THE UNIVERSITY OF HULL

The Proteomic Analysis of B-Cell Receptor Signaling in Chronic Lymphocytic Leukaemia

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By

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ABSTRACT

Chronic Lymphocytic Leukaemia (CLL) is the most common adult Leukaemia in the UK, Western Europe and America. It is a malignancy of naïve B-cells. The clinical course of patients with CLL is heterogeneous; some patients survive for years without treatment, others die of a chemotherapy resistant disease within two years of presentation. Genomic studies have found little variation in patients showing differing prognosis, suggesting that it is the same disease but with varying outcomes. At present there is no cost effective, reliable and routine clinical test which can distinguish patient prognosis and a "watch and wait" strategy is currently in clinical use.

Studies have shown that patients who express mutated IgV_H genes on the B-cell receptor (BCR) have a good prognosis, whereas patients who express unmutated IgV_H genes have a poor prognosis. However IgV_H gene mutational status is an expensive and time consuming test and would not be practical for routine clinical practice. If the B-cell has not been sensitised to a specific antigen (i.e. unmutated IgV_H genes on BCR) it is hyper-responsive to stimulation through the BCR by antigen. Stimulation of the BCR may prevent apoptosis of malignant cells; therefore a hyper-responsive BCR is linked to poor prognosis.

By artificially stimulating cells and using proteomic techniques we have investigated signaling pathways activated by the BCR to gain a greater understanding of the anti-apoptotic nature of the malignant B-cells and to find potential prognostic biomarkers related to a hyper-active BCR.

Protein was extracted from stimulated and unstimulated cells from CLL patients categorised as having a poor prognosis (unmutated IgV_H genes and hyper-responsive BCR). The extracts were separated using conventional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The gels were stained with coomassie blue total protein stain and analysed with statistical software. Proteins with a two-fold ($p<0.05$) change in expression between stimulated and unstimulated samples were excised from the gels and analysed by matrix assisted laser desorption/ionisation with time of flight mass spectrometry (MALDI-TOF-MS). Antibody microarrays were used as a complimentary method to 2D-PAGE and immunoblotting was applied as a verification technique.

Changes in protein expression were detected in response to prolonged BCR stimulation. Many of the proteins have had no previous connection with BCR signaling or leukaemia and give a greater insight to the mechanisms of the BCR. Targets found include ones which are associated with the activation of anaplastic lymphoma kinase (ALK), the plasma kallikrein-kinin system (KKS), the AKT-1 pathway, the MAPK pathways, the adenylate kinase system and involvement in the CD40-dependant activation of B-CLL cells. One of the protein targets found (kininogen) was increased by over two-fold in three independent clinical samples after sustained BCR stimulation. If we understand more about the BCR signaling pathways then we may be able to identify potential prognostic biomarkers and novel targets for therapeutic intervention that may inhibit survival of the malignant B-cells.

PUBLICATIONS

The Proteomic Analysis of B-Cell Signaling in Chronic Lymphocytic Leukaemia. G. L. Eagle, L. Cawkwell, D. Allsup. www.wiley-vch.de/vch/journals/2120/not2wis/HUPO_abstracts/POS-TUE-049-110.pdf, 2008, 10

A Proteomic Approach to Investigate Targets Associated with B-Cell Receptor Signaling in Chronic Lymphocytic Leukaemia. G. Eagle, L. Cawkwell, D. Allsup. Haematologica 2008, 93(s1), 31

Proteomics as a Tool to Investigate Signaling Pathways Activated by B-Cell Receptor Stimulation in Chronic Lymphocytic Leukaemia. G. L. Eagle, D. Allsup, L. Cawkwell. British Journal of Haematology, **141**, 2008, (Suppl. 1), 27

Proteomics as a Tool to Identify Potential Prognostic Biomarkers in Chronic Lymphocytic Leukemia. G. L. Eagle, K. Welham, D. Allsup, L. Cawkwell. In Press

B-Cell Receptor Signaling in Chronic Lymphocytic Leukemia (Review). G. L. Eagle, K. Welham, D. Allsup, L. Cawkwell. Manuscript in Preparation

The Proteomic Analysis of B-Cell Receptor Signaling in RAJI B-Cells. G. L. Eagle, K. Welham, D. Allsup, L. Cawkwell. Manuscript in Preparation

The Proteomic Analysis of B-Cell Receptor Signaling in Chronic Lymphocytic Leukemia. G. L. Eagle, K. Welham, D. Allsup, L. Cawkwell. Manuscript in Preparation

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ABBREVIATIONS

µg	Micrograms
µL	Microlitres
1D-PAGE	One dimensional polyacrylamide gel electrophoresis
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
2D-PAGE/MS	Two dimensional polyacrylamide gel electrophoresis mass spectrometry
AMBIC	Ammonium bicarbonate
ACN	Acetonitrile
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
B-cell	Bursa of fabricius derived cell
BCR	B-cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
Cells/L	Cells per litre
CHAPS	3[(3-cholamidopropyl) dimethylammonio]-propanesulphonate
CHCA	α-cyano-4-hydroxycinnamic acid
CID	Collision induced dissociation
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
DC	Direct current
DDH_2O	Double distilled water
DH_2O	Distilled water
DHB	2, 5-dihydroxybenzoic acid

DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
ERK	Extracellular regulated kinase
ESI	Electrospray ionisation
EtOH	Ethanol
FCS	Fetal calf serum
FISH	Fluorescence in-situ hybridisation
g	Grams
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
H_2O	Water
HB	Haemoglobin
HCl	Hydrochloric acid
HMDS	Haematological Malignancies Diagnostic Services
HPLC	High Performance liquid chromatography
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IEF	Isoelectric focusing
Ig	Immunoglobulin
IgV_H	Immunoglobin variable heavy chain
IPG	Immobilised pH gradient
KDa	Kilodaltons
LC	Liquid chromatography
LP	Linear positive Mode

MALDI	Matrix assisted laser desorption/ ionisation
MALDI-TOF-MS	Matrix assisted laser desorption/ ionisation - time of flight - mass spectrometry
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated ERK kinase
MeOH	Methanol
mg	Milligrams
mL	Millilitres
MLL	Myeloid/lymphoid or mixed lineage leukaemia gene
mM	Millimolars
mm^2	Millimetres squared
mm^3	Millimetres cubed
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MW	Molecular weight
m/z	Mass to charge ratio
ng	Nanograms
nL	Nanolitres
nm	Nanometers
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBMCs	Peripheral blood mononuclear cells
P-ERK	Phosphorylated extra cellular regulated kinase
pI	Isoelectric point
PMA	Phorbol 12-myristate-13-acetate
PMF	Peptide mass fingerprint

PMT	Photomultiplier tube
P-TYR	Phosphorylated tyrosine
QIT	Quadrupole ion trap
RB1	Retinoblastoma 1
RF	Radio frequency
RP	Reflector positive mode
SD	Solubilisation/denaturation
SDS	Sodium dodecyl sulphate ($CH_3(CH_2)_{11}SO_4^-Na^+$)
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TC	Tissue culture
TFA	Tetrafluoroacetic acid
TGS	Tris-glycine-SDS buffer
TOF	Time of Flight
Tris	Tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$)
TYR	Tyrosine
UV	Ultra violet
WBC	White Blood Count
ZAP-70	Zeta associated protein 70

Chapter 1 - A Clinical Introduction to Chronic Lymphocytic Leukaemia

A Clinical Introduction to CLL

1.1 Chronic Lymphocytic Leukaemia

Chronic lymphocytic leukaemia (CLL) is a malignancy of naïve B-cells (Hamblin *et al.* 1999). It is characterised by the progressive accumulation of mature B-cells with a characteristic immunophenotype (Hamblin *et al.* 1999; Lin *et al.* 2002). The clinical course of patients with B-cell CLL is heterogeneous; some patients survive for years without treatment, others die of a drug resistant disease in a year or two of presentation (Lin *et al.* 2002; Castro *et al.* 2005). However, it has been shown that CLL cases share a common mechanism of transformation and/or cell of origin, therefore CLL is one disease irrespective of the heterogeneous clinical course (Rosenwald *et al.* 2001).

1.1.1 Epidemiology

CLL is the most common adult leukaemia in the UK (Figure 1.1). An average of 2400 people in the UK are diagnosed with the disease every year (CancerBacup. 2004). Incidence is dramatically increased in the over 60 age range (Figure 1.2). From 1971 to 2001, deaths from CLL have shown a slight downward trend despite the increase in incidence, reflecting increased survival rates (Cancer Research UK. 2003).

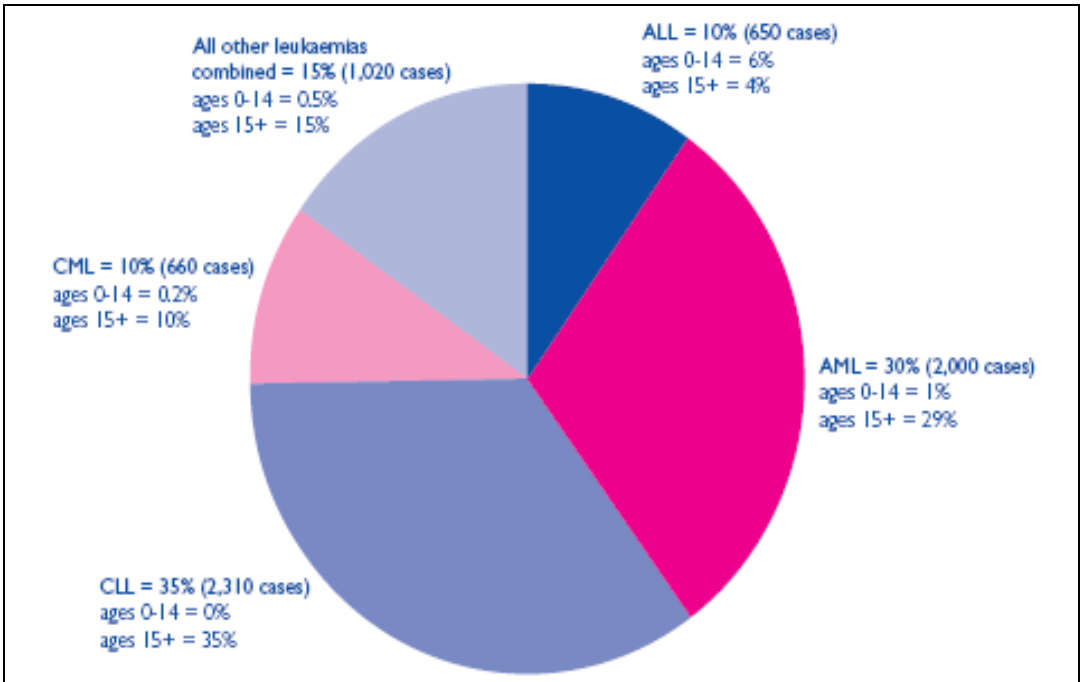


Figure 1.1: Chart to show numbers and proportions of leukaemias by type in the UK in 1999 (Cancer Research UK. 2003)

Pie chart to show incidence of CLL. CLL is observed as the most common leukaemia and constitutes the greater proportion of the chart with 2310 cases reported in the UK in 1999, accounting for 35% of all leukaemias reported. (Office for National Statistics. 2002; ISD Online: Information and Statistics Division NHS Scotland. 2003; Welsh Cancer Intelligence and Surveillance Unit. 2002; Northern Ireland Cancer Registry. 2003).

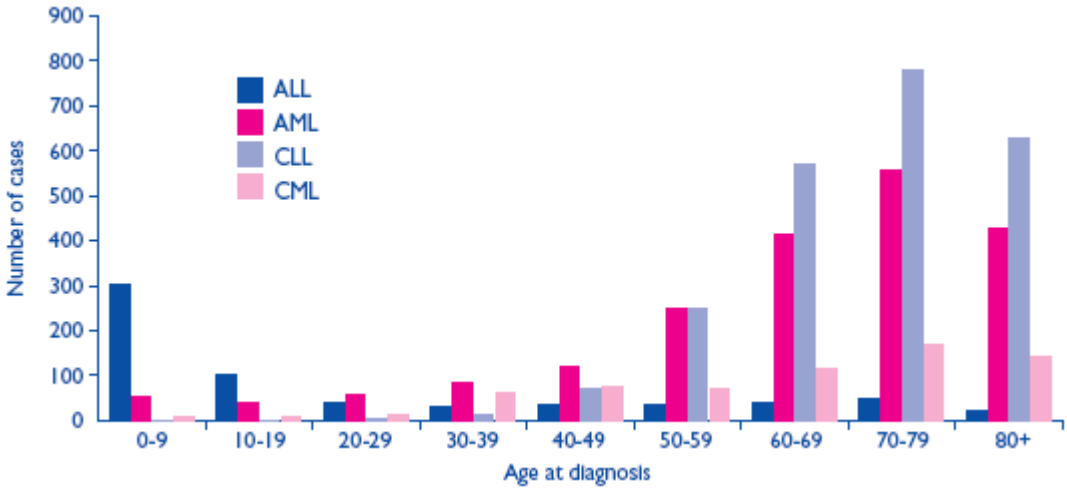


Figure 1.2: Chart to show numbers of cases by age and leukaemia type in the UK in 1999 (Cancer Research UK. 2003)

The chart shows that diagnosis of CLL increases with age. CLL is a disease of the elderly and rarely affects people under the age of 40 (Office for National Statistics. 2002; ISD Online: Information and Statistics Division NHS Scotland. 2003; Welsh Cancer Intelligence and Surveillance Unit. 2002; Northern Ireland Cancer Registry. 2003).

1.1.2 Aetiology

The aetiology of CLL is largely unknown, although it has been suggested that genetic factors may play a role (Chiorazzi *et al*. 2005; Crossen. 1997), it is primarily a disease of the elderly and it usually affects patients over the age of 55 (Kay *et al*. 2002). There is an overall excess of male diagnoses of CLL in a ratio of 2:1 to women (Leukaemia Research. 1996). Although the incidence in black and white populations are approximately equal, the disease in rarely seen in Asian origins irrespective of their residency or lifestyle. This suggests underlying differences in susceptibility (Leukaemia Research. 1996).

1.2 Diagnostic Markers

CLL cells share particular immunophenotypic features (Dighiero *et al*. 2008). The monoclonal population of B-cells in CLL express mature B-cell markers CD5 and CD23, weak expression of surface immunoglobulin and B-Lymphocyte surface antigen CD19 (Hamblin *et al.* 1999; Cochran *et al*. 2003; Chiorazzi *et al.* 2005). A positive expression of CD20 is also an indication of B-cell CLL. A total lymphocyte count of >5 x 10^9 L is required for diagnosis (3.5 x 10^9 L is classed as a normal count).

1.3 Clinical Staging

The disease is said to have a variable course, some patients die within one year after diagnosis while others live for longer than ten years (Rai *et al*. 1975). The clinical staging of CLL is set by two accepted and measurable standardised parameters. These are called the Rai clinical staging method (Rai *et al.* 1975), which is mainly used in the USA, and the Binet prognostic classification (Binet *et al*. 1982), which is used throughout Europe.

1.3.1 The Rai Clinical Staging Method

The Rai clinical staging method puts the disease into five main classifications; 0, I, II, III and IV (Table 1.1). Table 1.1 shows clinical features and median survival. In the study anaemia (haemoglobin (Hb) levels less than 11g/100ml) and thrombocytopenia (platelets count less than 100,000mm^3) were considered. The study was based on 125 patients (Rai et al. 1975).

