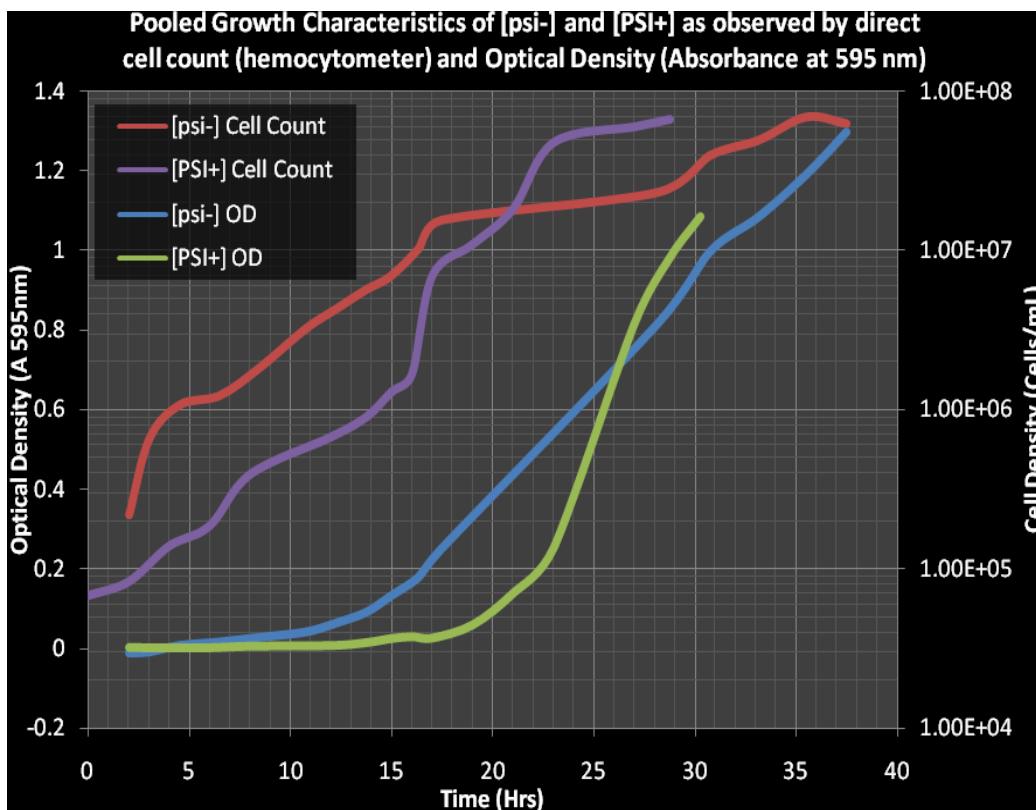


Figure 21. Pooled Growth Characteristics of [psi-] and [PSI+] as observed by direct cell count (hemocytometer) and Optical Density (Absorbance at 595nm)

Initial Sample Preparation via Lysis Buffer + 10% SDS

In order to achieve increased resolution, this sample preparation methodology will be employed in 17cm IPG strips with a pH of 4-7 since the protein spots on the proof of principle gels were well within the pH 4-7 range, thus narrowing down the range would increase resolution. In addition, 10% polyacrylamide gels will also be employed in the 17cm gels since the protein spots visualized encompass just about the entire 10% polyacrylamide gel when using

⁶ This growth curve data was used for triplicate analyses only, not for “Initial Sample Preparation via Lysis Buffer + 10% SDS”

7cm IPG strips. Increasing the SDS concentration would narrow the range in which molecular weight is detected and would result in decreased resolution.

Both [PSI+] and [psi-] protein extract samples were isolated via Lysis buffer and 10% SDS samples. Only the data for the [PSI+] sample is presented here; with the protein concentration depicted in Table 5 determined by using the equation of the line determined from the Modified Bradford Assay.

Table 5. Lysis Buffer and 10% SDS Methodology: Protein Extract Determination of Concentration

Sample	Protein concentration
[PSI+]	4.65 μ g/ μ L

The volume of sample to achieve a 250 μ g protein load was calculated and the sample was isoelectrically focused via the use of 17 cm pH 4-7 IPG strip. The IPG strip was focused according to the following parameters (Table 6):

Table 6. Lysis Buffer and 10% SDS Methodology: IEF Parameters for [PSI+] Sample

IPG strip size	17 cm
IPG strip range	pH 4-7
Rehydration	Active
Maximum Voltage	10,000V
Volt-hours	60,000 V-hr
Focus Temperature	20°C

The isoelectrically focused IPG strip was implemented into the second dimension between 10mA/gel (500V maximum) to 24mA/gel (500V maximum). The gel image is depicted in Appendix 3. The depicted image exhibited poor resolution, with minimal protein spots present. The experimentation with this methodology was repeated on two sets of gels; where each set included one [psi-] and one [PSI+] gel, with similar results (data not shown). Experimentation of variable sample preparation techniques was employed.

Protein Extraction Methodologies

Initial comparative experimentation was focused on protein extraction yield in which three cellular lysing techniques were compared with three [psi-] cultures which used 1000 μ L Sterile TE Buffer, 600 μ L Sterile TE Buffer, and 480 μ L Lysis Buffer + 48 μ L 10% SDS for cellular lysis. Cells were cultured and isolated as previously, with the addition of the above-mentioned components followed by a twenty-minute vortexing session to increase collision of molecules. Protein extraction was determined via the Modified Bradford Assay and calculated as previously, with the derived protein yields in Table 7.

Table 7. Protein Extraction Determination of Concentration of Comparative Cell Lysis Methodologies

Technique	Protein concentration
1000 μ L TE Buffer	> 8.0 μ g/ μ L [†]
480 μ L Lysis Buffer + 48 μ L 10% SDS	2.24 μ g/ μ L [‡]
600 μ L TE Buffer	0.67 μ g/ μ L

The 600 μ L Sterile TE Buffer and Lysis Buffer + 10% SDS methodologies yielded in poor results; i.e.: lower protein concentration yields. The optimal procedure was found to be 1000 μ L Sterile TE Buffer. To assure that this methodology would be reproducible, the procedure was repeated with the use of two [psi-] cultures; labeled [psi-] 1 and [psi-] 2. A Modified Bradford Assay was completed to determine the protein concentration of each of the samples in order to determine the protein concentration (Table 8).

[†]Two trials of 1000 μ L TE Buffer trials were completed, both of which were outside of the Bradford limits. Typically, the sample would be diluted and retested, however, this experimentation was implemented to determine the optimal protein extraction technique, and thus, a protein yield outside of the upper Bradford limit was desired and validated the procedure (as optimal).

[‡] This procedure was already determined to yield poor results and was used for comparative purposes only.

Table 8. Protein Concentration of Samples Prepared with 1000 μ L TE Buffer

Sample	Protein concentration
[psi-] 1	>8.00 μ g/ μ L
[psi-] 2	>8.00 μ g/ μ L

The protein yield in these samples was fairly consistent in comparison to the previous attempts discussed. The volume of [psi-] 1 and [psi-] 2 (refer to Table 8 above) to achieve a 250 μ g protein load was calculated and samples were rehydrated passively and then isoelectrically focused via the use of 17 cm pH 4-7 IPG strips. Strips were focused according to the following parameters (Table 9):

Table 9. IEF Parameters for [psi-] #1 and [psi-] #2 Samples

IPG strip size	17 cm
IPG strip range	pH 4-7
Rehydration	Passive
Maximum Voltage	10,000V
Volt-hours	60,000 V-hr
Focus Temperature	20 $^{\circ}$ C

Isoelectrically focused IPG strips were implemented into the second dimension between 10mA/gel (500V maximum) to 24mA/gel (500V maximum). The resultant gels yielded no protein spots; images are not shown.

TCA Precipitation

The results of the protein extraction methodology via the use of 1000 μ L TE Buffer were favorable (i.e.: protein concentration); disturbing agents were thought to be causing poor resolution of gels. In order to remove any disturbing agents from the samples, [psi-] samples lysed with 1000 μ L TE Buffer were precipitated via the Trichloroacetic acid (TCA) methodology discussed in the Materials & Methods section of this paper. The gels resulted in an optimal sample preparation methodology (overall) and will be discussed in the “Triplicate Analysis of [psi-] Samples” section, which follows.

Triplicate Analysis of [psi-] Samples

Three [psi-] samples obtained via the 1000 μ L TE Buffer (20 minute vortex) protocol were precipitated with TCA, with the addition of 0.2M NaOH. To verify the protein concentration, the Modified Bradford Assay was implemented and the Bradford Curve (not shown) was used to determine the protein concentration of each sample (Table 10).

Table 10. Protein Concentration of [psi-] Samples used for the Triplicate Analysis

Sample	Protein concentration
[psi-] 1	8.20 μ g/ μ L
[psi-] B	2.50 μ g/ μ L
[psi-] 2A	5.65 μ g/ μ L

The derived protein concentrations varied between 2.50 and 8.20 μ g/ μ L. The variation was most likely due to the amount of Rehydration Buffer (and ampholytes) added to resolubilise the sample. In order to account for any low abundance and/or low molecular weight proteins, the volume of sample to achieve a 300 μ g protein load was calculated (instead of 250 μ g as previously used). Samples were isoelectrically focused via the use of 17 cm pH 4-7 IPG strips according to the following parameters (Table 11):

Table 11. IEF Parameters for [psi-] 1, [psi-] B, and [psi-] 2A Samples

IPG strip size	17 cm
IPG strip range	pH 4-7
Rehydration	Passive
Maximum Voltage	10,000V
Volt-hours	60,000 V-hr
Focus Temperature	20 $^{\circ}$ C

Isoelectrically focused IPG strips were implemented into the second dimension between 10mA/gel (500V maximum) to 24mA/gel (500V maximum). Gel images are depicted in Figure 4.6 in Appendix 4.