Table 1.1: Table to show the five classification of CLL staging using the Rai staging method

Rai Stage	Clinical Feature	Median Survival (months)
0	Lymphocytosis (abnormal increase in number of lymphocytes)	>150
I	As 0 + enlargement of lymph nodes	101
II	As 1 + enlargement of spleen or liver	71
III	As 2 + progressive compromise of bone marrow function as revealed by anaemia	19
IV	As 3 + thrombocytopenia (abnormal decrease in number of circulating platelets)	19

1.3.2 Binet Prognostic Classification

The Binet prognostic classification stages the disease into three main classifications; A, B or C (Table 1.2). There are fewer stages than that of the Rai staging method and the system is simple, requiring only clinical examination and routine haematological analysis. Survivals of two series of CLL patients (99 from a retrospective series and 196 from a prospective series) were studied (Binet *et al.* 1982).

Group A was classed as patients with no anaemia (Hb levels above or equal to 10g/100ml), no thrombocytopenia (a platelet count of 100,000-400,000/mm^3) and less than three involved lymph node areas. Fifty-five percent of patients were categorised into group A and did not reach median survival after 10 years.

Group B was classed as patients with no anaemia, no thrombocytopenia and three or more involved lymph node areas. Thirty percent of patients were categorised into group B and reached median survival at 7 years.

Group C was classed as patients with anaemia (Hb less than 10g/100ml) and/or thrombocytopenia (platelets less than 100,000/mm^3). Fifteen percent of patients were categorised into group C and reached median survival at 2 years.

Table 1.2: Table to show the three classification of CLL staging using Binet staging method

Stage	Enlarged Lymph Node Areas	Haemoglobin Levels	Platelets Levels	Median Survival (years)
A	<3	≥10g/100ml	≥100,000mm^3	>10
B	≥3	≥10g/100ml	≥100,000mm^3	7
C	Any number	<10g/100ml	<100,000mm^3	2

1.4 Prognostic Factors

Indications of bad prognosis include:

1. Lymphocyte doubling time less than one year (Hamblin *et al.* 1999)
2. Zeta associated protein 70 (ZAP-70), a critical T-cell receptor molecule (Packham *et al*. 2005), found in over 20% of leukaemic B-cells
3. CD38, a cell surface enzyme involved in regulating B-cell activation (Hamblin. 2004), found in over 30% of leukaemic B-cells
4. Unmutated IgV_H genes on B-cell receptor (see section 2.3)

There is much controversy over using CD38 and ZAP-70 as prognostic markers. Expression of CD38 is known to change during the course of the disease (Chevallier *et al*. 2002) and CD38 expression conflicts with that of IgV_H gene mutational status as a prognostic marker (Hamblin *et al*. 2002). Expression of ZAP-70 has also shown to

conflict with IgV_H gene mutational status (Rassenti *et al*. 2004). It has been shown that some patients show cells that lack ZAP-70, but also show cells with hyper-active B-cell receptors (see section 2.2) (Deglesne *et al*. 2006). ZAP-70 and CD38 are not fully standardised (Hamblin. 2007). There are disagreement as to what defines positivity, and expression levels are suggested to change, therefore further work is required with larger cohorts (Hamblin. 2007). At present IgV_H status remains the strongest predictor of prognosis (Ghia *et al*. 2007; Hamblin *et al*. 1999; Maloum *et al*. 2000). To determine IgV_H status requires a time consuming and expensive test, so at present there is no routine, cost effective, reliable clinical test to show how a patient diagnosed with CLL will progress.

1.4.1 Chromosome Aberrations

In CLL chromosome aberrations are detected in approximately 40-50% of tumours when using conventional chromosome banding analysis (Dohner *et al*. 1999), while fluorescence in-situ hybridisation (FISH) and molecular techniques reveal an even higher incidence (Crossen. 1997).

- Chromosome 17 - Advanced disease is associated with a deletion in 17p. The tumour suppresser gene *p53* is located on chromosome 17p13. and analysis shows that 10-15% CLL patients have mutations in *p53* (Crossen. 1997).
- Chromosome 11 - A deletion in 11q is also associated with bad prognosis. The *myeloid/lymphoid or mixed-lineage leukaemia* (*MLL*) gene is located on the 11q chromosome and is involved in pattern development during embryogenesis (Stanulla *et al*. 2001).

- Chromosome 13 - Good prognosis is associated with 13q deletion as a sole aberration. 13q abnormalities are the most common structural abnormalities in B-CLL (Crossen. 1997). The gene encoding the retinoblastoma 1 (RB1) protein is located on the 13q chromosome and is a critical part of cell cycle control (Crossen. 1997).
- Chromosome 12 - Trisomy 12 is the most frequent numerical chromosome abnormality in B-CLL (Dohner *et al.* 1999). The segment duplicated includes bands 12q13-q21.2, indicating that this region contains genes involved with the pathogenesis of B-CLL tumours carrying the trisomy (Dohner *et al.* 1999).

1.5 Treatment

CLL is known as an incurable disease and patients are only recommended for treatment if the disease progresses or the symptoms become troublesome. Newly diagnosed patients with Binet stage A/B disease are monitored without therapy (Cheson *et al*. 1996). At present there is no clinical test to show how a patient will progress; therefore patients must show symptoms before undergoing any form of treatment. A “watch and wait” procedure is implied, which is often termed by the patients as “watch and worry”. The National Cancer Institute Sponsored Working Group has published recommended criteria for starting treatment (Cheson *et al*. 1996). These include weight loss (greater than 10%) within 6 months, fevers for 2 weeks, night sweats, or extreme fatigue, as well as worsening anaemia and/or thrombocytopenia, autoimmune cytopenias (deficiency of certain elements in the blood caused by an immune response), progressive enlargement of the spleen (splenomegaly), progressive enlargement of the lymph nodes (lymphadenopathy) and lymphocyte doubling time of 6 months (Abbott. 2006).

Oral chemotherapy is usually the first treatment recommended. Chlorambucil and Fludarabine are the most common drugs used. Table 1.3 shows the names and types of the most common drugs used in CLL. Some patients may need to be treated with intravenous chemotherapy, and if patients do not respond to a treatment, combination therapies can be used. Table 1.4 shows the most common combination therapies in CLL.

Table 1.3: Table showing chemotherapy drugs used to treat CLL and mode of action

Drug	Type
Chlorambucil	Alkylating Agent
Fludarabine	Purine Analogue
Cyclophosphamide	Alkylating Agent
Cladribine	Purine Analogue
Vincristine	Vinca Alkaloid
Prednisone	Steroid
Prednisolone	Steroid
Doxorubicin	Anthracycline Antibiotic
Rituximab	Monoclonal Antibody
Alemtuzumab	Monoclonal Antibody

Table 1.4: Table showing drugs used in chemotherapy combinations to treat CLL

Combinations	Drugs
COP	Cyclophosphamide, Vincristine and Prednisolone
CHOP	Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone
CAP	Cyclophosphamide, Doxorubicin, and Prednisolone
FC	Fludarabine and Cyclophosphamide
FCR	Fludarabine, Cyclophosphamide and Rituximab

Chapter 2 - A Molecular Introduction to Chronic Lymphocytic Leukaemia

A Molecular Introduction to Chronic Lymphocytic Leukaemia

CLL is an accumulation of mature B-cells (Hamblin et al. 1999) that have escaped apoptosis but do not proliferate (Bernal et al. 2001).

2.1 The B-Cell

The abbreviation “B” in B-cell stands for bursa-derived cells from the Bursa of Fabricius, which is a lymphoid organ found in birds were B-cells were first found to mature (Glick *et al*. 1964). B-cells are lymphocytes that mature in the bone marrow, travel through the lymphatic system in the blood and enter the lymph node. When in the germinal centre of the lymph node each cell is sensitised to a specific antigen (Stevenson *et al*. 2004), such as a toxin, bacterium or enzyme derived from outside the cell and from foreign cells (Figure 2.1). When an antigen is introduced into the body it stimulates the B-cell sensitised specifically to it which produces antibodies specific to that antigen.

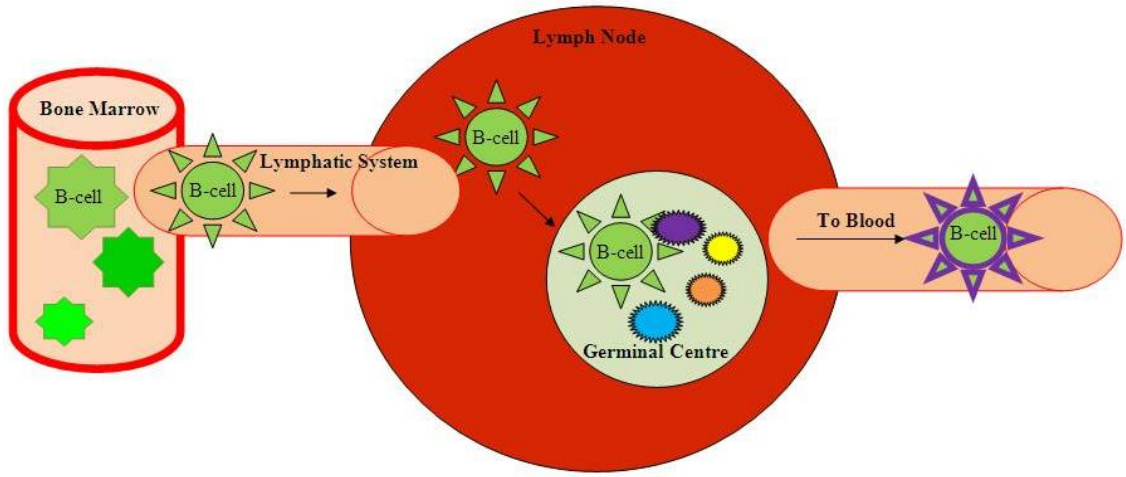


Figure 2.1: Diagram to show development of normal B-cells

The B-cells become mature in the bone marrow, travel through the lymphatic system in the blood and enter the lymph node, where, in the germinal centre each cell is sensitised to a specific antigen.

CLL is a tumour of mature B-cells (Hamblin et al. 1999) that have escaped apoptosis and do not proliferate (Bernal et al. 2001). The CLL B-cells are inert *in vitro* (Dighiero *et al*. 2008), suggesting that the cells rely on their microenvironment for survival (Burger. 2007). Most CLL research is therefore based on established Burkitt's lymphoma B-cell lines such as RAJI, DAUDI and RAMOS.

2.2 B-Cell Receptor

B-cells express thousands of receptors on their surface, which bind to a targeted antigen if encountered to initiate cellular signaling pathways. Data has shown that stimulation of the B-cell receptor (BCR) (Figure 2.2) by antigen may prevent apoptosis of the malignant B-cells, and thus tumour behaviour relates to the differential ability of the BCR to respond to antigen (Stevenson *et al*. 2004). Good prognosis is associated with a BCR which is unresponsiveness (only responsive to it's specific antigen) and bad

prognosis is associated with a BCR, which maintains the full capacity to respond to any antigen which the cells may be chronically exposed to (Scielzo *et al.* 2005).

2.3 Mutations in the Variable Region of the Heavy Chain Immunoglobulin Genes

The BCR consists of membrane bound Immunoglobulin (Ig) arranged in heavy and light chain loci (Figure 2.2).

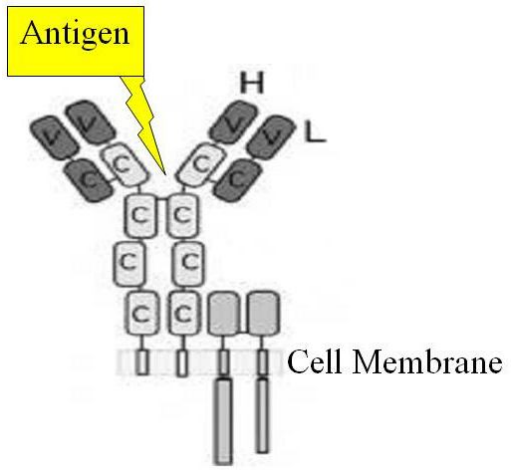


Figure 2.2: Diagram showing the BCR structure and membrane bound immunoglobulin

B-cells express thousands of receptors on their surface, which bind to a targeted antigen to initiate cellular signaling pathways.
Key: H: Heavy chain, L: Light chain V: Variable region, C: Constant region

CLL may be divided into clinically distinct groups depending on whether the gene that controls the shape of the variable region of the heavy chain surface immunoglobin (IgV_H) has under gone mutation or not (Cochran *et al.* 2003). The mutational status of the IgV_H genes is an excellent prognostic indicator (Hamblin *et al.* 1999), but the analysis of the IgV_H genes is a very time consuming expensive test and cannot routinely

be done in the clinic. CLL patients with mutated IgV_H genes have a favourable prognosis over those with unmutated IgV_H genes (Cochran *et al.* 2003).

There are a few possible theories to explain IgV_H gene mutational status as an indication of prognosis. One reason may be as follows: Genetic mutations occur in the germinal centre, which is situated in the lymph node. Here the cell is bombarded with antigens and the gene that controls the shape of the IgV_H is mutated, so that the cell is sensitised to a specific antigen. A BCR which expresses unmutated IgV_H genes has not been sensitised to a specific antigen. This suggests that it may not have entered the germinal centre, and therefore has not had the shape of the IgV_H gene altered and is able to respond to a variety of non-specific antigens (Figure 2.3). Alternatively, the cell may have been exposed to antigen in the germinal centre but with insufficient stimulus (Stevenson *et al.* 2004).

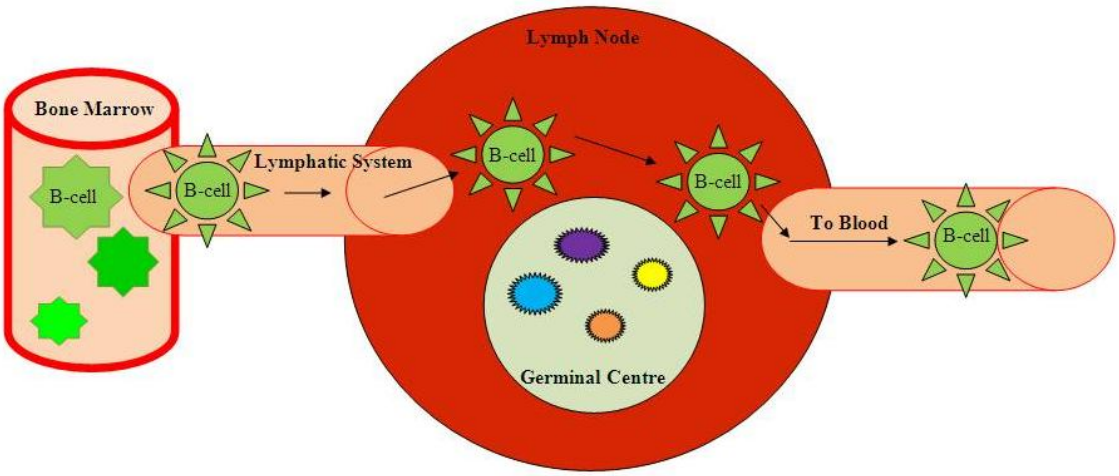


Figure 2.3: Diagram to show theoretical development of the CLL B-cell which lacks IgV_H mutation

The B-cell has not entered the germinal centre in the lymph node, and has not had the shape of the IgV_H gene altered. Therefore the BCR is not sensitised to a specific antigen and can trigger cell signaling in response to a variety of non-specific stimuli.

Because the BCR is not sensitised to a specific antigen, it is hyper-responsive to stimulation from a variety of non-specific antigens. The over-active BCR will incessantly initiate cascades of cellular signaling pathways, which may support the survival of malignant B-cells (Bernal *et al.* 2001). This may be the reason why unmutated IgV_H genes is related to a poor prognosis (Hamblin *et al.* 1999). It has also been shown that CLL patients share a common gene expression "signature" irrespective of IgV_H gene mutational status (Rosenwald *et al.* 2001).

2.4 B-Cell Receptor Signaling

When an antigen stimulates the BCR, it translocates to lipid-enriched microdomains in the membrane termed lipid rafts (Figure 2.4) (Allsup *et al.* 2005). The lipid rafts are cholesterol-rich plasma membrane microdomains, and serve as a platform for B-cell

signaling (Cheng *et al*. 2001). Receptor stimulation induces phosphorylation of the receptor linked tyrosine (TYR) kinases (Stevenson *et al.* 2004). This leads to recruitment of other kinases that become phosphorylated. This results in the activation of signaling cascades (Figure 2.5) that control cell proliferation, differentiation and apoptosis.

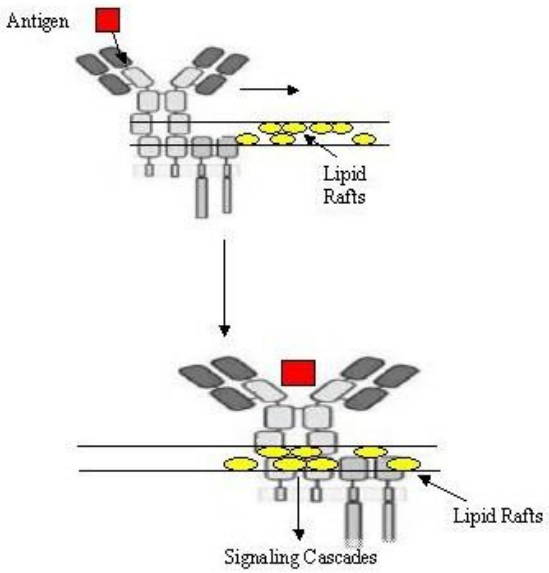


Figure 2.4: Figure to show BCR ligation into lipid rafts

When an antigen stimulates the BCR, it translocates to lipid-enriched microdomains in the membrane. The lipid rafts act as a platform for B-cell signaling.

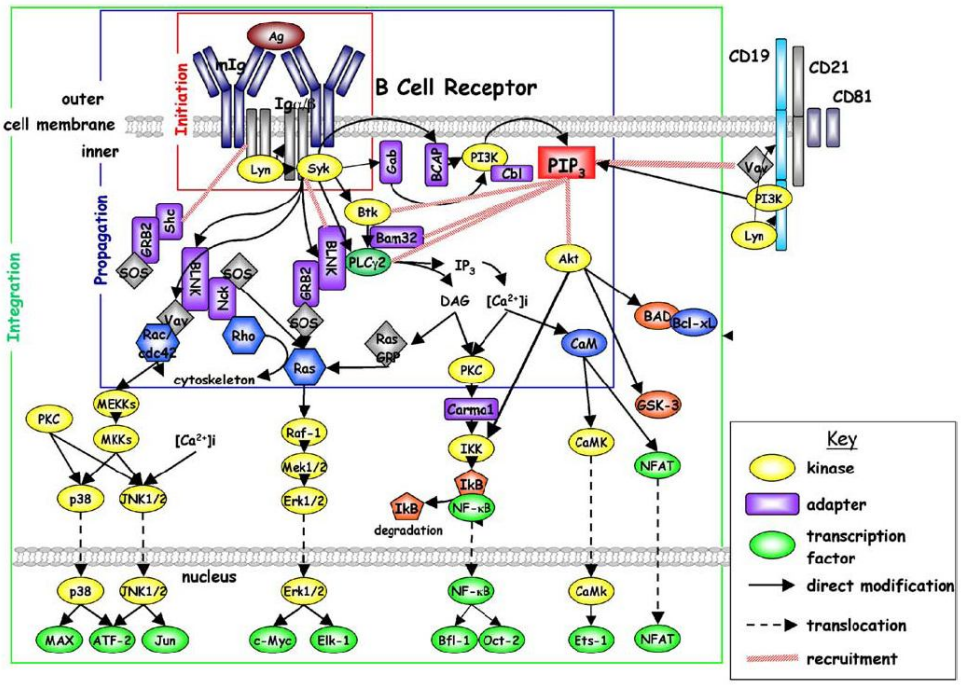


Figure 2.5: The complex signaling pathways associated with BCR activation (Dal Porto *et al*. 2004)

BCR stimulation induces phosphorylation of kinases. This results in the activation of complex inter-connected signaling pathways which control cellular functions such as proliferation, differentiation and apoptosis.

The mitogen activated protein kinase (MAPK) pathway is an example of one of the signaling pathways induced by BCR stimulation (Figure 2.6). When an antigen stimulates the BCR, auto-phosphorylation is triggered on the TYR (TYR) residues. Signaling molecules attach to the phosphorylated TYR (P-TYR). This binding stimulates the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), which activates a G protein (Kolch. 2000). The G protein is a protein found in the inner surface of the plasma membrane. They are called G proteins because they bind guanine nucleotides. The G protein activates a kinase cascade (Kolch. 2000).

The MAPK/extra cellular regulated kinase (MAPK/ERK) pathway (Figure 2.6) is an example of a MAPK signal transduction pathway. The pathway is very complex and regulates cellular activities such as cell proliferation, differentiation and importantly, apoptosis. This pathway has important implications in CLL due to survival capabilities of mature CLL B-cells in the blood. The G protein, Ras, is activated after BCR stimulation by an antigen. Ras recruits Raf, a cytosolic protein kinase (MAPKKK) (Dumaz *et al.* 2005) from the cytosol to the membrane. Raf is activated by kinases that phosphorylate the activating sites and de-phosphorylate the inhibitory sites. Activated Raf phosphorylates and activates mitogen activated ERK protein kinase (MEK). Phosphorylated MEK (P-MEK) activates ERK. Activated ERK then distributes into the cytosol and the nucleus. The activation of these proteins initiates other signaling pathways making the cascades interconnected and very complex. The result is the activation of various cellular processes essential for cell survival.

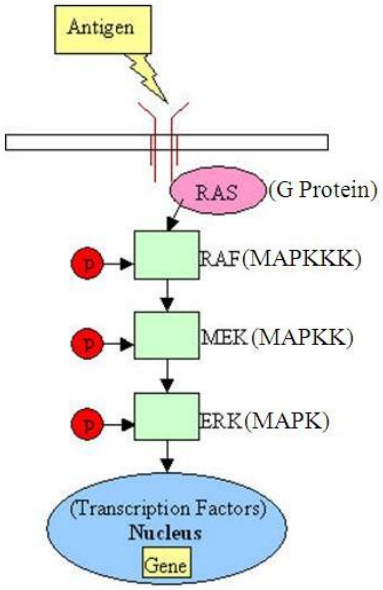


Figure 2.6: Diagram of the MAPK pathway

Upon antigen stimulation, kinases are phosphorylated resulting in the initiated of signaling pathways.
Key
p: Phosphorylation
MAPKKK: Mitogen Activated Protein Kinase Kinase Kinase
MAPKK: Mitogen Activated Protein Kinase Kinase
MAPK: Mitogen Activated Protein Kinase
MEK: Mitogen Activated ERK Protein Kinase
ERK: Extra Cellular Regulated Kinase

The BCR can be artificially stimulated to produce cascades of signals. Antibodies can be used that react with the light chain immunoglobulin (Ig) on the surface of the BCR, resulting in the clustering of BCR and initiating activation. Naïve and mature B-cells express the IgM antibody molecule, which has five base units covalently bonded with disulfide bonds. Each base unit has two binding sites. The B-cell can be artificially activated by cross-linking the BCR with an anti-IgM antibody in vitro (Haas et al. 2000). By analysing the expression status of phosphoproteins downstream of the pathways, such as P-ERK, activation of the BCR can be confirmed. Chemicals such as phorbol 12-myristate 13-acetate (PMA) can also be used to stimulate B-cells. PMA

activates protein kinase C, which will subsequently lead to the activation of the MAPK pathway. Sole expression of IgG (an antibody with one base unit) is rare in CLL B-cells (Potter *et al*. 2006), therefore it can be used as an isotype control when activating cells with IgM (Silver *et al*. 2003).

2.5 Summary

Chronic lymphocytic leukaemia is the commonest leukaemia in the UK. The clinical course of patients with B-cell CLL is heterogeneous; some patients survive for decades without treatment, others die of a drug resistant disease in a year or two of presentation. There is no routine, cost effective clinical test to show how a patient will progress. IgV_H gene mutational status has proven to be an excellent indication of prognosis, but the test is time consuming and expensive. The mutational status is related to the BCR responsiveness, which therefore can also be linked to prognosis. A bad prognosis is associated with unmutated IgV_H genes and consequently a hyper-responsive BCR that is not sensitised to a specific antigen. A good prognosis is associated with mutated IgV_H genes and a BCR that has been sensitised to a specific antigen to trigger a specific pathway. The signaling pathways which the hyper-active BCR is activating may be the key to understanding the survival skills of the mature B-cells in CLL.

Chapter 3 - An Introduction to Proteomic Techniques

An Introduction to Proteomic Techniques

3.1 Proteomics

The search for cancer-causing alterations within the whole genome is complicated by the different ways genes may be transcribed into a variety of functionally different proteins which can themselves undergo essential functional changes (Baak *et al*. 2005). The expression levels of all proteins would arguably provide the most relevant single data set characterising a biological system (Cox *et al*. 2007). There are many reasons why protein expression analysis (proteomics) is considered an alternative method to mRNA-based measurements (transcriptomics). For example proteins of interest in blood are mainly extracellular and transcriptomics is not directly relevant. Also proteomics delivers the desired end point, namely the protein expression level of a gene of interest (Cox *et al.* 2007). Proteins can also undergo post-translational modifications which affect the protein's stability, localisation and function (Aebersold *et al*. 2003). Therefore proteomics gives a better reflection of the current state of the cell's microenvironment than gene expression profiling (genomics) or transcriptomics. In 2001 Rosenwold et al used genomics to distinguish between CLL patients with good and bad prognosis. They found that CLL is characterised by a common gene expression "signature" irrespective of IgV_H mutational status which is an accepted prognostic marker (see section 2.3) (Rosenwald *et al.* 2001). However, changes in protein expression have been found between CLL patients with differing prognosis (Scielzo *et al.* 2005; Cochran *et al.* 2003), therefore, we have used proteomics as a technique to investigate B-cell receptor signaling, an indication of prognosis (see section 2.4) in CLL.

Proteomics has been an important developing area of research for the past two decades (Aldred et al. 2004). The recent developments in mass spectrometry, such as high sensitivity and automation of protein identification has significantly increased the number of large scale proteomics projects (Stasyk *et al*. 2004). Although previously called 'fishing expeditions', today the term 'discovery science' is respectfully used for such large scale studies (Baak *et al.* 2005). Initial experiments using cell lines are now giving rise to the analysis of complex tissue and biological fluids to establish protein changes associated with disease (Aldred *et al.* 2004).

3.1.1 One Dimensional Polyacrylamide Gel Electrophoresis

One dimensional (1D) polyacrylamide gel electrophoresis (PAGE) is used to separate proteins in one dimension by molecular weight into a polyacrylamide gel in a tris-glycine-sodium dodecyl sulphate (SDS) buffer (TGS buffer).

Protein samples must be extracted or resuspended in a suitable buffer such as Laemmli buffer. Components of a Laemmli buffer are:

- Detergent – (e.g. SDS) to solubilise membrane proteins and break down protein-protein interactions
- Reducing Agent – (e.g. β-mercaptoethanol) to reduce protein disulphide bonds prior to SDS-PAGE
- Buffer – e.g. tris (hydroxymethyl) aminomethane-hydrochloride (Tris-HCl pH6.8)

- Glycerol – to increase the density of a sample so that it sinks into the well when loading onto a gel
- Protease Inhibitors – to inhibit the function of protease enzymes, which break down proteins. Protease inhibitor cocktails are available commercially
- Phosphatase Inhibitors – to inhibit phosphates enzymes, which remove phosphate groups from proteins by hydrolysing phosphoric acid esters. It is important to use phosphatase inhibitors if studying phosphoproteins. Phosphatase inhibitor cocktails are available commercially
- Dye – (e.g. bromophenol blue) to colour protein sample to enable visualisation

The protein mixture is loaded into the gel and separated out into individual protein bands dependant on molecular weight (Figure 3.2). The proteins are then stained to visualise them using a dye such as coomassie blue (see section 3.1.3). A molecular weight marker is run in the gel. These are available to purchase ready made and contain a "ladder" of known proteins of specific weights, which are measured in Kilodaltons (KDa). The protein bands are compared to the known standard in the ladder to confirm their weight. Proteins can then be excised; digested and analysed as described in section 3.1.4. It is an excellent technique if there are only a small number of proteins to be separated. However, if there are too many proteins, the bands will be masked by other proteins of a similar weight. By separating the proteins in a second dimension according to pH (see section 3.1.2) resolution is improved and a large number of proteins can be visualised and accurately excised.

3.1.2 Two Dimensional Polyacrylamide Gel Electrophoresis

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a method widely used to separate proteins. Proteins are first separated horizontally onto an immobilised pH gradient (IPG) strip on the basis of their pH dependant, net charges (pI) by isoelectric focusing (IEF). This is known as the first dimension of separation. They are then separated vertically on the basis of their molecular masses by electrophoresis into a polyacrylamide gel in a TGS buffer. This is the second dimension of separation. Proteins spots on the gel are visualised using a stain (Figure 3.1).

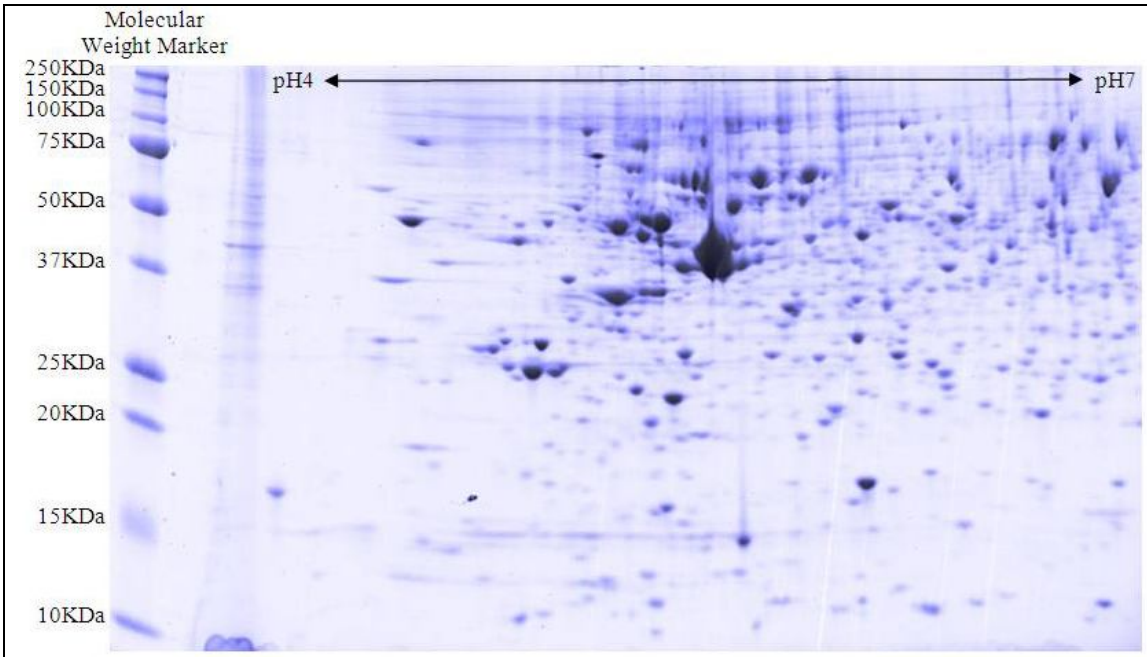


Figure 3.1: Picture of a 2D gel stained with coomassie blue protein stain

The picture is from a complex protein mixture extracted from a blood sample, which has been separated out into individual protein spots. Proteins where first separated horizontally dependant on there pI on an IPG strip. The IPG strip was then placed into a gel, where and the proteins ran out of the IPG strip and separated vertically dependant on their molecular weight. A molecular weight marker was included in the gel and the gel was stained with coomassie blue to visualise the proteins.