The samples precipitated with TCA and the addition of 0.2M NaOH increased gel resolution and the qualitative abundance of protein spots represented on the gel. These gels were used for a comparative analysis (against [PSI+] triplicate gels) that will be discussed later.

Triplicate Analysis of [PSI+] Samples

Three [PSI+] samples obtained via the 1000µL TE Buffer (20 minute vortex) protocol were precipitated with TCA and the addition of 0.2M NaOH. To verify the protein concentration, the Modified Bradford Assay was implemented and the Bradford Curve (not shown) was used to determine the protein concentration of each sample (Table 12).

Table 12. Protein Concentration of [PSI+] Samples used for the Triplicate Analysis

Sample	Protein concentration
[PSI+]Y2	7.50µg/µL
[PSI+]ZZ	6.50µg/µL
[PSI+]I2	6.50µg/µL

The derived protein concentrations varied between 6.50 and 7.50µg/µL. In order to account for any low abundance and/or low molecular weight proteins and to remain consistent, the volume of sample to achieve a 300µg protein load was calculated. Samples were rehydrated passively and isoelectrically focused via the use of 17 cm pH 4-7 IPG strips according to the following parameters (Table 13):

Table 13. IEF Parameters for [PSI+] Y2, [PSI+] ZZ, and [PSI+] I2 Samples

IPG strip size	17 cm
IPG strip range	pH 4-7
Rehydration	Passive
Maximum Voltage	10,000V
Volt-hours	60,000 V-hr
Focus Temperature	20°C

Isoelectrically focused IPG strips were implemented into the second dimension between 16mA/gel (500V maximum) to 26mA/gel (500V maximum). Gel images are depicted in Figure 4.7 in Appendix 4.

PDQuest Analysis

Three independent replicate trials of each experimental condition ([psi-] versus [PSI+]) strains) were imported into the PDQuest software to create individual MatchSets (master gels) depicted in Appendix 5. The automated detection and matching tools were selected in order to select the faintest spot and the largest spot cluster. Smoothing parameters were increased to a 5x5 kernel size. Next, landmark spots were chosen and the default parameters were used for matching the spots between gels. Once the MatchSet was created, each spot on the master gel was manually checked; any missing spots were added and erroneous spots were removed. The local regression model normalized each condition. The overall mean coefficient of variation (CV) between [psi-] gels was 0.494. For [PSI+], the mean coefficient of variation was 0.456. A group consensus was obtained, with the matching summary represented in Figure 22 and Figure 23. Match Rate 1 represents the percent of matched spots on the gel relative to the total number of spots on that gel. Match Rate 2 represents the percent of matched spots on the gel relative to the total number of spots on the master. The correlation coefficients represent the correlation between the master gel versus remaining gels, in which the master gel is annotated with * in the figures below, and as such, has a correlation coefficient of 1.000.

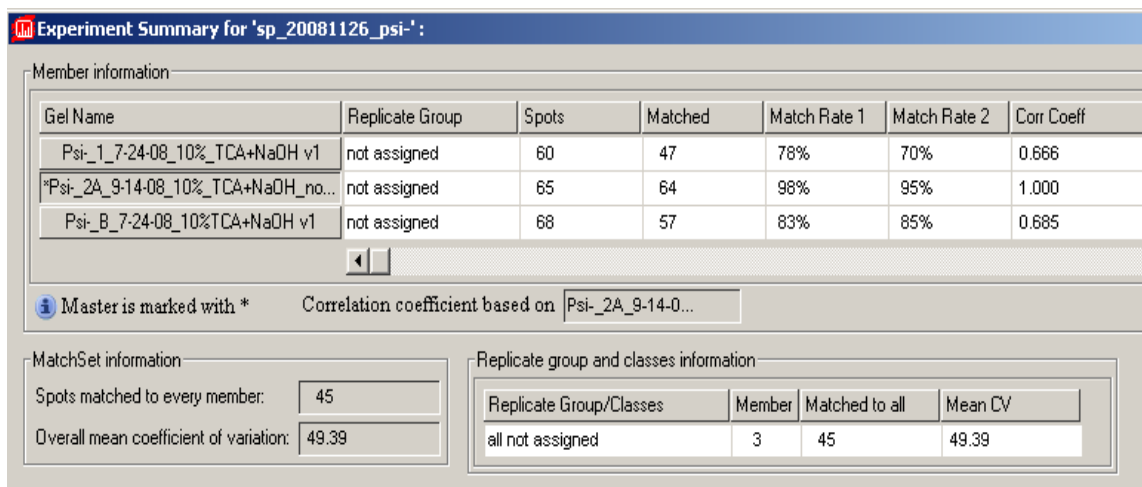


Figure 22. Experiment Summary for [psi-] triplicate analysis (excerpt from PDQuest)

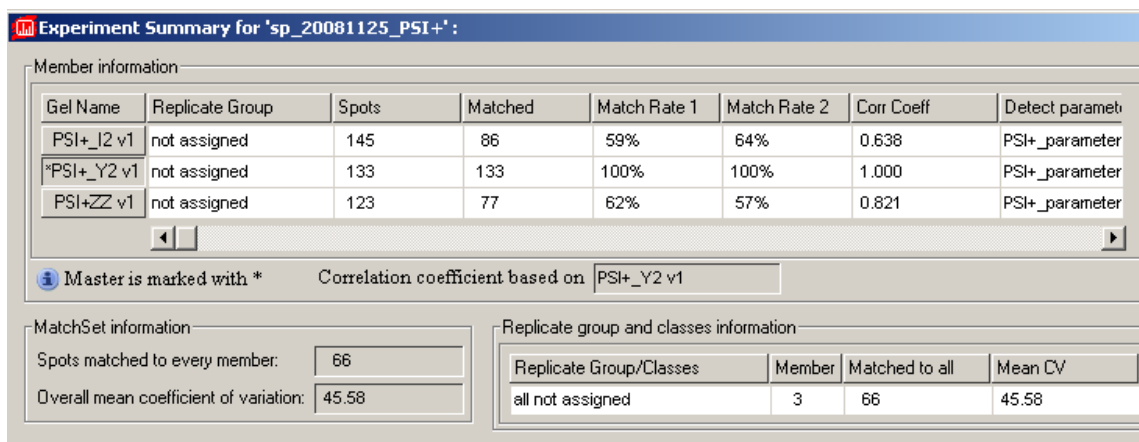


Figure 23. Experiment Summary for [PSI+] triplicate analysis (excerpt from PDQuest)

Each of the individual MatchSets (depicted in Appendix 5) were imported into PDQuest to create a High Level MatchSet for the final comparative analysis. Two High Level MatchSets were created. The first High Level MatchSet created used the [psi-] master gel as the master gel image for the comparative analysis ([psi-] versus [PSI+]). The second High Level MatchSet created used the [PSI+] master gel as the master gel image for the comparative analysis ([PSI+] versus [psi-]). In order to ensure that MatchSets (master gels) were aligned properly; landmark spots were set and were manually checked for alignment. The master gel images are depicted in Appendix 6 Figures 6.10 and 6.11. The following summaries were derived (Figures 24 and 25):

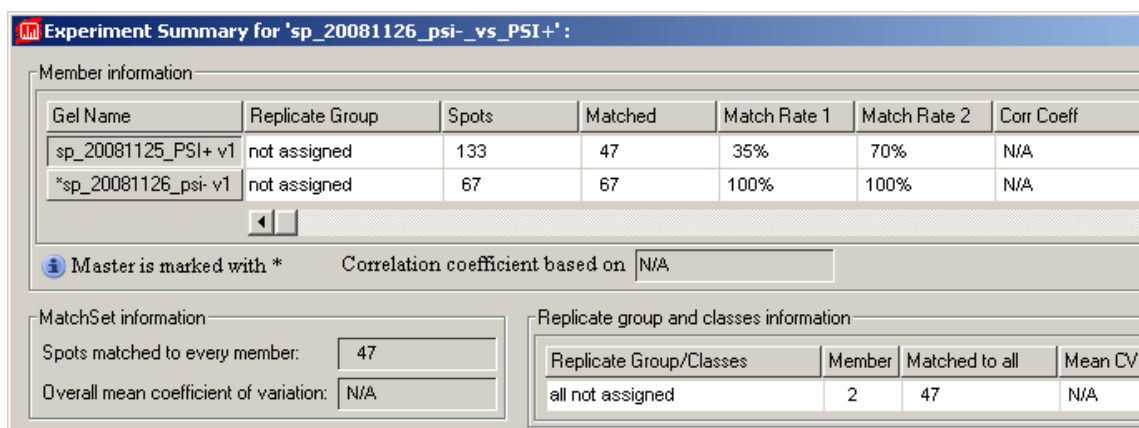


Figure 24. Experiment Summary for High Level MatchSet [psi-] as master (excerpt from PDQuest)

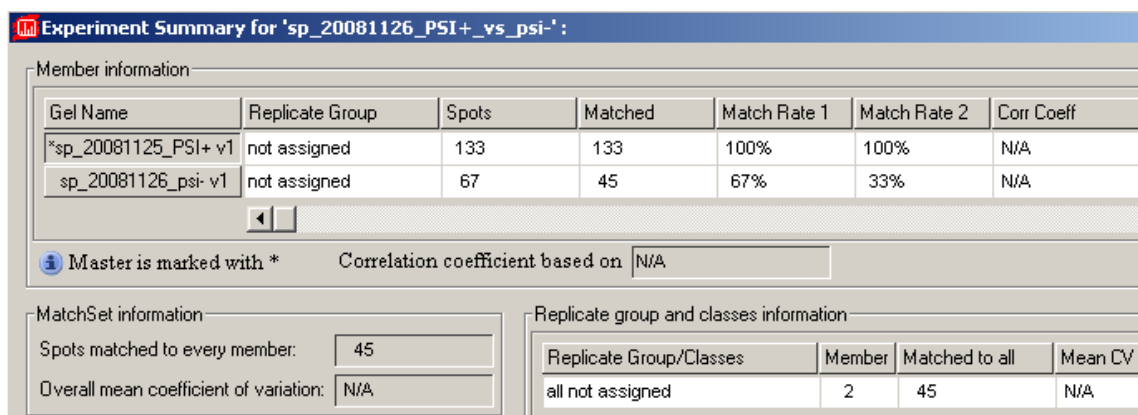


Figure 25. Experiment Summary for High Level MatchSet [PSI+] as master (excerpt from PDQuest)

Refer to Appendix 7 for the overlapped images used to check for differentiation.