When the protein spots are visible, they are cut from the gel, digested into peptides using an enzyme such as trypsin, and mass spectrometry (MS) is then used to generate a peptide mass fingerprint (PMF) which can be used to identify the protein.

Throughout the 2D-PAGE experimental workflow it is vital that the sample is not contaminated by foreign proteins. The sample must be handled with nitrile gloves to prevent polymer contaminations from latex gloves, and extra care must be taken to avoid keratin contamination from skin and hair throughout the experiment. New, clean equipment should be used. Proteins samples should be frozen at -80^{o}C or in liquid nitrogen in the presence of protease inhibitors (Beranova-Giorgianni. 2003).

3.1.2.1 First Dimension: Separation by pH

To prepare the sample for gel electrophoresis the protein sample needs to be extracted in, or resuspended in a solubilisation/denaturation (SD) buffer (Beranova-Giorgianni. 2003). This is used to ensure reliable running of the IEF. Components of the SD buffer are:

- Chaotrophe – (e.g urea/thiourea) to disrupt hydrogen bonded structures in the sample protein and increase solubility
- Reducing Agent – (e.g. dithiothreitol (DTT)) to reduce disulphide bonds, so that all proteins in the sample have the same linear shape
- Detergent – (e.g 3-((3-Cholamidopropyl)Dimethylammonio)-1-propanesulphonate (CHAPS)) to solubilise membrane proteins and break down protein-protein interactions

- Ampholytes – (e.g. Bio-Lyte® (Bio-Rad)) to establish a stable pH gradient for use in IEF
- Protease Inhibitors – to inhibit the function of protease enzymes
- Phosphatase Inhibitors – to inhibit phosphatase enzymes
- Dye – (e.g. bromophenol blue) to colour protein sample

In order to separate out the proteins, a pH gradient must be established. The proteins are placed in a medium with a pH gradient and an electric field is applied. The proteins will migrate towards the electrode with the opposite charge and will pick up or lose protons. The net charge and the mobility will decrease and eventually the proteins will slow down. Each protein will stop at the point in the pH gradient that is equal to its pI.

An IPG is commonly used for IEF. Immobilines are buffering acrylamide derivatives immobilised within a polyacrylamide gel, which produce a pH gradient that does not drift. An IPG is stable for IEF as the gel is plastic backed and the pH gradient is fixed. Also an IPG can be applied to larger protein samples without gradient degeneration and decreased batch variability. The disadvantage of an IPG is that membrane and hydrophobic proteins can be poorly represented in SDS-PAGE. This could be due to protein/gel interactions during IEF. However the advantages of using an IPG outnumber the disadvantages.

IPG strips are pre-packed from suppliers and arrive dehydrated and in a variety of pH ranges. Before they are used they must be rehydrated. If the strip is rehydrated in the presence of the sample (in-gel rehydration) the resolution is improved, and large sample volumes can be handled (Rabilloud *et al*. 1994).

3.1.2.2 Second Dimension: Separation by Molecular Weight

When the proteins have been separated by IEF they can be further separated by molecular weight by SDS-PAGE. The IPG strips must be equilibrated before they can be used for SDS-PAGE to further denature proteins, maintain solubility, and establish an appropriate pH for SDS-PAGE analysis. DTT is added to reduce any re-formed disulphide bonds and iodoacetamide (IAA) is added to alkylate the sulphydryls, converting them to carboxyamidocysteine residues so that they cannot recombine to form disulfides. It is important that in the second dimension the proteins are treated with SDS to give them a negative charge and to denature them (Garfin. 2003). The equilibrated IPG strip is placed onto the top of the polyacrylamide gel and an electric field is applied. The proteins migrate into the gel and separate out according to their molecular weight.

3.1.3 Visualising Proteins

After the SDS-PAGE has been performed the proteins must be visualised in order to excise them, so the proteins are stained. There are various different staining methods, the most popular being coomassie blue staining as it is relatively cheap, quick and easy. This stain can detect down to 80ng of protein (Figure 3.2) and it is compatible with MS.

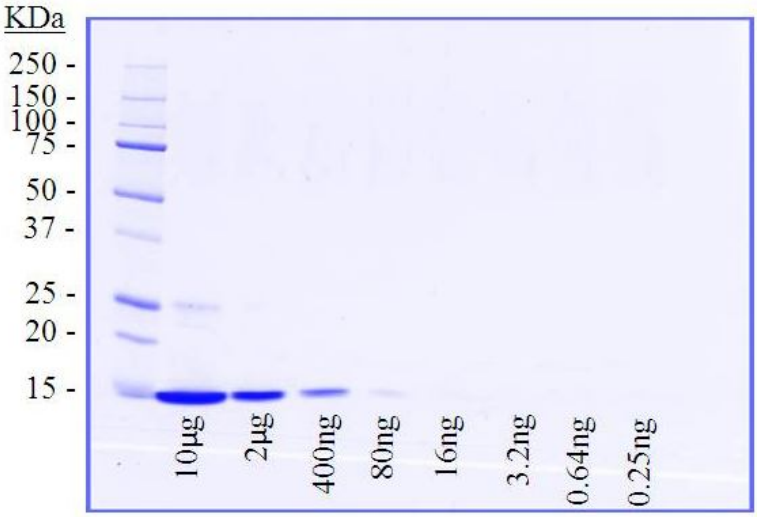


Figure 3.2: Picture of a 1D gel showing the sensitivity of coomassie blue protein stain

The picture shows a 1D gel loaded with different concentrations of pure protein standard, cytochrome C (12KDa). The protein was stained with coomasie blue to visualise and a molecular weight ladder was included to confirm protein band weights. The result shows that coomassie can detect down to 80ng of protein.

Silver staining is another popular option. The stain can detect 0.5-1.2ng protein, which makes it more sensitive than coomassie. However, it is relatively time consuming, messy and expensive. Silver stain is not usually compatible with MS; however, some suppliers now produce a range suitable for MS. The quantification of protein spots is unreliable with silver staining because of its lack of dynamic range.

Fluorescent stains are becoming more popular. This is because they can detect 1-2ng of protein, they are selective and quantitative. They range in price and most are quick and easy to use. Commercially available total protein fluorescent stains include Sypro Ruby (Molecular Probes), Flamingo (Bio-Rad) and Krypton (Pierce) (Table 3.1)

Table 3.1: Table to show cited ranges and excitation/emission maxima of total protein fluorescent stains.

Fluorescent Stain	Protein Detection	Excitation/Emission Maxima
Sypro Ruby	0.25ng-1ng	450/610nm
Flamingo	0.5ng-1µg	532/605nm
Krypton	>0.25ng	520/580nm

These are total protein stains but there are fluorescent stains which only stain specific proteins, such as phosphoproteins, membrane proteins and glycoproteins. Pro-Q Diamond phosphoprotein gel stain (Molecular Probes) is a selective stain for phosphorylated proteins. Pro-Q Diamond stain can detect 1-16ng of protein and has an excitation/ emission maxima of ~555/580nm. A robotic system is an advantage for spot excision as visualising fluorescently stained proteins can be a problem. However, the fluorescent stain can be excited by a UV light source and the spots can then be cut out manually. Fluorescent stains are light sensitive which makes handling them a problem. Fluorescent stains are excellent for visualising low amounts of proteins, however, if the proteins are to be identified, the technique used for identification must retain a similar level of sensitivity.

3.1.4 Protein Identification

The protein spots of interest are individually cut out of the gel. The proteins must then be de-stained to prevent the stain interfering with the analysis. The proteins are too big to be removed from the gel; therefore they are digested within the gel into small

peptides for analysis. This can be done using an enzyme, such as trypsin, which cleaves the protein at the C-terminal side of lysine and arginine (Beranova-Giorgianni. 2003).

The sample may need to be de-salted before MS analysis. This can be done by diluting the sample with buffer or with the use of desalting tips such as Zip Tips (Millipore). The pipette tips contain immobilised C18, C4 or another resin attached to the tip. The sample is drawn into the tip and the peptides bind to the resin. The peptides can then be washed and eluted. However, 100% recovery of peptides cannot be guaranteed. It is not always necessary to desalt samples when using a MALDI-TOF-MS and it may depend on the instrument.

When the samples have been prepared they can then be analysed by MS to determine the peptide mass fingerprint. The sequenced peptides are traced back to the parent protein by using bioinformatics.

2D-PAGE coupled with MS is a very popular method for identifying biomarkers and differences in protein expression between samples; however, it does have its limitations. The main problem is contaminations from abundant proteins such as keratins. Hair and skins cells can fall into tubes and samples, which will be digested with the sample when trypsin is added. The result is a mixture of sample peptides and contaminating peptides which cause high intensity MS peaks. These peaks can shroud lower abundant proteins resulting in insignificant identifications. However, an experienced analyst will be able to edit the MS data to disregard the contaminating peaks (see Appendix H). Co-migration of proteins causes problems when excising and identifying as there maybe more than one protein present in the gel piece excised. In addition 2D-PAGE may exclude highly hydrophobic, very acidic, very basic, very small, very large, and low

abundant proteins. However, apart from the restrictions mentioned, the technique is global and there is no method at present which is equivalent.

3.2 Mass Spectrometry

The two main types of ionisation techniques used in proteomics for MS are:

- Matrix assisted laser desorption ionisation (MALDI) (Aebersold *et al*. 2001; Lin *et al*. 2003)
- Electrospray ionisation (ESI) (Aebersold *et al.* 2001; Lin *et al.* 2003)

3.2.1 Matrix Assisted Laser Desorption Ionisation

MALDI uses energy from a laser to ionise biomolecules (Lin *et al.* 2003). The sample to be analysed is mixed with small, energy absorbing, organic matrix molecules, such as 2, 5 dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA). The matrix and sample are mixed in an appropriate solvent to prevent aggregation of the sample. The mixture is spotted onto a MALDI sample plate, which is a flat stainless steel surface containing hydrophilic anchors on the surface (Figure 3.3). The solvent is removed under vacuum conditions or by evaporation, leaving co-crystallized peptides homogeneously dispersed within matrix molecules.

Figure 3.3: Picture of a MALDI sample plate

Picture of an AnchorChip™ MALDI Target (#209515; Bruker Daltronics). The sample plate is a made from stainless steel and contains spotting areas with hydrophilic anchors on the surface which are arranged on a coordinate grid. The spotting areas and hydrophilic anchors come in a range of sizes and shapes dependant on the amount and type of sample used.

Ionisation occurs when the matrix molecules absorb energy from the photon energy provided by the laser and transfers it into excitation energy (Figure 3.4) (Lin *et al.* 2003). When the pulsed laser beam is tuned to the appropriate frequency, the energy is transferred to the matrix, which is partially vaporised, carrying intact peptides into the vapour phase and charging the amino acid chains. MALDI produces mainly singly charged ions, which allows the mass to charge (m/z) value to be determined.

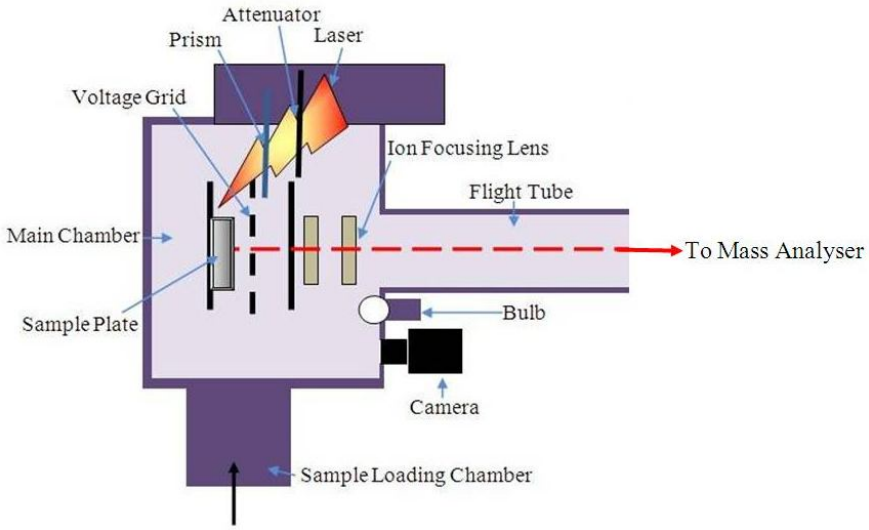


Figure 3.4: Schematic of a MALDI source

A laser is used to transfer energy to the matrix carrying it and the peptides towards the mass analyser.

Multiple laser shots are used to improve the signal-to-noise ratio and the peak shapes, which increases the accuracy of the molecular mass determination. The user can determine how many shots the laser will fire per acquisition. Usually this range is between twenty and fifty laser shots per spectra acquirement. Multiple spectra are acquired and summed to give “averaged” spectra. Good quality spectra should have a minimum of 200 laser shots combined from each acquirement to give the final spectra. However, if more spectra are acquired and combined, the peaks will be better defined making peak picking/editing and the protein identification process easier. This is more relevant to DHB matrix rather than CHCA, as the DHB/sample mixture is not a homogenous mixture due to the large size of the DHB crystals. Peptides are not distributed equally in the co-crystallisation on the sample plate, which means that the user may need to “hunt” for the peptides. CHCA dries to form a fine powder, making it easier to gain spectra as the mixture is homogenous.

3.2.2 MALDI-TOF Mass Analyser

Time of flight (TOF) mass analysers are used with MALDI-MS (Aebersold *et al.* 2001). MALDI-TOF-MS is a favoured technique for generating peptide mass fingerprinting to allow the identification of unknown proteins (Beranova-Giorgianni. 2003) as it is a robust, sensitive technique (to femtomole levels) which can analyse a large mass range. The mass analyser is responsible for separating the ions by their mass to charge ratios (Lin *et al.* 2003). The ions travel the same distance down an evacuated field-free flight tube; the smaller ions arrive at the detector in a shorter amount of time than the larger ions. Separated ion fractions arriving at the end of the drift region are detected by a recorder that produces a signal upon impact of each ion group. By adding an ion reflector at the end of the flight tube, the mass resolution can be improved. The ion reflector is essentially a mirror which increases the distance at which the ions have to travel, therefore correcting for small energy differences among ions (Figure 3.5). The digitised data generated from successive laser shots are summed yielding a TOF mass spectrum.

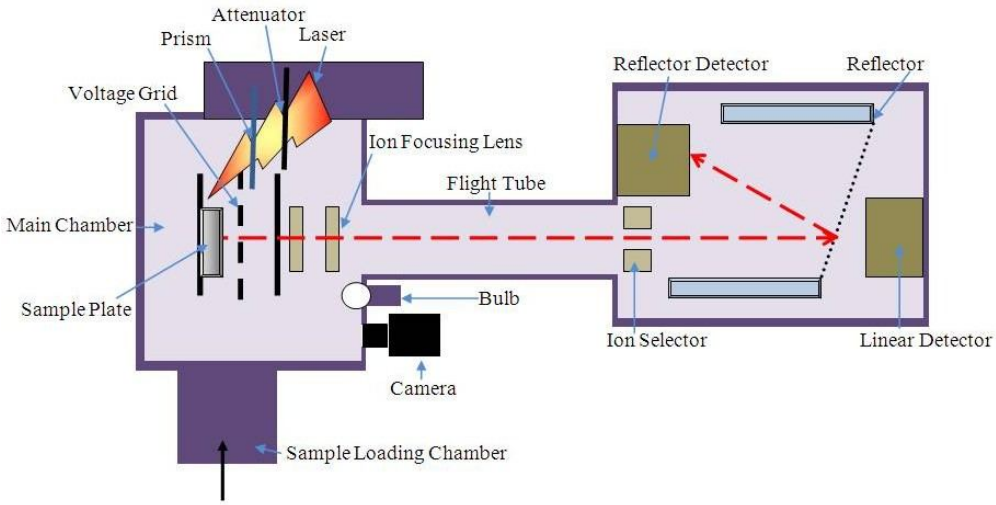


Figure 3.5: Schematic of a MALDI-TOF mass spectrometer

Ions travel through the flight tube at different speeds according to their mass. The detector records the data produced and send the information to a computer.

The TOF mass spectrum is a recording of the detector signal as a function of time. The time of flight for a molecule of mass (m) and charge (z) to travel this distance is proportional to (m/z) ½. Therefore, t ~ (m/z) ½ can be used to calculate the mass of the ion. By using this calculation, the conversion of the TOF mass spectrum to a conventional spectrum of m/z vs. ion intensity can be achieved (Figure 3.6).

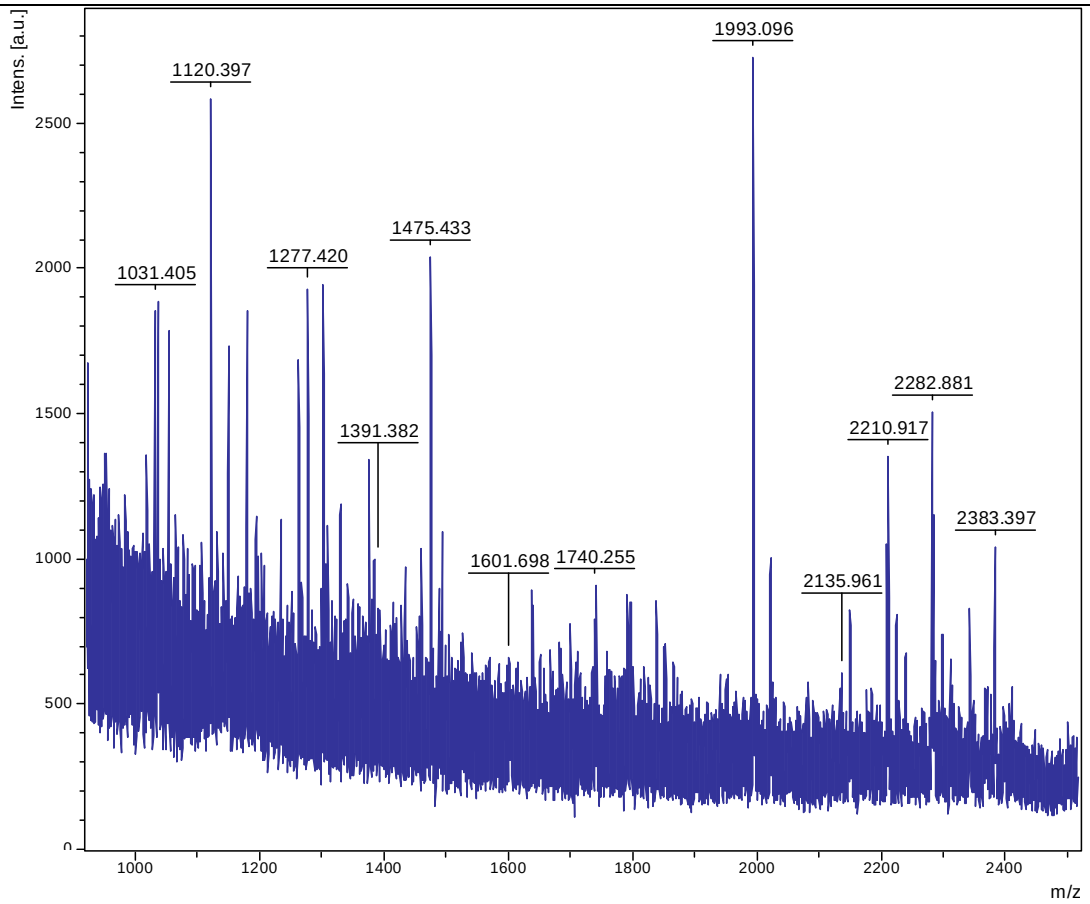


Figure 3.6: PMF of glyoxalase domain containing 4 (GLOD4).

Spectra shows intensity (y-axis) vs. m/z (x-axis).

3.2.3 MALDI-TOF-MS Analysis

Differentially expressed proteins in biological research are often low abundant proteins, the identification of which can be shrouded by more abundant contaminants such as keratins and trypsin autolysis peaks; even if the analyst has taken precautions to avoid contaminations (see section 3.1.2) (Keller *et al*. 2008). Common contaminating peaks can be determined by performing experimental controls by digesting blank pieces of gel and digesting known proteins standards. The resultant peptides can then be analysed by MS to obtain the background peaks which are individual to each user and to each

laboratory. When the analyst has performed this analysis multiple times and is confident that the peaks obtained are true background peaks, they can then be taken out of future spectra by editing the peak list in the MS software. It is also important to carefully check the spectra and make sure the software had correctly selected peaks and to edit accordingly. It is important to spend time inspecting the PMF as the risk of false positive results will be decreased if this is done correctly.

The PMF peak list is submitted to a database search, which compares the peptide in the sample to the peptides in the database of theoretically digested proteins. If enough peptides from the PMF match the database PMF then a protein identification will be given based on the ion score and the percentage of sequence covered by the PMF to the database PMF (see Appendix H: Mascot Search Results – Beta Galactosidase (LacZ Protein)). The ion score is a calculation based on probability:

-10*log(P)

Where **P** is the probability that the match is a random event. Protein scores that are classed as significant will have $p<0.05$ (see Appendix H: Mascot Search Results – Beta Galactosidase (LacZ Protein)). The theoretical protein's pI and molecular weight can then be matched to location of the protein spot on the 2D gel.

One disadvantage of using a PMF database is that proteins which have undergone post-translational modifications (e.g. phosphorylation) may not be identified. This is because peptides from a modified protein will not match those of the unmodified protein in the database, making a positive identification very difficult. Another problem is the presence of more than one protein in the digested sample. For example, if two proteins migrate to the same point on the gel they will be excised together and digested together.

The result would be a PMF mixture from more than protein, making a positive identification unfeasible. It is also worth remembering that a large percentage of proteins are not yet in the databases, and that small proteins may not generate a sufficient amount of peptides to gain a significant identification. However, in these cases the amino acid sequence for an individual peptide in the PMF can be gained by subjecting selected ions to further fragmentation by tandem mass spectrometry (MS/MS) (see section 3.2.6).

3.2.4 Electrospray Ionisation

ESI-MS/MS is often used in a two-tier protein identification strategy (Beranova-Giorgianni. 2003), MALDI-TOF-MS is used primarily to identify the peptide mass fingerprint data, and database searching is used to try and identify the parent protein. However, if the protein is still not identified then ESI-MS/MS or MALDI-TOF-MS/MS can be used to generate product ion data (see section 3.2.6) (Beranova-Giorgianni. 2003).

ESI produces charged solvent droplets when a high electric potential is set between a capillary and the inlet to a mass spectrometer (Lin *et al.* 2003). Typically peptides or proteins are analysed as positive ions using the capillary as an anode and the MS inlet as the cathode. The sample to be analysed is suspended in an appropriate solvent such as 50:50 acetonitrile: water with 1% acetic acid and injected directly into the instrument. ESI produces mainly doubly charged ions which allows for the determination of the m/z value. The ions are generated directly from the solution by a fine spray. As the droplet size decreases, the electric charge density on the surface increases. Mutual repulsion between the like charges on the surface exceeds the forces of the surface tension, and

ions begin to leave the droplet through a "Taylor cone" (a jet of charged particles emanating above a threshold voltage) (Figure 3.7). Ions are electrostatically directed into mass analyser. ESI is readily coupled to high performance liquid chromatography (HPLC) as it uses a steady stream of solvent to produce a continuous beam of ions (Lin *et al.* 2003). The peptide mixture is first separated by liquid chromatography (LC), which is coupled on-line to the ESI-MS. This technique is used to gain product ion data (Beranova-Giorgianni. 2003). The flow rate on the ESI-MS is usually set to µl per minute, but if the flow rate is lowered to nanolitres per minute, the signal is greatly improved. This technique is called nano-electrospray.

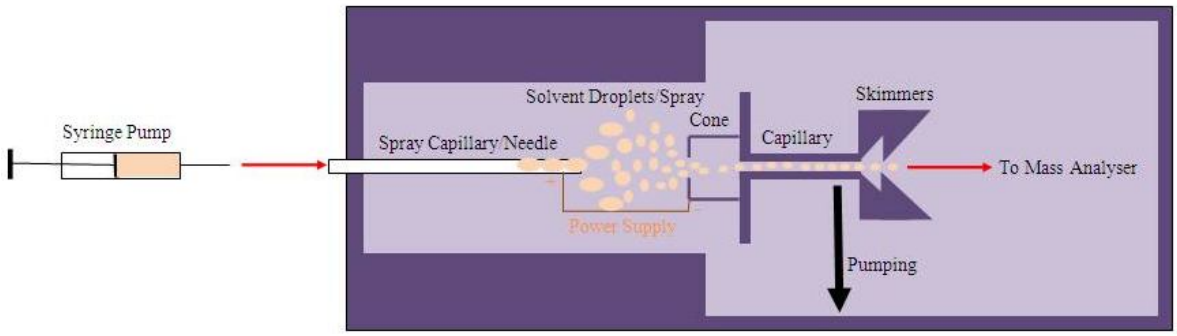


Figure 3.7: Schematic of an ESI Source.

The sample is injected through the syringe pump directly into the machine. Ions are generated by applying a high electric potential, and are sprayed from the capillary to the mass analyser.

3.2.5 ESI Mass Analyser

The ESI source is readily coupled to a quadrupole ion trap (QIT) mass spectrometer and tandem mass spectrometry (see section 3.2.6). A QIT mass analyser uses a combination of radio frequency (RF) and direct current (DC) voltages to select ions of a particular m/z (Khalsa-Moyers *et al.* 2006). The ions are then trapped in an electric or magnetic

field and stored by decreasing their kinetic energy. The ions are sequentially ejected into a detector by increasing their kinetic energy by ramping RF voltages (Khalsa-Moyers *et al.* 2006). A population of variable charged ions produced by ESI generate the spectra (Figure 3.8). The peaks observed are due to multiple charging effects. The spectra can then be used to pick individual peptides to be subjected to tandem mass spectrometry.

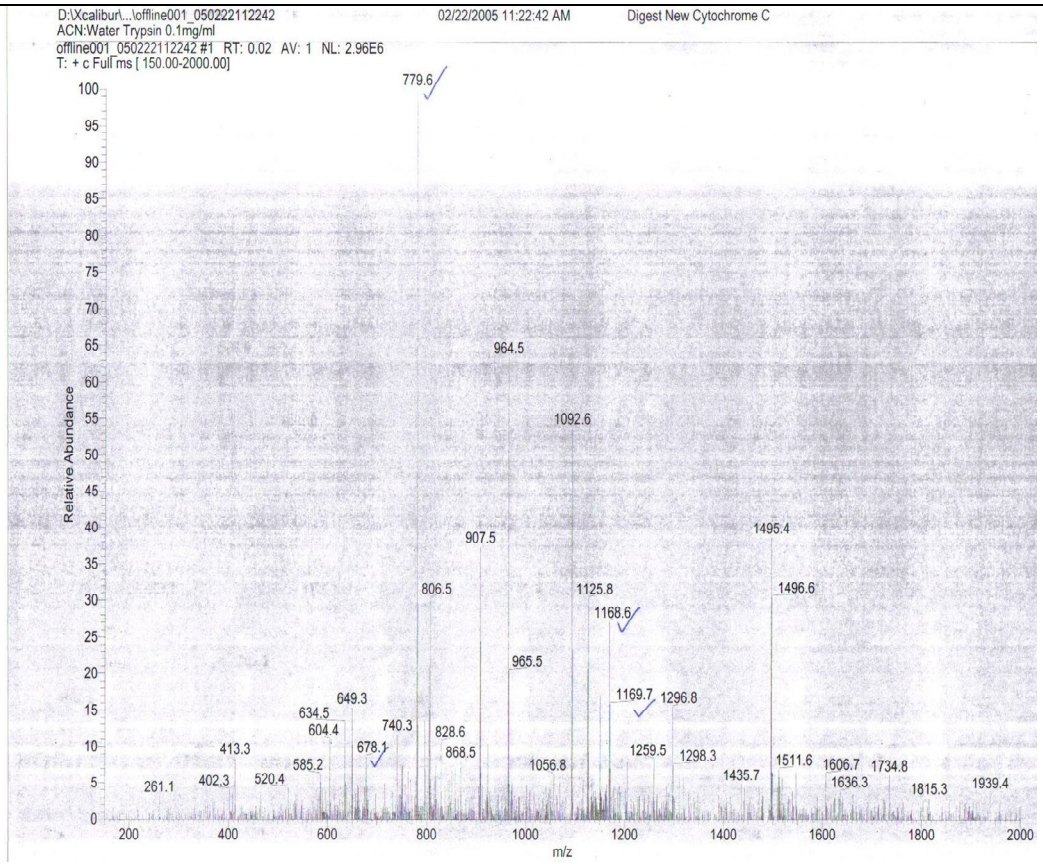


Figure 3.8: Spectra obtained from ESI-MS of cytochrome C digested

The spectra shows relative intensity (y-axis) (where the highest peak is 100%) vs. m/z (x-axis).

3.2.6 Tandem Mass Spectrometry

Tandem mass spectrometry is brought about by multiple series of mass spectrometry. The first course of MS isolates individual peptides. The second round fragments the selected peptides further. The fragmentation is brought about when the precursor ion collides with a gas in the vacuum of the mass spectrometer. This is called collision-induced dissociation (CID). The peptide sequence tag generated by MS/MS can be used to identify the peptide in a database.

3.3 Antibody Microarrays

An antibody microarray is a collection of antibodies spotted onto a nitrocellulose coated glass slide. Antibody microarrays can be used to compare protein expression profiles of two samples (e.g. test vs. control). Each protein sample is labelled with a different dye (cy3 or cy5) and is incubated on the antibody microarray. The expression of a protein can be detected when it binds to its corresponding antibody spotted on the slide (Figure 3.9). The fluorescent signal intensity for each sample is recorded individually at the wavelength corresponding to the dye label of the sample and control. The protein expression for the two samples can then be compared.