Discussion

The initial growth curve comparing [psi-] and [PSI+] *S. cerevisiae* illustrated the growth cycle of each strain. It was apparent, that the growth cycle of each strain was not equivalent; log phase occurred between 15 and 25 hours in [psi-] and between 10 and 20 hours in [PSI+]. Each protein sample was harvested when the cellular concentration reached approximately 1×10^7 cells/mL to ensure that differential protein expression between strains reflected strain differences instead of proteome changes due to the influence of the phase in the growth cycle it was encountering, as well as to have consistent sample preparation guidelines, and thus enhance reproducibility.

Initial proof of principle sample preparation methodology depicted distinct differences between strains. Superior sample preparation quality, and thus overall gel quality, was observed when the samples were centrifuged for 60 minutes prior to rehydration and IEF, thus, being the justification for centrifuging samples prior to isoelectric focusing. When attempting to employ the same methodology in 17cm gels, the results were far from comparable. Samples lysed via the Lysis buffer and SDS methodology resulted in the appearance of a thin white layer floating

on top, thought to be a lipid layer. In addition, SDS is not compatible with IEF and was thought to be at a low concentration that would not interfere with IEF, however, initial concerns of using SDS may have held to be true. Resultant images exhibited poor resolution; streaking spot morphologies, minimal number of spots, etc. Additional attempts were implemented, with consistently poor gel resolution. As a result, comparative sample preparation methodologies were implemented.

Comparative sample preparation methodologies introduced a host of obstacles in this study. One of the most critical steps in 2D electrophoresis is sample preparation, which starts with protein extraction. After determining the optimal protein extraction technique to be by the addition of 1000 μ L Sterile TE Buffer to the sample followed by vortexing for 20 minutes to lyse cells, the resultant gel images were far from anticipated (although no floating layer was present in the sample). It was postulated that disturbing agents within the sample were causing interference with isoelectric focusing. Disturbing agents were removed from the samples via the TCA precipitation procedure because the pI of the protein is not altered by TCA. By implementing the TCA precipitation, resolution on resultant gels were improved due to the removal of salts, lipids, ionic detergents, etc. that are known to interfere with isoelectric focusing. 0.2M NaOH and rehydration buffer was added to the sample to resolubilise the protein pellet, as well as to neutralize the charge of the acid to avoid protein precipitation during the IEF, and thus, minimizing the appearance of streaking on resultant gels. The importance of the neutralization step of the protein sample prior to IEF was confirmed and is supported by Candiano et. al (37).

Over time, mutations in [PSI+] samples used in the initial proof of principle experimentation and comparative sample preparation methodologies were introduced. Ancillary

testing by Dr. Irene Evans' Undergraduate Research Students at Rochester Institute of Technology (RIT) could not revert [PSI+] strains to [psi-] strains with the use of guanidine hydrochloride, and as such, could not conclude that the [PSI+] strain was genetically equivalent to the [psi-] strain with the addition of the [PSI+] prion. However, it must be noted that at the time of initial testing (approximately one and one half years prior) the strains may have obtained the expected integrity, however, the authors decided to obtain additional samples by Dr. Susan Liebman at the University of Illinois to ensure the integrity of this study⁷. The Undergraduate Research students at RIT successfully reverted the newly obtained [PSI+] samples to [psi-] via the use of guanidine hydrochloride. An additional growth curve, with the newly obtained samples, was completed in order to determine log phase of growth (as done previously), with similar results. Log phase occurred between 7 and 25 hours in [psi-] and between 5 and 22 hours in [PSI+], indicating slightly different rates of growth when compared to historical observations. However, these variations between the historical measurements and current experimental procedure are of little consequence, as there are several years between the sets of observations and minor deviations are bound to persist. Each protein sample was harvested when the cellular concentration reached approximately 1×10^7 cells/mL, as verified by Optical Density. In congruence with the literature, cultures were allowed to maintain log phase until a sufficient density of cells is achieved, occurring at an absorbance of approximately 1.0 at 595 nm⁸. This cell density allows for the observation of proteome expression during normal log phase, as well as maximizing cell load during harvesting, and subsequently providing high protein yields.

⁷ The PSI+ samples used in the final triplicate analysis were the newly obtained (and confirmed) PSI+ samples from Dr. Susan Liebman at the University of Illinois.

⁸ Referencing Figure 19, the approximate cell density corresponding to this absorbance is 4×10^7 and 3×10^7 cells/ml for [PSI+] and [psi-] respectively.

After resolving the potential strain inconsistency, higher-level analysis of the resulting gels could then be performed with a much higher degree of confidence. Once a complete set of gels (three each) of each strain was generated, analysis in PDQuest was performed. Upon completion of creating the individual MatchSets (master gels) for [psi-] and [PSI+] triplicate images, the average gel-to-gel coefficient of variation was determined to be 0.494 and 0.456 respectively. The coefficient of variation is useful in comparing variation between multiple triplicate gels within the same strain as well as to its counterpart. Inherent variation may be caused by biological and experimental variability even when following consistent methodology from sample to sample in the attempt to reduce variability. Any reagents, buffers, polyacrylamide gels, etc. were not obtained commercially, but were made in the laboratory and thus, variation from experiment to experiment could occur with the use of varying preparations of each of these materials. Even slight variation in concentration in a reagent could cause variation in the final gel image. In addition, the yeast genome is complex and can undergo posttranslational modifications, such as glycosylation, phosphorylation, etc. that causes differences in higher order protein folding, and as such, would alter the protein expression depicted in a proteome map derived from 2D electrophoresis. When introducing a sample to IEF, more specifically during passive rehydration when no current is being applied to the IPG strip, proteins not diffused into the strip will not be represented in the subsequent proteome map. More specific to the PDQuest analysis, the algorithm may not recognize low intensity spots particularly if there is a large degree of background noise due to differences in staining and destaining between replicate gels. There may also be a slight offset of spot positioning, intensity, and pattern in replicate gels due to slight differences during either the first or second dimension resolution, which would be inherent differences if replicate gels were not resolved during the

same experimental run. However there is one major drawback to this analysis type which is directly attributable to the presence or absence of a data point in the individual gel data set. The absence of a spot on one gel significantly increases the associated variability in the data set, with the likelihood of this occurrence increasing as the sample size increases. These observations are congruent with those exhibited by Challapalli et. al (36) and are comparable between triplicate images of each strain.

After taking this into account and examining the master gel overlay for each strain (Appendix 7 Figures 7.12 and 7.13), there are obvious differences between the strains that are statistically significant. When examining one strain's protein expression characteristics to the other there are essentially three observable results in these types of analyses. Firstly, the real presence or absence of a spot from one gel to another, indicating an obvious expression difference between the two. A more subtle difference entails the slight movement of a spot on one strains gel as compared to its relative position on another (taking into regard the overall experimental resolving deviations between the gels). These changes are more difficult to quantify, as they are prone to inherent error of automated analysis regarding them as a unique protein, rather than as the same protein with a change in the posttranslational modification schema or other tertiary/quaternary structure change causing a difference in migration. The third principle type of quantifiable change from strain to strain is the increased or decreased (differential) expression of one particular protein between the two gels. Of the three, this particular form of protein expression change is of the greatest interest as it represents a statistically significant, quantifiable alteration in the cell's proteome expression resultant of the presence or absence of the prion. Regardless of the type of expression change, any of these events allow for greater experimental scrutiny via excision of the spot of interest from one or

both gels, and proceeding with a mass spectroscopy analysis of the protein to determine its amino acid sequence and subsequent identity. The results elicited from both overlay master gels indicate that such an analysis could proceed and would be of benefit to further elucidate the nature of effect conveyed by the prion itself. However, for the purposes of this analysis, it is sufficient to state that there are differences between the two strains with respect to proteome expression.

Conclusions

Through continued development, the two-dimensional PAGE gel technique has emerged as the hallmark technology utilized in virtually all protein expression analyses. One part of the primary objective of this study was the determination of quantifiable proteome differences between [psi-] and [PSI+] strains of *Saccharomyces cerevisiae* using this technique. Examining the overlay master gel images, which seem to elicit similar patterns, there are clearly differences in spot intensities and expression between the two strains that warrant further study. However, for the purposes of this analysis it is clear that the overall proteome expression characteristics are markedly different between the two strains, proving the initial hypothesis that there are proteomic differences between the strains correct.

While this has been accomplished, of greater importance is the development and validation of a protein extraction protocol (recalling the initial component of the primary objective) that yields highly reproducible whole proteome isolates from both strains, a crucial component for the success of the analysis proper. Via coupling of a precipitation of Trichloroacetic acid to the TE buffer sample extract, gel resolution was significantly increased, allowing for a more accurate interpretation of the 2DE map by PDQuest. The successful validation of the protein extraction methodology has significant implications for continued work,

which is clearly justified given the differences in proteome expression between the two strains. This is particularly of interest as it has subsequently been verified by ancillary work⁹ that the only significant difference between [PSI+] and [psi-] strains is the presence of the prion in the former.

This study concludes that there is a quantifiable and reproducible effect on protein expression conveyed by the prion state in yeast, which warrants future studies to realize this implication within the context of epigenetics.

⁹ Completed by Dr. Evans' Undergraduate Research Students at the Rochester Institute of Technology.