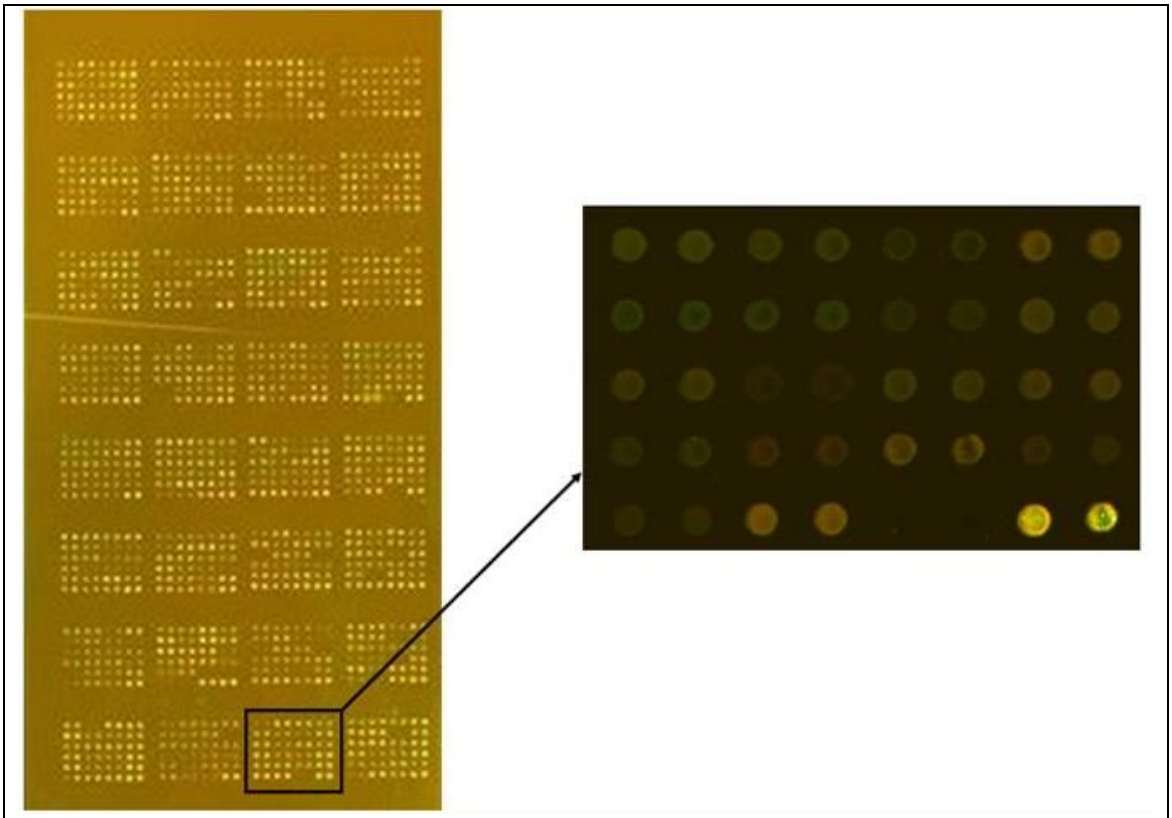


Figure 3.9: Picture of an antibody microarray slide

Left: picture of an antibody microarray slide spotted with 725 antibodies. The slide has been incubated with two separately labelled protein samples.
Right: enlarged image of the highlighted area. The protein expression is represented by the colour of the spot. If more protein is expressed in the control sample (labelled with cy3) then the spot will be green, if more protein is expressed in the more test sample (labelled cy5) then the spot will be red. If equal amounts of protein are expressed in both samples the spot will be yellow.

Antibody microarrays are a quick way of generating vast amounts of data, but replicates can become expensive. However, by purchasing some of the antibodies separately to the array kit, results can be confirmed using immunoblotting. This allows the antibody microarray to be used as a preliminary screening method. The array only takes approximately 3 days to run, which is an improvement to 2D-PAGE which takes 5-6 weeks; however, protein identifications will always be limited to what antibodies are spotted on the slide. There are many antibody microarrays commercially available on

the market, each with a different number of antibodies and different protein targets. Pathway specific arrays are also available for more focused research.

3.4 Western Blot Analysis

Western Blot analysis is a method to detect a particular protein in a biological sample and can be used to confirm identifications by MS.

One dimensional gel electrophoresis is used to separate proteins in a gel (3.1.1 One Dimensional Polyacrylamide Gel Electrophoresis). The proteins are then transferred to a nitrocellulose membrane by applying a current between two electrodes and placing the gel and the membrane in between the electrodes. The charged proteins move from the gel onto the membrane in the same orientation as they were on the gel. The membrane is then washed with a blocking solution to prevent non-specific protein interactions when it is probed with an antibody. Bovine serum albumin (BSA) or non-fat dried milk powder can be used to block non-specific binding sites on the proteins.

The proteins immobilised on the membrane are then probed with a specific primary antibody. If the primary antibody is not horseradish peroxidase (HRP) conjugated, a secondary HRP conjugated antibody is needed to visualise the proteins. Secondary antibodies bind to the primary antibody. A chemiluminescent agent is used to initiate a reaction with the HRP, which produces luminescence in proportion to the amount of protein. The luminescence can be used to visualise the protein bands by exposing the membrane to a photographic film. Densitometry can then be used to give a ratio of the level of protein between samples. To ensure that equal amounts of protein have been

loaded onto the gel the membrane is probed for a “housekeeping” such as GAPDH or beta-actin, which should demonstrate constant levels of expression and acts as a loading control (Figure 3.10). Densitometry can be used to compare the intensity of the bands from each sample. If the bands have an equal level of intensity then it can be assumed that equal amounts of protein have been loaded. Western blotting can be used as a confirmation method for targets found by 2D-PAGE/MS or as a technique to identify levels of a specific protein.

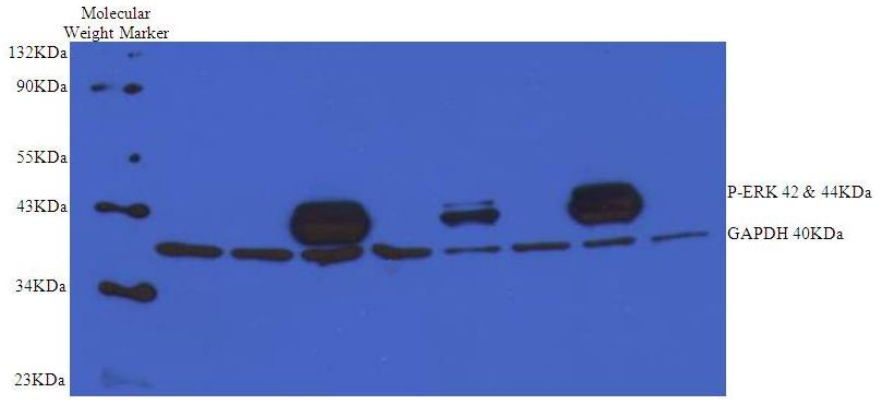


Figure 3.10: Western blot of phosphorylated ERK (P-ERK) with a GAPDH loading control.

The picture shows that each lane has a similar amount of protein loaded as the GAPDH band (40KDa) shows a similar intensity for all samples. However, the samples have varying amounts of P-ERK protein (42/44KDa) between them.

AIMS

The clinical course of patients with CLL is heterogeneous; some patients survive for years without treatment, others die of a chemotherapy resistant disease within two years of presentation. There is no routine, cost effective clinical test to show how a patient will progress.

The aim of this project is to identify potential biomarkers which could be used in a clinical test to predict patient prognosis. IgV_H gene mutational status has proven to be a good prognostic marker but the test is expensive and time consuming and can not be used routinely in clinical practice. If the B-cell has not been sensitised to a specific antigen (i.e. unmutated IgV_H genes on BCR) it is hyper-responsive to stimulation through the BCR by antigen. Stimulation of the BCR may prevent apoptosis of malignant cells; therefore a hyper-responsive BCR is linked to poor prognosis.

By using proteomics we will investigate signaling pathways activated by the BCR to gain a greater understanding of the anti-apoptotic nature of the malignant B-cells and to find potential prognostic biomarkers related to a hyper-active BCR. If we understand more about the BCR signaling pathways then we may be able to identify novel targets for therapeutic intervention. These may inhibit survival of the malignant B-cells.

Intravenous blood samples will be collected from patients with CLL who have not received treatment for three months prior to sampling. Peripheral blood mononuclear cells (PBMCs) will be extracted from the blood and the samples will be classified according to IgV_H genes mutational status and BCR responsiveness. Cells will be

artificially stimulated to induce BCR signaling cascades and to categorise BCR responsiveness by western blotting. RAJI B-cells will be used for optimisation and to obtain preliminary results.

Selected samples which have been categorised as having a poor prognosis (hyper-active BCR and unmutated IgV_H genes) will be taken forward for further analysis. Protein will be extracted from stimulated and unstimulated cells and the protein profiles of the two groups will be compared using 2D-PAGE. Protein spot editing software will be used to identify statistical changes between the two groups and these proteins will be excised from the gel and digested into peptides using trypsin. MALDI-TOF-MS will be used to generate a PMF and identify the excised proteins. Potential protein targets will be confirmed by western blotting and antibody microarrays will be used as a complementary method to 2D-PAGE. Proteins found may be utilised as potential prognostic biomarkers related to a hyper-active BCR, and data obtained should give a greater understanding of the anti-apoptotic nature of the malignant B-cells.

Chapter 4 - Materials and Methods

Materials and Methods

All chemicals were of the highest quality or proteomics grade. All solution components and details of suppliers can be found in Appendix A.

4.1 Culturing B-Cells Lines

Established suspension B-cell lines RAJI, DAUDI and RAMOS-EHRB derived from patients with Burkett's lymphoma were cultured for preliminary experiments and to optimise techniques.

When culturing cells it is vitally important that everything is sterile and clean to avoid contaminations. All work was carried out in a sterile tissue culture (TC) hood and 70% alcohol was used to sterilise equipment before it was placed in the hood. Cells were cultured in an incubator set at 37°C with 5% CO_2. On average cells were passaged three times a week. Cells were grown in a T25 or a T75 tissue culture flask (Sarstedt) depending on the number of cells. Contaminated waste was soaked in Virkon disinfectant for 24hrs before discarding.

4.1.1 Passaging Cells

Cells were taken out of the flask with a pipette and transferred into a 30ml screw top tube (Scientific Laboratory Supplies). The cells were centrifuged (Sigma-Aldrich 2-5; SciQuip centrifuge) at 1600rpm for 3mins. The media was discarded to leave the pellet of cells.

Cells were resuspended in at least 5ml of tissue culture media (see Appendix C) pre-warmed to 37°C, and transferred to appropriate number of new tissue culture flasks. Each flask was made up to an appropriate volume (dependant on the number of cells) with warmed tissue culture media. Flasks were placed in an incubator maintained at 37°C with 5% CO_2.

4.1.2 Cryopreservation of Cells

Cells were resuspended slowly (2-3mins) in tissue culture freezing media, which contained 10% dimethyl-sulphoxide (DMSO) (see Appendix C), at a density of between $1x10^6$ cells/ml to $1x10^8$ cells/ml, and transferred into 2ml cryovials (Scientific Laboratory Supplies) and frozen at -80°C. Cells were later transferred to liquid nitrogen for long term storage.

4.1.3 Thawing Cells

Samples were taken out of the freezer, thawed quickly in a water bath set to 37°C and transferred to a 30ml screw top tube. The DMSO was diluted out with 10ml of tissue culture media warmed to 37°C. DMSO is toxic to thawed cells if left in too long, however, it needs diluting out slowly (dropping media in over a period of 2-3mins) to avoid the cells going into shock. The cells were centrifuged (Sigma-Aldrich 2-5; SciQuip centrifuge) for 3mins at 1600rpm and the supernatant was discarded. Fresh tissue culture was added and the cells were transferred to a T25 or T75 tissue culture flask and placed in an incubator maintained at 37°C with 5% CO_2.

4.2 Clinical Sample Collection

4.2.1 Ethical Approval

The study was approved by Hull and the East Riding Local Research Ethics committee and Hull and East Yorkshire NHS Trust Research and Development department 05/Q1104/33.

4.2.2 Mononuclear Cell Isolation

Following informed consent 50ml of blood was taken from patients diagnosed with CLL who had not received chemotherapy treatment in the previous 3 months. The peripheral blood mononuclear cells (PBMCs) were separated using Histopaque® (#10771; Sigma-Aldrich). Histopaque® contains polysucrose and sodium diatrizoate adjusted to a density of 1.007g/ml. By centrifuging whole blood, layered onto Histopaque®, the PBMCs form a layer at the plasma/Histopaque® interface. The white blood count (WBC) from the patient was needed before the PBMCs were stored. Each cryovial of cells stored contained at least $1x10^7$cells/ml. For example, if the WBC was $80x10^9$cells/L then 100 vials at a concentration of $4x10^7$cells/ml were stored. If the WBC was $20x10^9$cells/L then 50 vials at a concentration of $1x10^7$cells/ml were stored.

Six ml of ice cold Histopaque® was added to four 50ml centrifuge tubes (Sarstedt). One 50ml blood sample was split between the four tubes, i.e. 12.5ml of blood was layered on top of the Histopaque®. The tubes were centrifuged (Rotanta 96R; Hettich centrifuge) at 1700rpm for 20mins at 4°C and set to ramp down slowly to avoid the interfaces

mixing (Figure 4.1). The cloudy mononuclear layer in the middle of the tube was carefully removed using a 1ml pipette and the PBMCs from the four tubes were combined into a new 50ml centrifuge tube. The combined cells were washed in 10-20ml of ice-cold tissue culture media and centrifuged (Rotanta 96R; Hettich centrifuge) at 1700rpm for 10mins at 4°C. The supernatant was discarded and the PBMCs were resuspended in an appropriate amount of ice-cold tissue culture media (see Appendix C) (1/2 the total volume needed to freeze). For example, if a patient had a WBC of $80x10^9$cells/L, and the sample was to be stored into 100 vials, the cells would be resuspended into 50 ml of ice-cold tissue culture media and stored on ice.

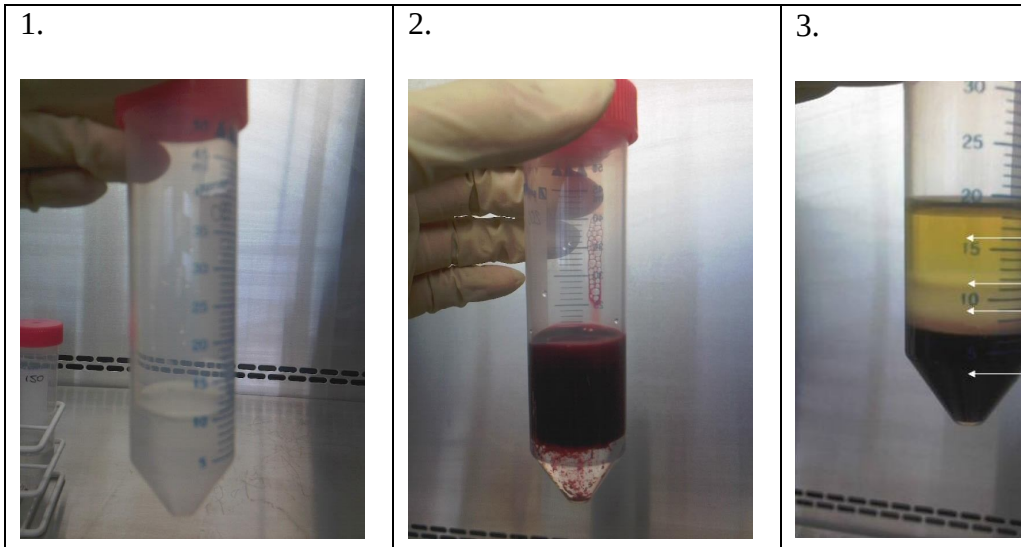


Figure 4.1: Picture of cellular layers obtained by centrifuging whole blood, layered onto Histopaque®

The pictures show, from left, Histopaque is placed in tube (1), blood is layer on to the Histopaque (2), tube is centrifuged and four layers are produced, plasma, PBMCs, Histopaque and red cells (3). The PBMCs can then be extracted with a pipette.

Fifty ml of 20% DMSO freezing media (Appendix C) was then added slowly (over 3-5mins) to make the total volume to 100ml. The sample was mixed thoroughly and aliquoted into 1ml cryovials. As DMSO is toxic to the cells, once added the samples had

to be frozen within 15mins. Vials were stored at -80°C and transferred to liquid nitrogen for long term storage.

4.2.3 IgV_H Gene analysis

Approximately $1x10^7$ cells from each sample were sent to the Haematological Malignancy Diagnostic Service (HMDS) at St. James's Institute of Oncology, Leeds for IgV_H gene analysis.

RNA was extracted from PBMCs using the Qiagen RNeasy mini kit (Qiagen, Crawley, UK) and complementary DNA was synthesised using the Reverse-iT kit according to the manufacturer's instructions (ABgene). The IgV_H gene was amplified using polymerise chain reaction (PCR) (Hamblin *et al.* 1999) and sequenced using the BIG dye terminator sequencing kit version 3 according to the manufacturer's instructions (Applied Biosystems). Sequences were compared to accepted germline sequences using IgBLAST (National Centre for Biotechnology Information, Bethesda, Maryland; http://ncbi.nih.gov/igblast/). A <5% difference in standard germline sequence was classed as an unmutated IgV_H gene and a >5% difference in standard germline sequence was classed as a mutated IgV_H gene (Allsup *et al.* 2005).

4.3 Cell Counting

A Neubauer haemocytometer was used to determine the cell concentration per millilitre of tissue culture media in cell lines and in clinical samples.

Cells were centrifuged to remove dead cells. The cell pellet was resuspended in a volume of tissue culture appropriate for the size of the pellet. The volume could be adjusted later if there were too many or too few cells in the counting chamber.

Twenty-five µl of thoroughly mixed cells were taken and added to 25µl of 0.4% trypan blue (Sigma-Aldrich). Trypan blue stains non-viable cells (dead cells) blue because the chromophore is negatively charged and reacts with damaged cell membranes. The solution was thoroughly mixed by pipetting and left for 5mins to allow the cells to absorb the dye. Twenty-five µl of the solution was then carefully pipetted into the haemocytometer chamber underneath a glass cover slip. The haemocytometer was then placed onto a microscope stage.

The haemocytometer consists of nine 1mm x 1mm squares, which can each hold a volume of 10^{-4}ml (Figure 4.2). It is important that each 1mm square has between 70-150 cells as this will give a more accurate cell count. If there are too many or too few cells on the haemocytometer, the original stock solution must be diluted or resuspended in the correct volume and the procedure repeated. Cells in five of the squares (Figure 4.2) were counted separately. The cell numbers were then averaged.

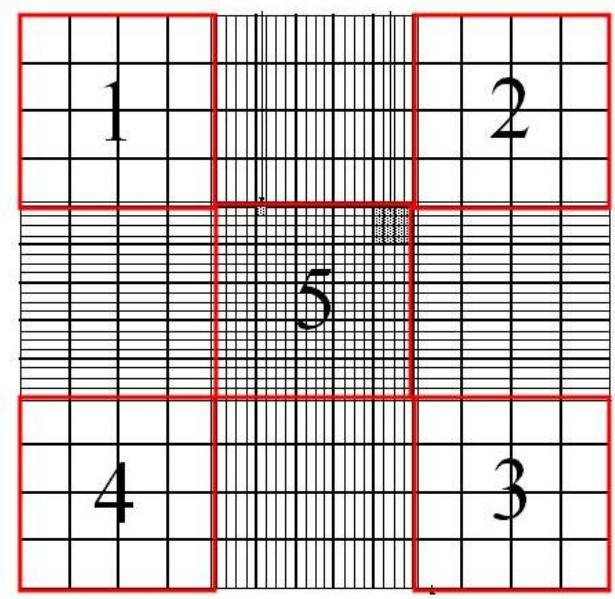


Figure 4.2: Figure showing Neubauer haemocytometer grids

Grid system of a Neubauer haemocytometer showing the five 1mm squares counted to determine cell concentration.

The following equation was used to calculate the cell concentration:

$$\text{Cell Concentration (Cells/ml)} = \frac{\text{Average Number of Cells per 1mm}^2}{\text{Volume } (10^{-4}\text{ml})}$$

For example:

Square 1 = 118 cells

Square 2 = 114 cells

Square 3 = 120 cells

Square 4 = 116 cells

Square 5 = 122 cells

Average = 118 cells per 10^{-4}ml

The trypan blue dilution factor needs to be taken into account (solution is 50% trypan blue and 50% cells) therefore this number must be multiplied by 2.

118 cells x 2 = 236 cells per 10^{-4}ml

= 236 x 10^4 cells/ml

4.4 BCR Stimulation

The stimulation protocol was followed for both B-cell lines and clinical cells.

Cells were taken from the freezer and thawed as described in 4.1.3 Thawing Cells. Cells were centrifuged for 3mins at 1600rpm and resuspended in an appropriate amount of tissue culture media (dependant on number of cells). They were transferred into a T25 or T75 flask and left in an incubator for 3 hours at 37°C with 5% CO_2 to acclimatise.

After the incubation period cells were centrifuged for 3mins at 1600rpm and resuspended in 10-20ml of tissue culture media. A cell count was then performed on the sample (4.3 Cell Counting). Cells were resuspended at a final concentration of $5x10^6$/mL for CLL cells and $5x10^5$/mL for B-cell lines (the cells of the B-cell lines were bigger than the PBMCs and fewer cells were needed). An appropriate volume of cells (at least 1ml for Western blot analysis, and 15ml for 2D-PAGE analysis) were transferred into four separate T25 or T75 tissue culture flasks containing respectively:

1. **Stimulant**

   10μg/ml of AffiniPure F(ab')$_2$ fragment goat anti-human IgM, Fc$_{5\mu}$ fragment specific (#109-006-129 Jacksons Immuno Research Laboratories Inc)

2. **Isotype Control**

   10μg/ml ChromPure Goat IgG, F(ab')$_2$ fragment (#005-000-006 Jacksons Immuno Research Laboratories Inc)

3. **Positive Control**

   100nmol/L of phorbol 12-myristate 13-acetate (PMA) (#P1585 Sigma-Aldrich) (which activates protein kinase C and causes widespread protein phosphorylation)

4. **Neutral/Negative Control**

   Nothing added

All the tubes were incubated at 37°C with 5% CO_2 for either 5 minutes (see section 7.1), 8hrs (see section 7.3), or 5.5hr (see sections 9.3 and 10.1). Cells were transferred to a 30ml screw top tube and centrifuged for 3mins at 1600rpm. The supernatant was discarded and the cells were washed in ice cold phosphate buffered saline (PBS). At least three cycles of PBS washes and centrifugations were completed in order to wash away all of the stimulant and media. Samples which were to be analysed by 2D-PAGE were washed at least five times to remove any proteins from the supernatant such as fetal calf serum (FCS) in the media. On the final wash the samples were transferred to 1.5ml polypropylene microfuge tubes (Scientific Laboratory Supplies). All of the PBS was removed by using a fine pipette and the stimulation was terminated by adding Laemmli buffer (see Appendix F) or 2D-PAGE lysis buffer (see Appendix D).

4.5 Western Blot Analysis

4.5.1 Protein Extraction

Cells were resuspended in Laemmli buffer (125µl-300µl dependant on number of cells) (see Appendix F). Samples were vortexed for 5mins at room temperature and sonicated for 5mins at room temperature to disrupt the cells. The tubes were then placed on an end-over-end rotator at 4°C overnight. Samples were centrifuged at 16,000xg for 15mins at 4°C to pellet cell debris and the supernatant was transferred into new, cold 1.5ml polypropylene microfuge tubes. Extracts were stored at -80°C.

4.5.2 Protein Quantification for Western Blot

The RCDC kit (#500-0122; Bio-Rad) was used for the quantification, which allows proteins to be quantitatively precipitated whilst washing away contaminants. The technique is based on the Lowry assay, which is a colorimetric assay for determining the protein concentration (Lowry *et al*. 1951). Copper (II) ions react with the protein samples in alkaline conditions to form complexes which react with folin phenol (phospho-molybdic-phosphotungstic) reagent. The product is reduced to molybdenum/tungsten blue. The higher the concentration of protein, the more molybdenum/tungsten blue is produced, and the darker the solution (Lowry *et al.* 1951). All samples must be in the same volume and protein standards of known concentrations are required to produce a graph of optical density vs. protein concentration. The RCDC kit is compatible with protein extraction buffer containing detergents such as SDS, and reducing agents such as β-mercaptoethanol.

Four dilutions of protein standards were prepared using a 2mgml stock of bovine serum albumin (BSA) (Amersham Biosciences). Dilutions were as follows:

- 1.5mg/ml
- 1mg/ml
- 0.5mg/ml
- 0.25mg/ml

Some samples required dilution (1:2, 1:5 or 1:10 in DH_2O) as the detection limits of the kit range from 0.2mg/ml to 1.5mg/ml.

RC Precipitation/Clean Up

Twenty-five µl of BSA standards and samples were pipetted into 1.5ml microfuge tubes. One hundred and twenty-five µl of RC Reagent 1 (from the kit) was added. Samples were vortexed and incubated at room temperature for 1 min. One hundred and twenty-five µl of RC Reagent 2 was added and the samples were vortexed. Samples were centrifuged at 15,000xg for 5mins and the supernatant was discarded. Tubes were inverted on absorbent tissue paper to allow the liquid to completely drain leaving only the protein pellet.

DC Quantification Assay

Reagent A' (Working Colour Reagent) was prepared following the manufacturers instruction by adding 5µl of DC Reagent s to every 250µl of DC Reagent A.

One hundred and twenty seven µl of Reagent A' was added to each tube. The samples were vortexed for 5mins at room temperature to ensure the pellet had fully dissolved. One ml of DC Reagent B was added to each tube, samples were vortexed immediately and tubes were incubated at room temperature for 15mins. Two hundred µl of each sample and standard were placed into individual wells of a 96 well plate. Two hundred µl of DH_2O was used as a blank. The absorbance was read at 690nm on a Multiscan MS plate reader (Thermoelectron, UK).

The BSA standard protein optical density was used to produce an optical density vs. concentration graph, which was then use to determine the protein concentrations of the samples (Figure 4.3).

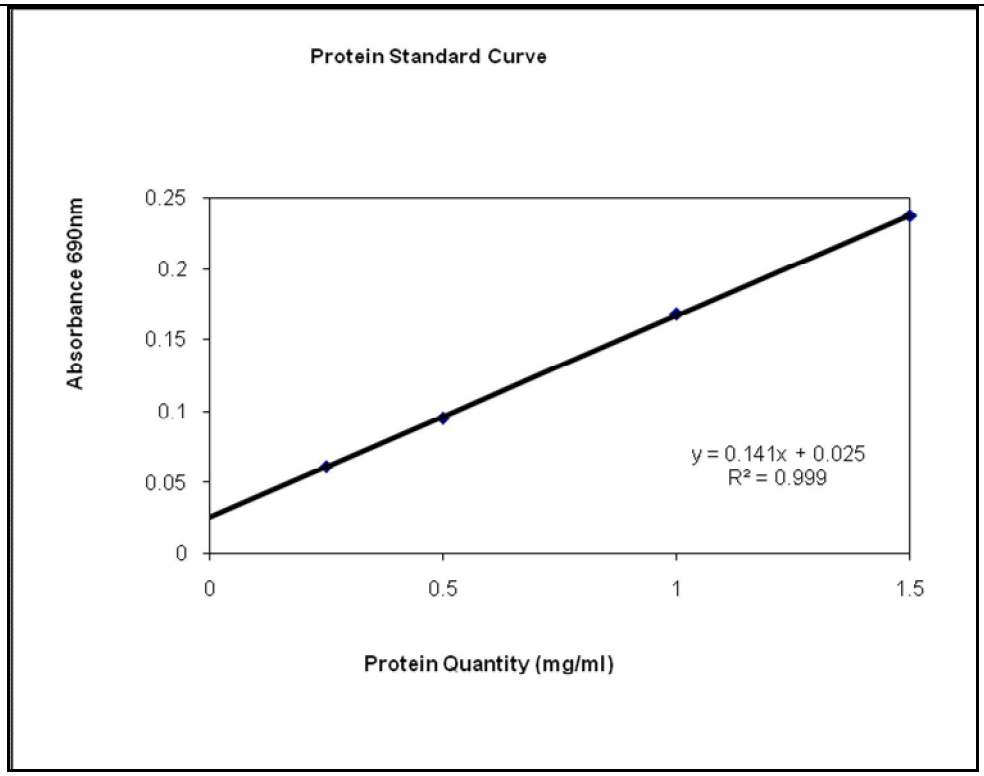


Figure 4.3: Graph of optical density vs. protein concentration using a BSA protein standard and the RCDC Kit (Bio-Rad)

The equation from this graph can be used to calculate the protein concentration of a test sample when the optical density is known.

4.5.3 SDS-PAGE

Twenty µg of protein was loaded into one well on a 1D gel as this amount generally gave visible bands that were not saturated.

Protein samples (20µg) were diluted with Laemmli buffer to a volume of 25µl in order to ensure that equal amounts were loaded. The phosphatase and protease inhibitors were not added to the Laemmli buffer, as these cocktails are only beneficial when storing samples. Twenty-five µl of diluted protein sample was placed in 0.5ml microfuge tube. The samples were heated at 95°C in a thermocycler for 5mins to denature. The β-

mercaptoethanol in the Laemmli buffer cleaves the disulphide bonds in the proteins, thus allowing peptides to migrate to the correct point in the gel according to its chain length, with no influence from the secondary structure. The tubes were placed immediately on ice, vortexed for 30sec and centrifuged briefly to collect sample at bottom of tube. A homogenous Novex® 12% Tris-Glycine gel (1.0mm, 8x8cm, 10 well) (#EC6005box; Invitrogen) was placed in a vertical electrophoresis unit and approximately 120ml and 1120ml of 1x running buffer (see Appendix F) was added to the upper and lower chambers respectively. One or two pre-cast gels could be run simultaneously in the electrophoresis unit.

Ten µl of Precision Plus Dual Colour Molecular Protein Standard (#161-0374; Bio-Rad) was added to the last well of the gel for visibility on the gel and membrane (Figure 4.4). Ten µl of Cruz Marker™ molecular weight standards (#sc-2035; Santa Cruz) was added to the first well for visibility after chemiluminescence (Figure 4.5). Twenty µl of the 25µl protein sample was loaded into each well and SDS-PAGE was performed at a constant 200V for 60mins at room temperature.

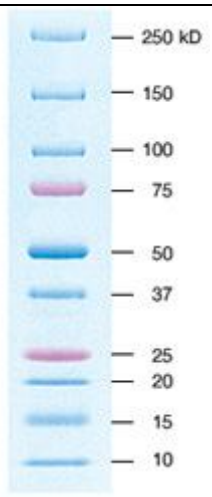


Figure 4.4: Precision Plus Dual Colour Protein Standard

The Dual Colour Protein standard consists of ten precise recombinant pre-stained protein standards (10-250KDa) for electrophoresis and western blotting. The marker is visible on the gel and on the membrane.

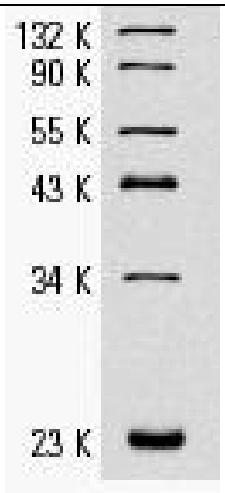


Figure 4.5: Cruz Marker™ Molecular Weight Standards

The Cruz Marker ladder consists of six bands (23-132KDa) which appear on the final western blot film after incubation with Cruz Marker™ compatible western blotting secondary antibodies.