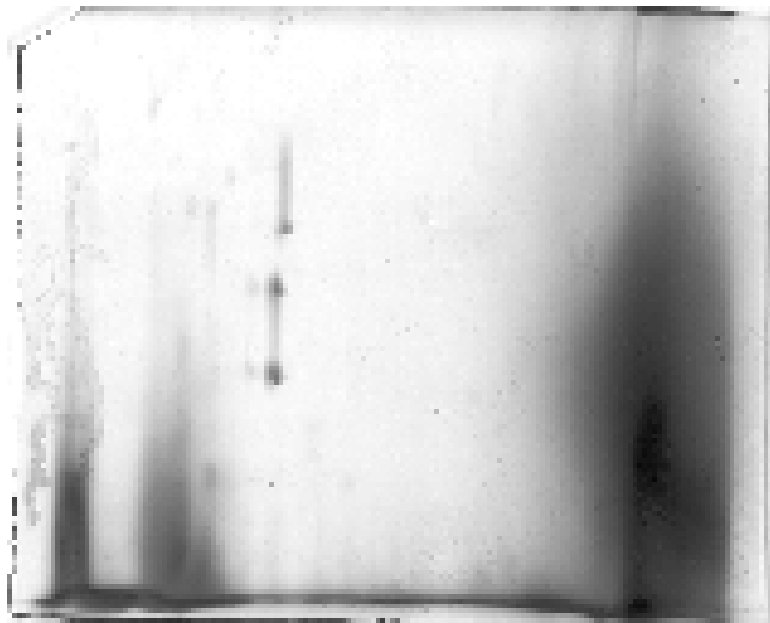
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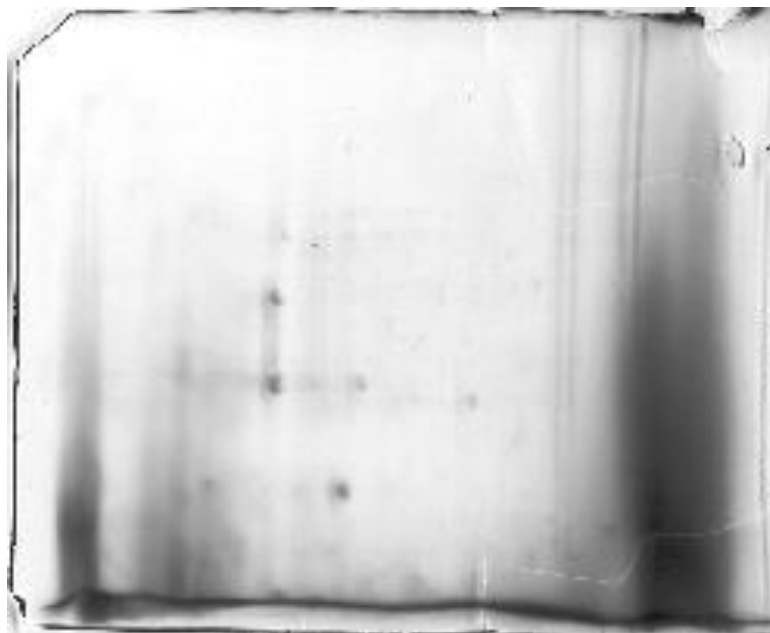
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Appendix 1

Figure 1.1 Initial Proof of Principle Gel Images of (a) [psi-] and (b) [PSI+] *S. cerevisiae* (pH 3-10 IPG Strips, 10% polyacrylamide) before centrifugation of sample

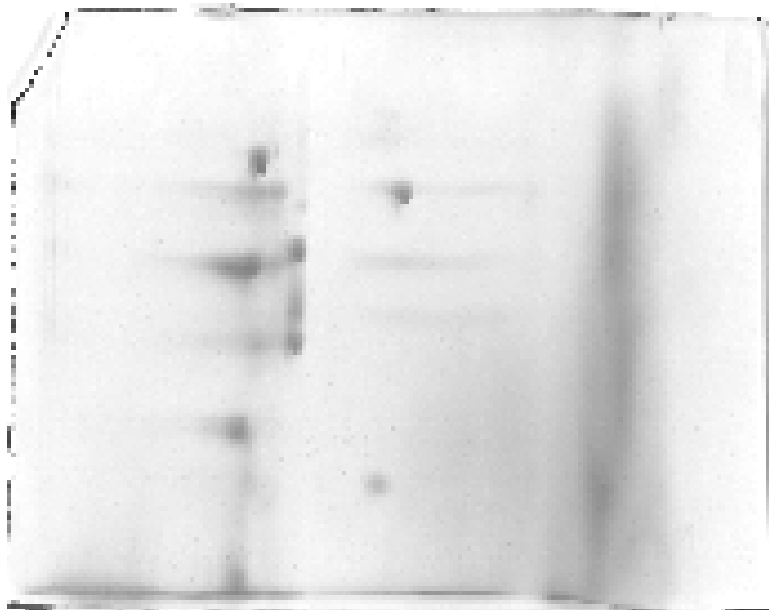
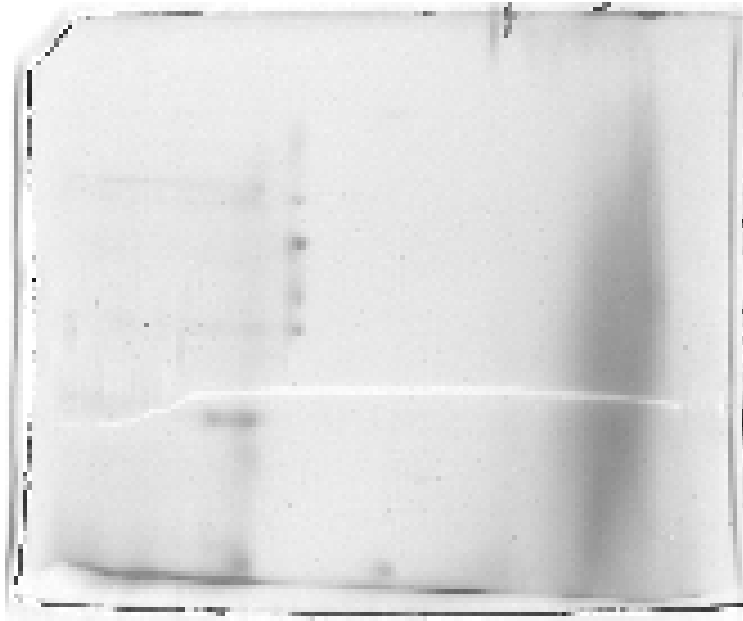


(a)



(b)

Figure 1.2 Initial Proof of Principle Gel Images of (a) [psi-] and (b) [PSI+] *S. cerevisiae* after centrifugation of samples (pH 3-10 IPG Strips, 10% polyacrylamide)



(b)

Appendix 2

Figure 2.3 [psi-] observed and pooled growth characteristics as observed by direct count (hemacytometer) and Absorbance at 595nm

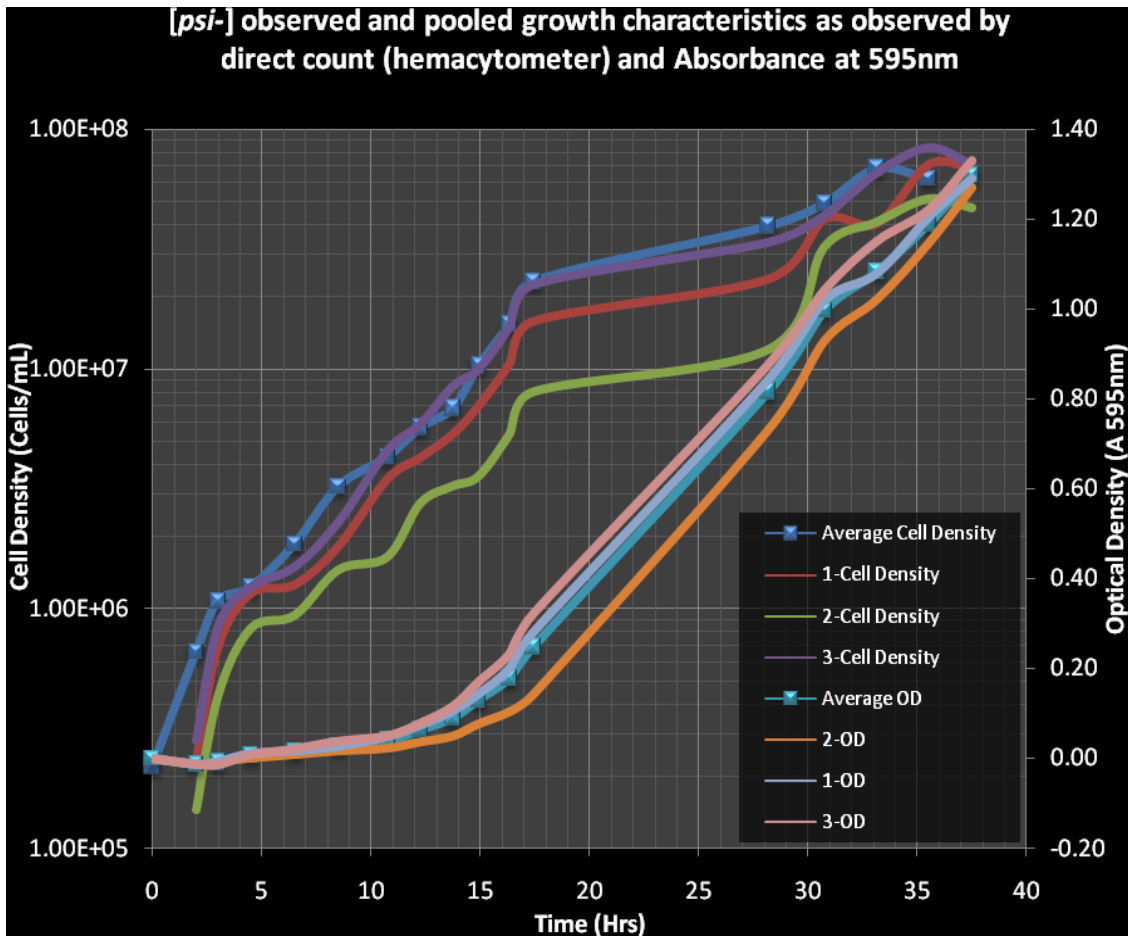
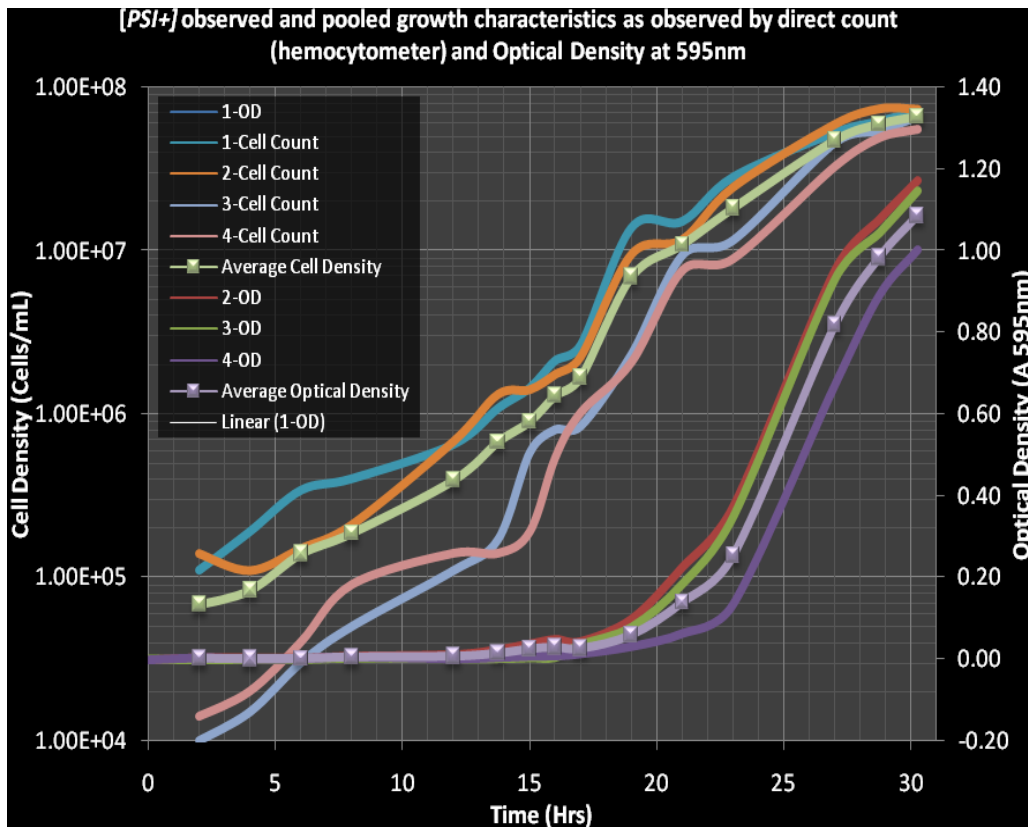
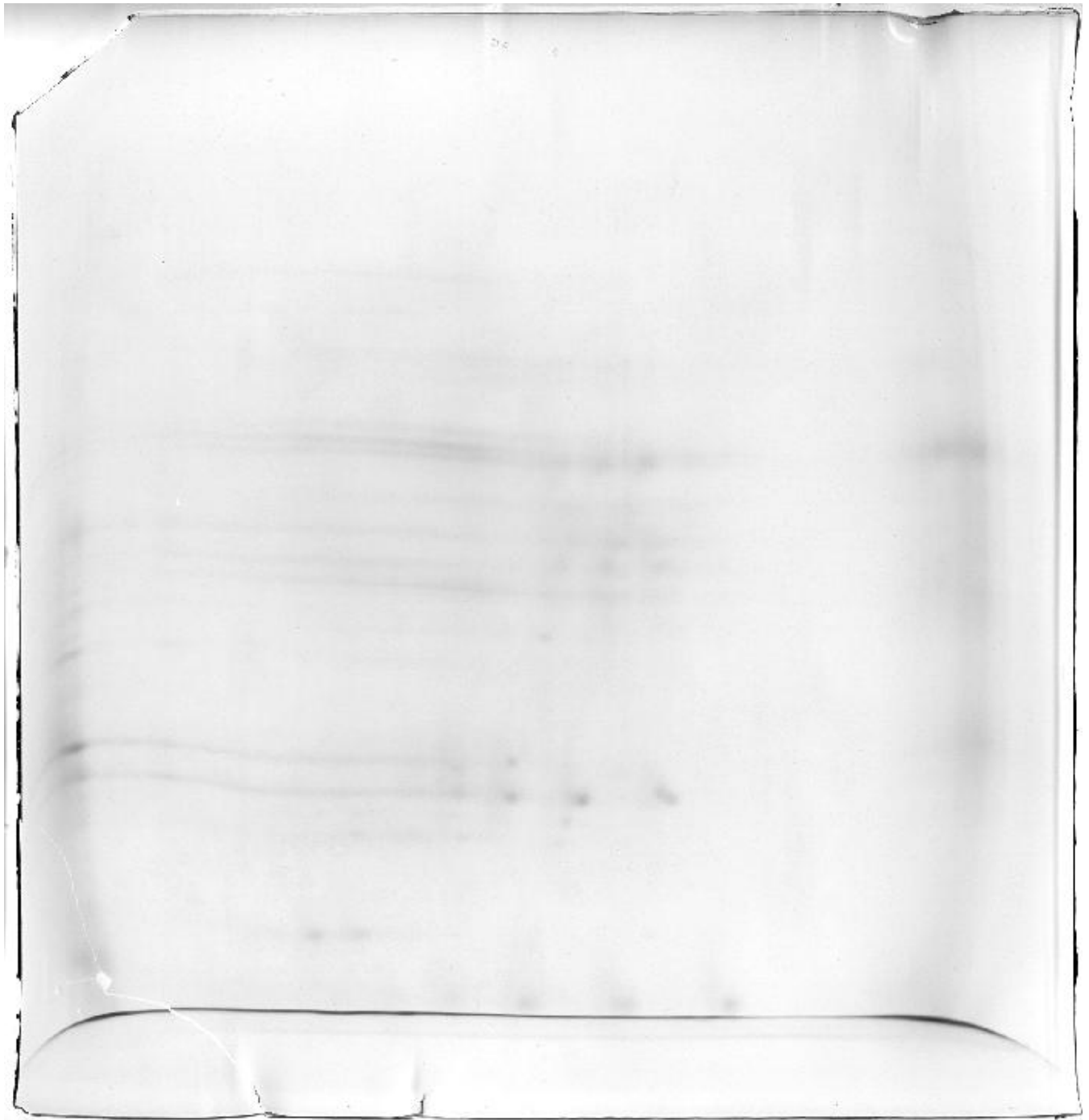


Figure 2.4 [PSI+] observed and pooled growth characteristics as observed by direct count (hemacytometer) and Absorbance at 595nm



Appendix 3

Figure 3.5 Gel Image of [psi-] sample prepared with Lysis Buffer + 10% SDS yielding poor resolution (pH 4-7 IPG strips, 10% Polyacrylamide)

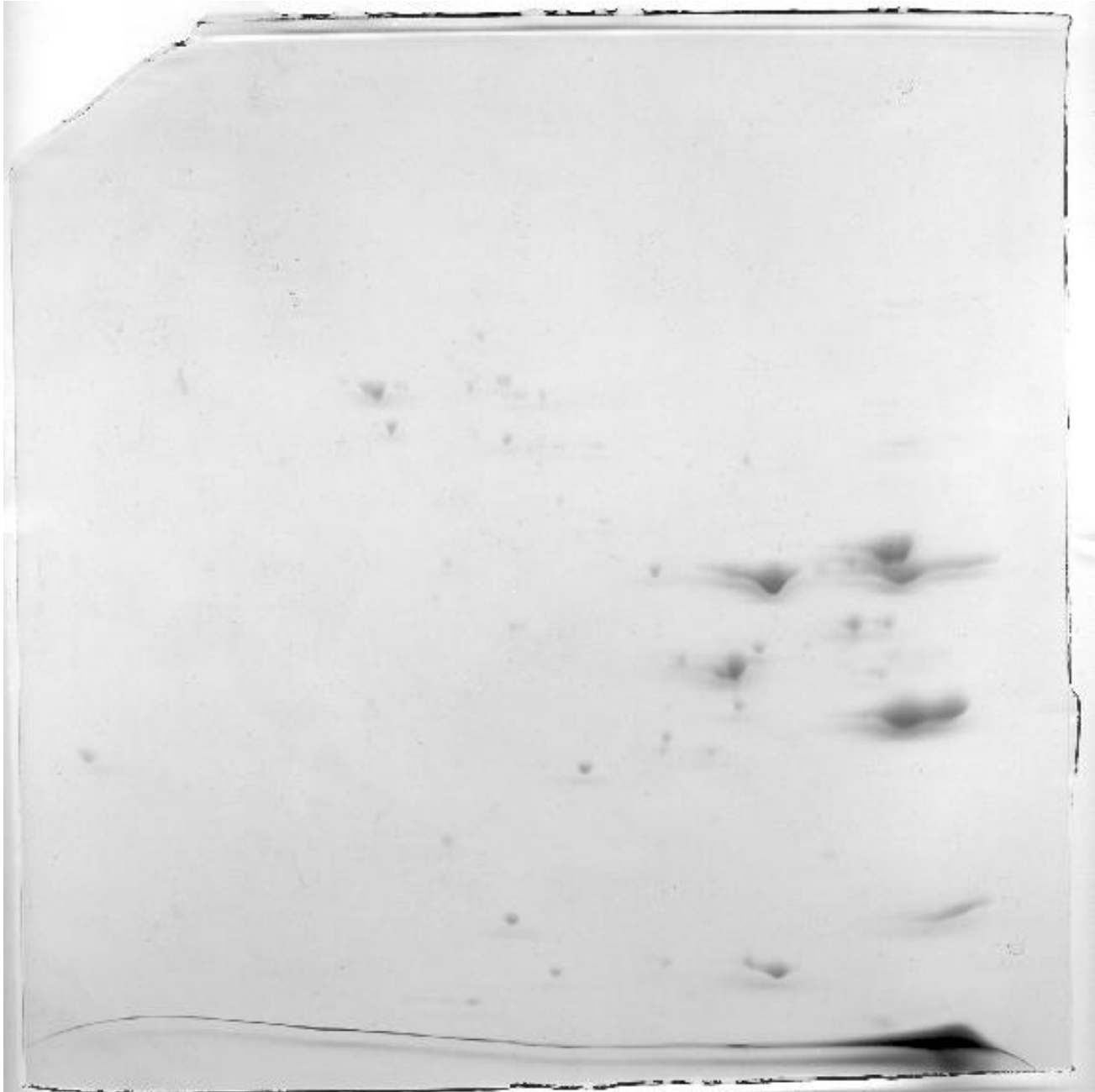


Appendix 4

Figure 4.6 Triplicate Gel Images of [psi-] *S. cerevisiae* (a) [psi-] B, (b) [psi-] 1 (c) [psi-] 2A
(pH 4-7 IPG strips, 10% Polyacrylamide)



(a)

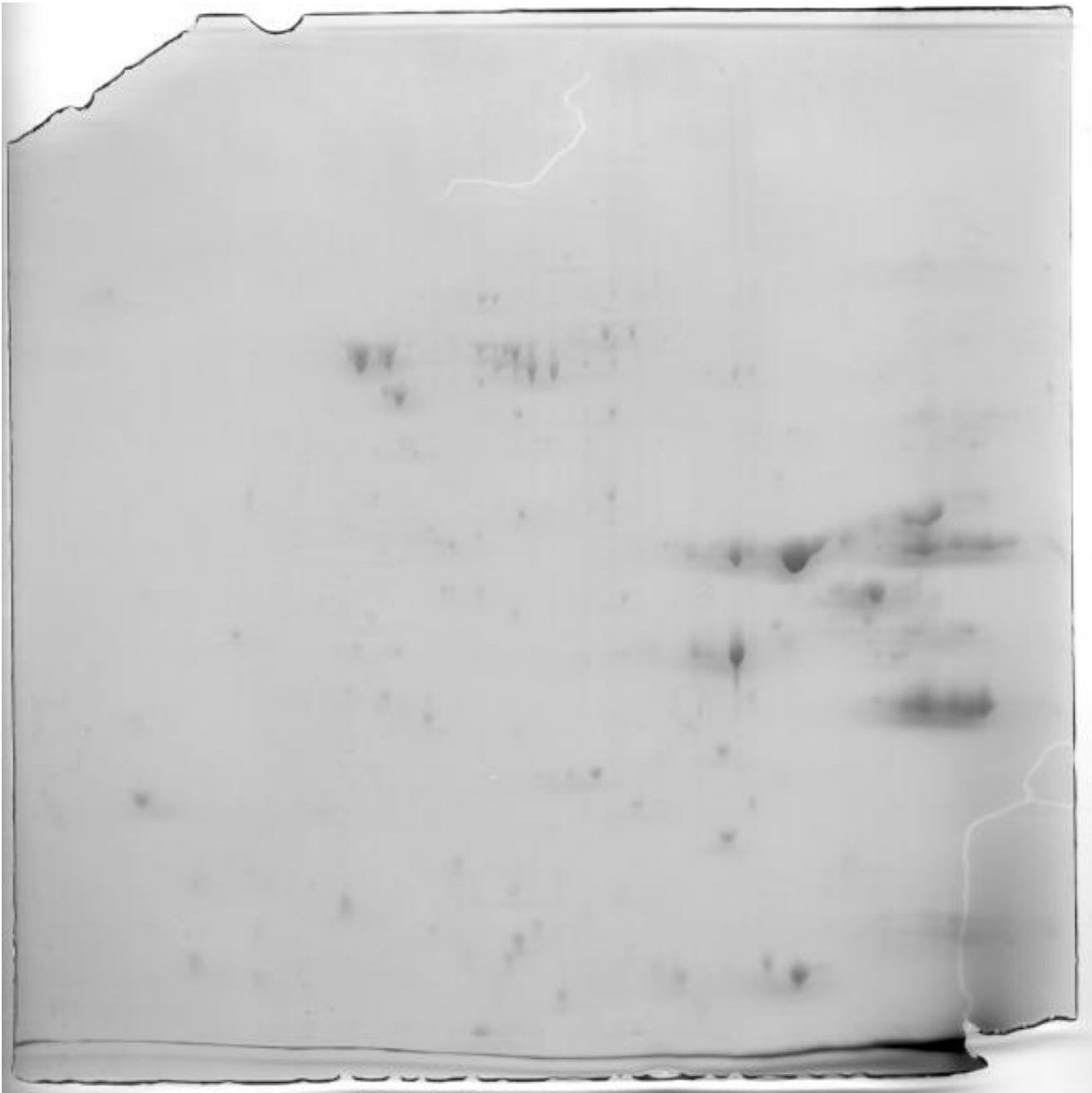


(b)



(c)

Figure 4.7 Triplicate Gel Images of [PSI+] *S. cerevisiae* (a) [PSI+] I2 (b) [PSI+] ZZ (c) [PSI+] Y2
(pH 4-7 IPG strips, 10% Polyacrylamide)



(a)



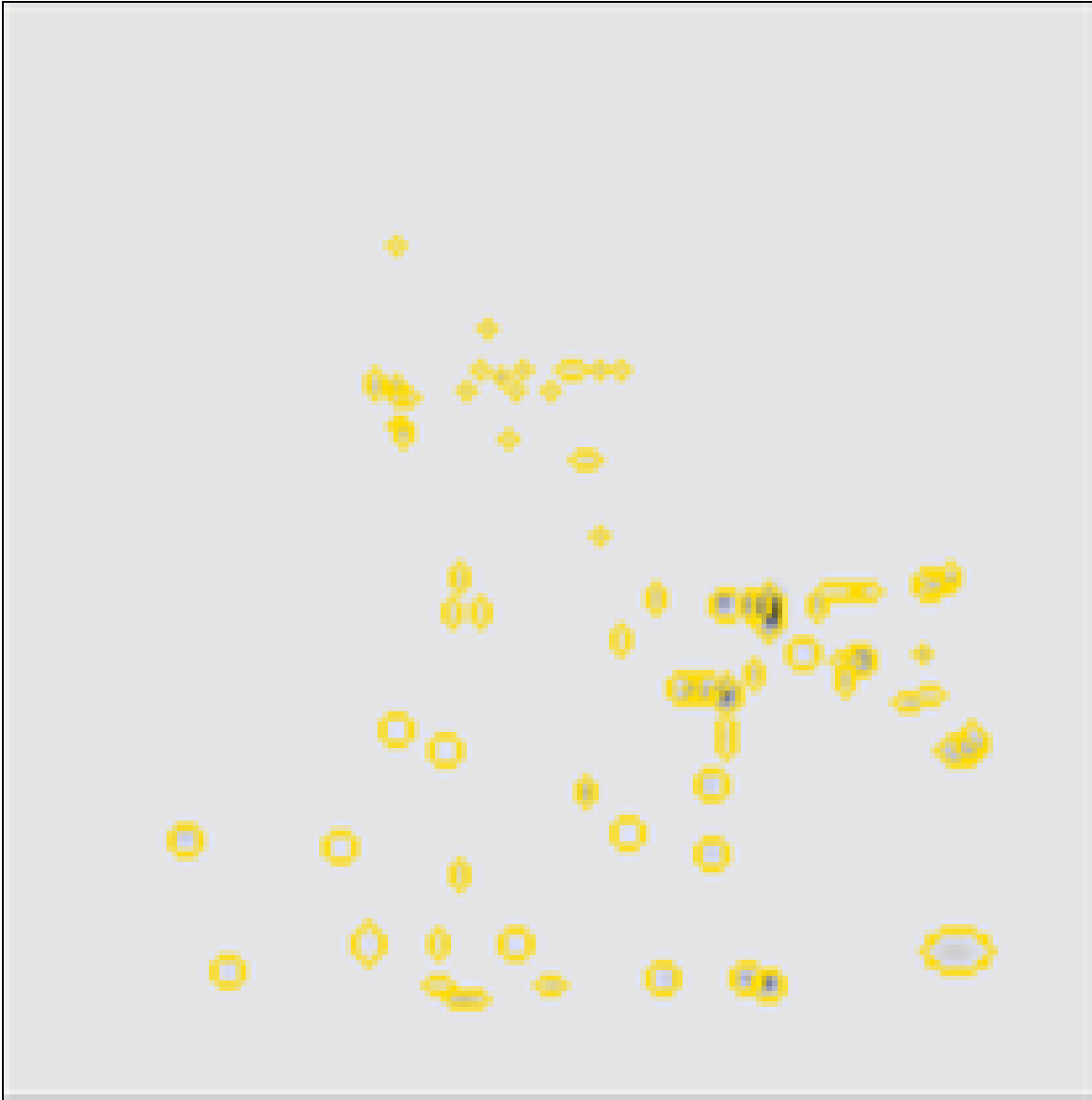
(b)



(c)

Appendix 5

Figure 5.8 [psi-] Master Gel Images (a) with overlays (b) without overlays

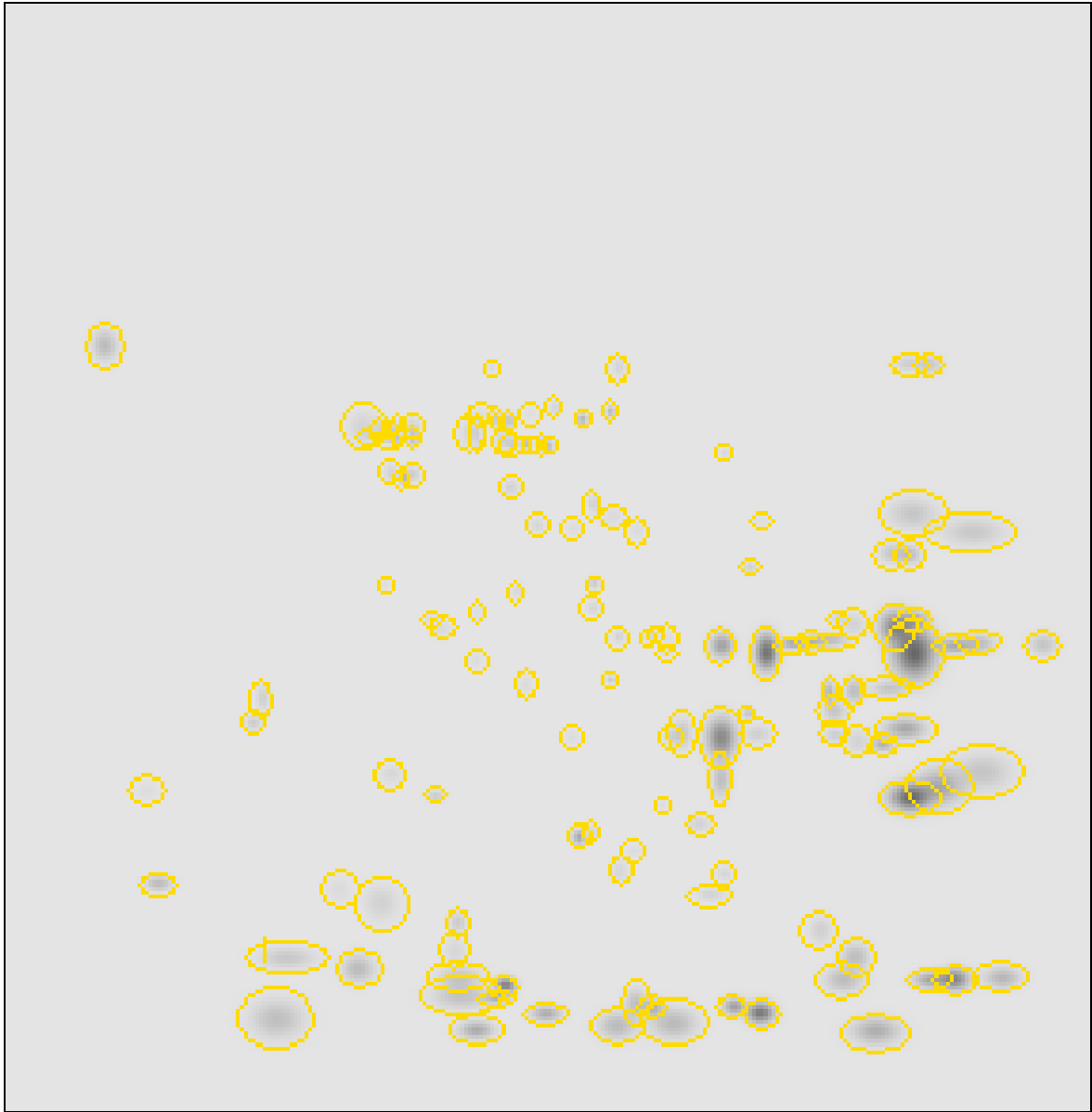


(a)



(b)

Figure 5.9 [PSI+] Master Gel Images (a) with overlays (b) without overlays



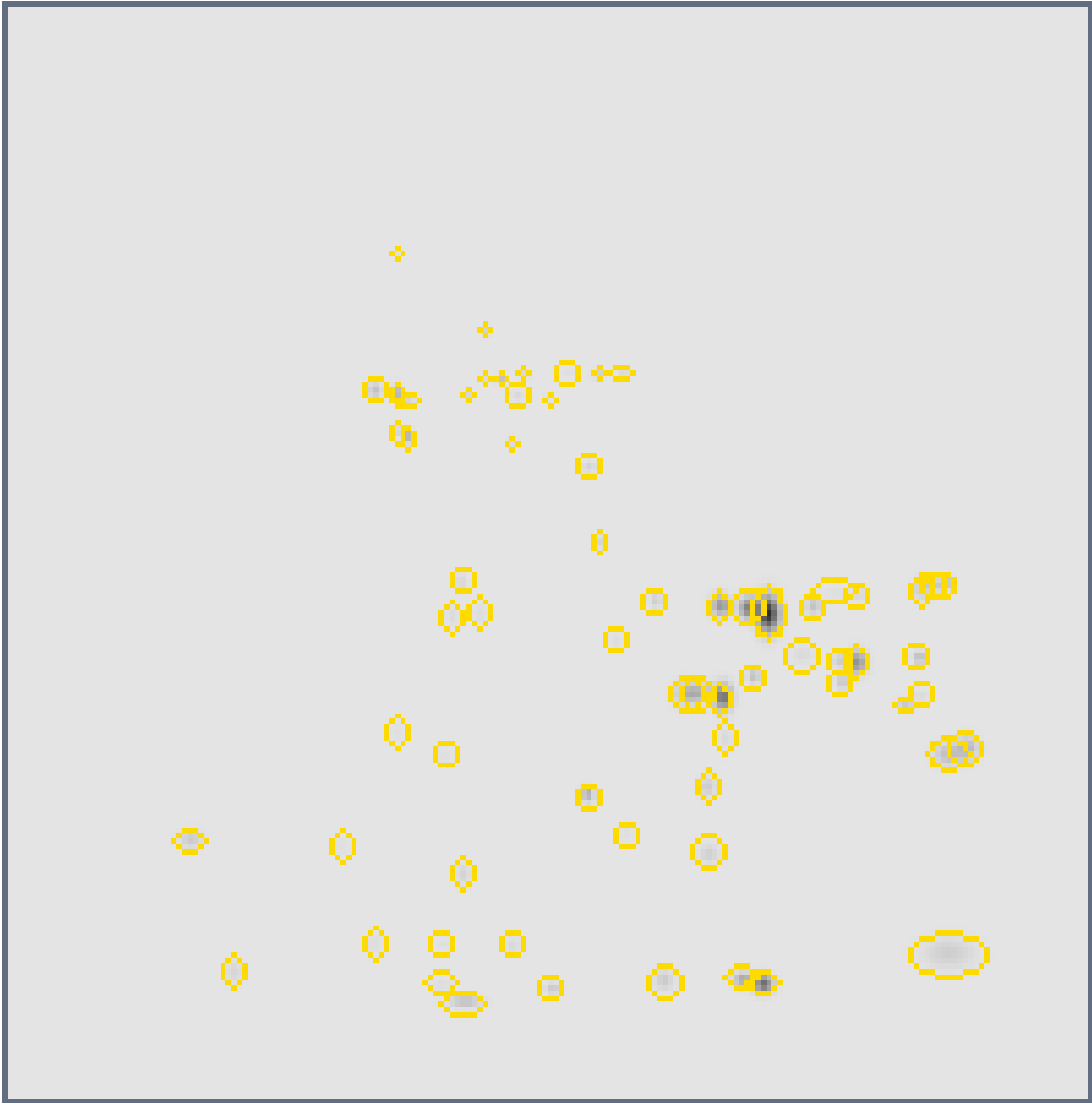
(a)



(b)

Appendix 6

Figure 6.10 High Level MatchSet.
[psi-] as master.
(a) with overlays (b) without overlays

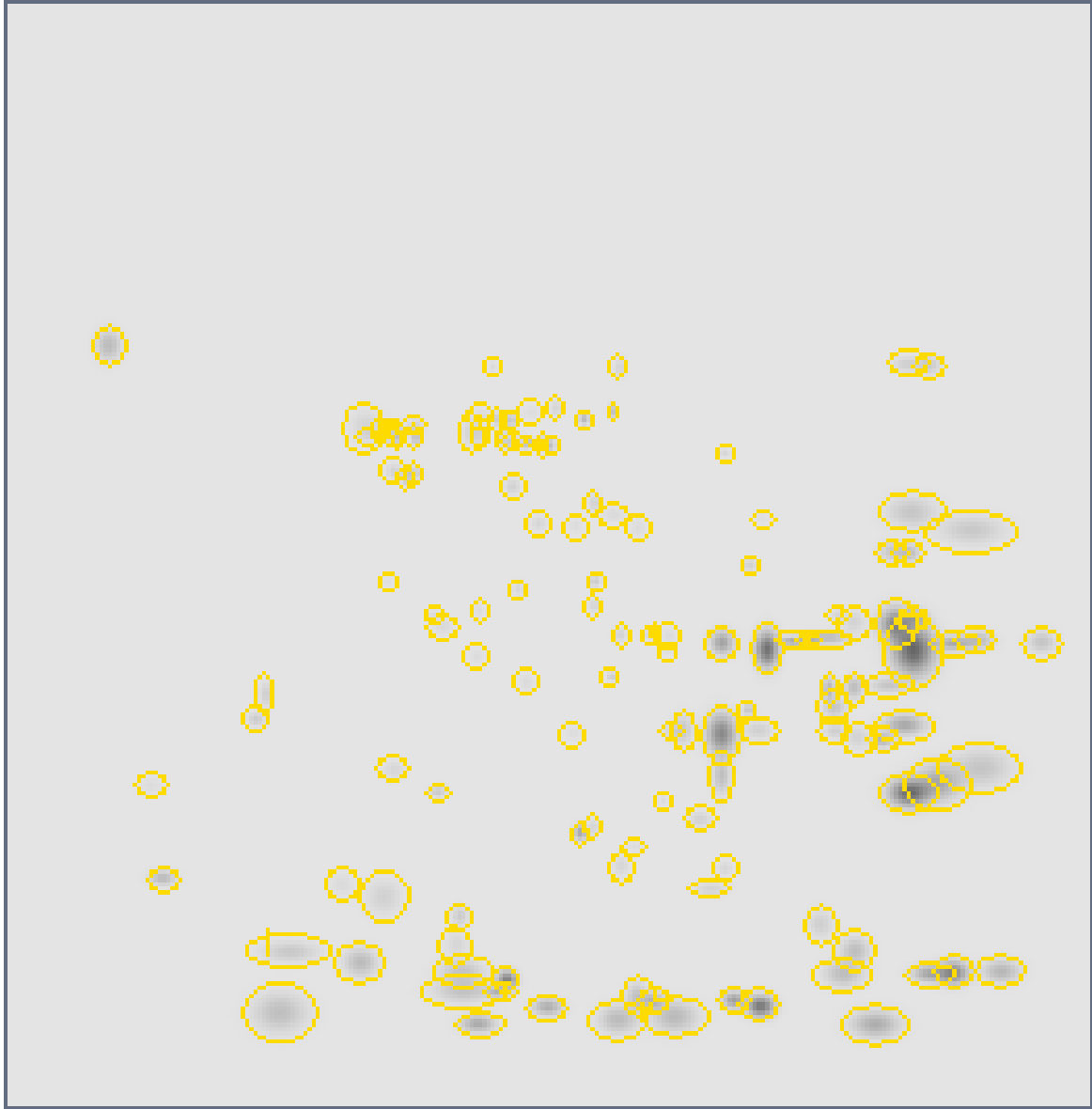


(a)



(b)

Figure 6.11 High Level MatchSet.
[PSI+] as master.
(a) with overlays (b) without overlays



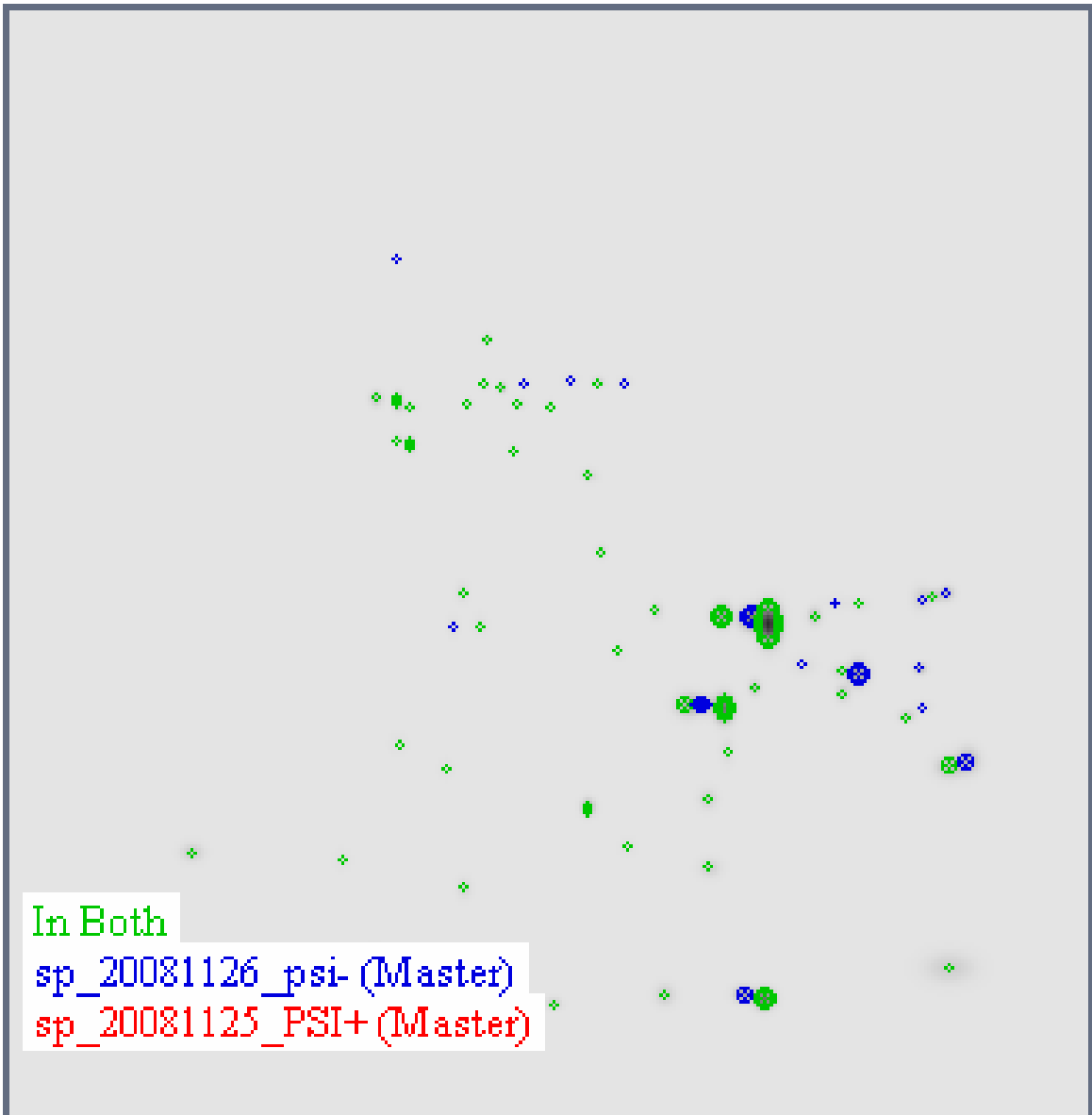
(a)



(b)

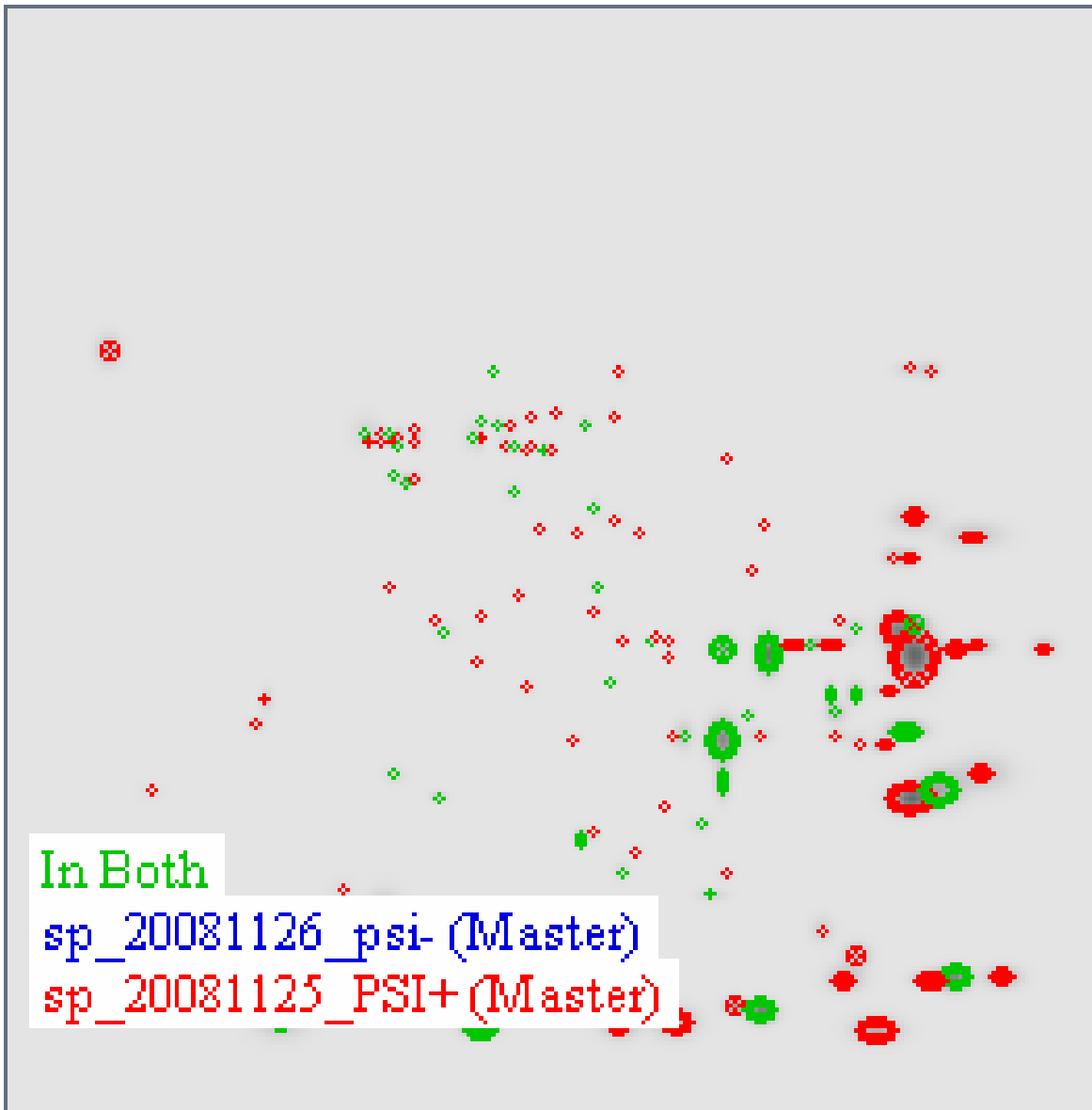
Appendix 7

Figure 7.12 Overlay Image ([psi-] master with [PSI+] matches) used to check for differentiation



Note: No sp_20081125_PSI+ (Master) points are apparent in this figure; however, these matches are represented in the next figure below.

Figure 7.13 Overlay Image ([PSI+] master with [psi-] matches) used to check for differentiation



Note: No sp_20081126_psi- (Master) points are apparent in this figure; however, these matches are represented in the figure above.