4.5.4 Transfer to Membrane

The gels were taken out of their plastic cassettes and placed in a tray containing chilled transfer buffer (see Appendix F) to equilibrate them and to wash off any running buffer. For each gel to be transferred, two Mini Trans-Blot filter papers (#1703932; Bio-Rad), a Trans-Blot nitrocellulose membrane (0.45µm) (#162-0115; Bio-Rad) cut to the size of the gel and two foam pads were also soaked in chilled transfer buffer. The fibre pads, filter papers, gel and membrane were layered on the transfer cassette as shown in Figure 4.6. Care was taken to ensure no air bubbles were trapped between the gel and the membrane, as they would interfere with the transfer.

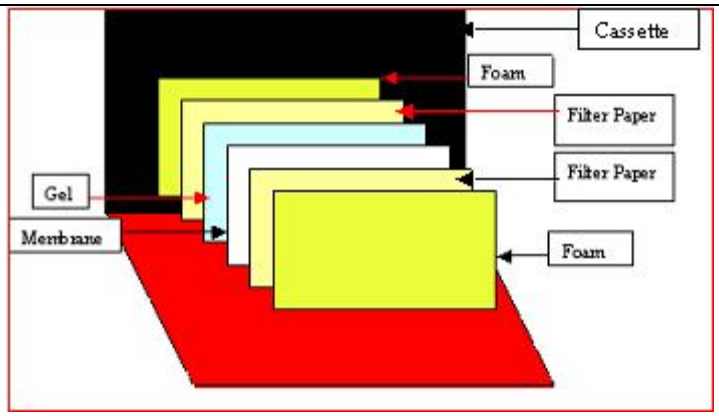


Figure 4.6: Schematic of the Transfer Layers in a Western Blot

The transfer cassette has a black side and a red side. To ensure a successful transfer of proteins from the gel to the membrane the first foam pad must be layered onto the black side. This is followed by filter paper followed by the gel. The nitrocellulose membrane is placed directly onto the gel and filter paper is placed onto the membrane. At this point it is important to get out any air bubbles trapped between the membrane and the gel. This can be done by using a glass pipe or pipette to roll over the filter paper. The final foam pad is placed onto the filter paper before closing the cassette.

The cassette was closed and placed in the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The transfer cell could transfer one or two gels onto a membrane

simultaneously. The transfer cell was filled with approx 900ml of chilled transfer buffer which was stirred throughout the transfer to keep cool. Electrophoretic transfer was performed at 400mA for 1hr at 4°C. The transfer works on the same principle as that of electrophoresis. A charged protein can travel through an electric field whether that is through a gel, or from a gel onto a membrane support. The negatively charged proteins travel to the positive electrode (from black side of cassette to the red side), but they are blocked by the membrane which binds them. After the transfer the gel can be stained to make sure the proteins have completely transferred. In an efficient transfer there should be no protein left on the gel.

4.5.5 Blocking

Proteins, including antibodies, bind readily to the nitrocellulose membrane. To ensure the antibodies do not bind non-specifically, the membrane is incubated in a solution of protein such as BSA or casein (milk protein). This blocks all the binding sites on the membrane leaving only the bound transferred proteins exposed to the antibodies. The membrane was transferred to a Nalgene staining box (Scientific Laboratory Supplies) containing 20ml of 5% milk blocking solution (see Appendix F) and incubated for 1hr on an orbital rocker at room temperature. Nalgene staining boxes were used to prevent the antibodies from sticking onto the surface of the plastic. It was important that the membrane did not dry out at any time during the protocol.

4.5.6 Antibody Probing

Each antibody needs optimising for concentration and incubation time before use. The optimised amount of primary antibody was added to 10ml of 5% milk blocking solution (Table 4.1). The membrane was incubated in the antibody for the optimised time on an orbital rocker. Incubations over 2hrs were performed at 4C°. The membrane was washed 3 times for 5mins each wash in tris buffered saline mixed with 0.05% TWEEN 20 detergent (TBS/TWEEN) (see Appendix F). It was important to ensure that all unbound primary antibody was washed off to avoid non-specific binding of the secondary antibody. The primary antibody was not HRP conjugated therefore a secondary HRP conjugated antibody, which is specific to the primary antibody's species, is required. The secondary antibody was used at a 1:1000 dilution in 10ml 5% milk blocking solution (Table 4.2). The membrane was incubated in the secondary antibody for 1hr on an orbital rocker at room temperature.

Table 4.1: Table of western blot primary antibody details

Antibody	Type	Catalogue Number & Supplier	Dilution	Molecular Weight
P-ERK	Mouse Monoclonal	#sc-7383 Santa Cruz	1:100	P-ERK 1: 44KDa P-ERK 2: 42KDa
ERK	Rabbit Polyclonal	#sc-154 Santa Cruz	1:1000	ERK 2: 42KDa
GAPDH	Rabbit Polyclonal	#ab9485 Abcam	1:10000	40KDa
B-Actin	Rabbit Polyclonal	#ab8227 Abcam	1:2500	47KDa
Bcl-2α AB-3	Mouse Monoclonal	#MS-597-P0 Thermo Scientific	1:200	25/26KDa
AK5	Rabbit Polyclonal	#Ap8131a Abgent	1:100	22KDa
DARS	Mouse Monoclonal	#H00001615-M01 Abnova	1:200	57KDa

Table 4.2: Table of western blot secondary HRP conjugated antibody details

Antibody	Supplier	Dilution
Goat anti mouse IgG HRP	#sc-2031 Santa Cruz	1:1000
Goat anti Rabbit IgG HRP	#sc-2030 Santa Cruz	1:1000

4.5.7 Developing

The membrane was washed three times (5mins each) on an orbital rocker in TBS/TWEEN before the two components of the Supersignal West Pico Chemiluminescence kit (#34078; Pierce Biotechnology) were added. Eight ml of Stable Peroxide Solution and 8ml Luminol/Enhancer Solution were mixed to obtain the West Pico Supersignal Working Solution. The membrane was incubated in the working solution in the dark for 5mins with gentle agitation. The chemiluminescence agents react with the HRP (conjugated to the secondary antibody) to produce light in proportion to the amount of target protein bound to the membrane (Figure 4.7).

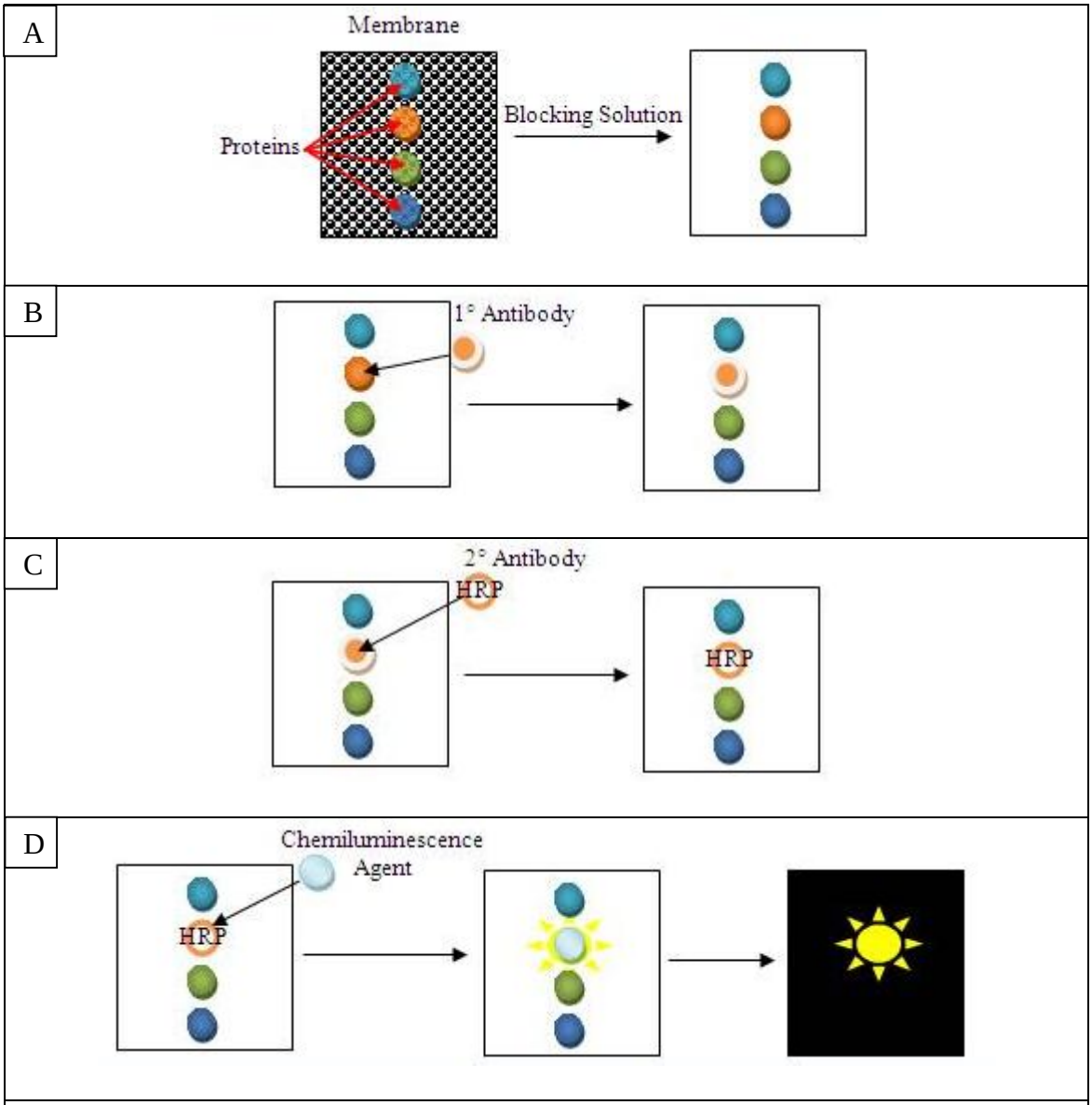


Figure 4.7: Schematic of antibody probing on a Western blot membrane

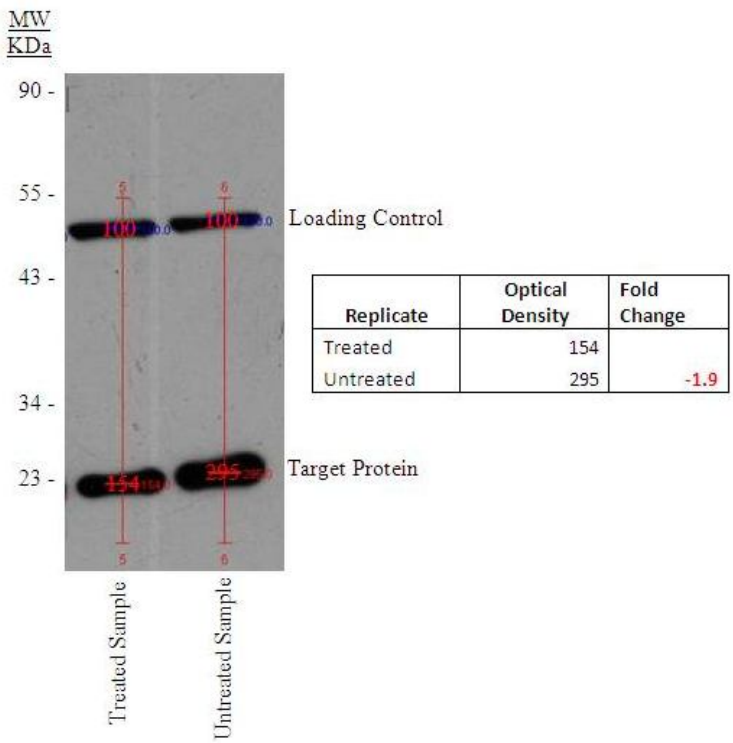
The membrane is first incubated with a dilute solution of protein such as casein or BSA to block any non-specific binding sites (A). The membrane is then incubated with a primary antibody (B), followed by incubation with a HRP conjugated, species specific secondary antibody (C). A chemiluminescence agent is then exposed to the HRP to produce luminescence in proportion to the amount of target protein bound to the membrane (D).

Membranes were exposed to SuperSignal CL-Xposure film (Pierce Biotechnology) for 5mins. Films were developed by gentle agitation in GBX developer (Kodak), then 2% acetic acid (Fisher) and finally GBX Fixer (Kodak) for about 30secs each. The film was

rinsed with water and air-dried. The membrane was stored in PBS at 4°C prior to stripping of the antibodies (see section 4.5.8) and re-probing.

4.5.8 Densitometry

Films were scanned using a GS-800 laser densitometer (Bio-Rad) into the Quantity One software (Bio-Rad). Target bands were normalised to a loading control such as GAPDH or β-actin to account for protein loading variability, and the optical density of the target band was recorded (Figure 4.8).



Replicate	Optical Density	Fold Change
Treated	154	
Untreated	295	-1.9

Figure 4.8 Normalised western blot image scanned into Quantity One software (Bio-Rad)

The picture shows a western blot of a 22KDa protein target and a 47KDa protein loading control. The target bands have been normalised to the loading control bands. The western blot is of a protein extract from treated cells and a untreated cells. The normalised optical density of the target bands is shown so the target protein expression can be compared in the treated sample and the untreated sample. This result shows a fold change of -1.9 of the target protein in the treated sample.

4.5.9 Stripping Membranes

Sometimes membranes need to be re-probed with different antibodies. Other antibodies can include loading controls or other related proteins of interest. If the original protein is a similar molecular weight to the new protein of interest, then the antibody from the first experiment has to be removed to avoid overlapping bands. Stripping buffers are available commercially.

The membrane was incubated in 15ml of Restore Plus Western Blot Stripping Buffer (#46430; Pierce) for 10mins at room temperature for a mouse antibody or 15mins at 37°C for a rabbit antibody on an orbital rocker.

The membranes were then washed, briefly re-blocked (see section 4.5.5) for 30mins and incubated in the 2° antibody used prior to stripping. Films were developed as described in 4.5.6 and 4.5.7 to ensure that the primary antibody had been completely removed. If the antibodies had been successfully removed then the membrane was re-probed with a new primary antibody. Repeated stripping of a membrane will eventually remove bound proteins, therefore membranes were stripped a maximum of three times.

4.6 Two Dimensional Gel Electrophoresis

Protein extracts intended for 2D-PAGE/MS analysis must be handled correctly to avoid contaminations and to prevent loss of sample. Polypropylene tubes were used to avoid proteins and peptides sticking inside, nitrile gloves were worn as opposed to latex gloves which cause protein contaminations and clothing and hair were covered to avoid keratin contaminations. All reagents used were proteomic/MS grade.

It is important that a minimum of three technical replicates of the same sample are done to acquire reliable results. For cell line work, protein should be extracted on three consecutive days to account for day to day variation in the cells, and a clinical sample should be divided into three and at processed the same time.

4.6.1 Protein Extraction

Cells were resuspended in 2D-PAGE lysis buffer (1ml) (see Appendix D). Samples were vortexed for 5mins at room temperature and sonicated for 5mins at room temperature to disrupt the cells. The tubes were then placed on an end-over-end rotator at 4°C overnight. Samples were centrifuged at 16,000xg for 15mins at 4°C to pellet cell debris and the supernatant was transferred into new, cold 1.5ml polypropylene microfuge tubes. Extracts were stored at -80°C.

4.6.2 Protein Quantification for 2D-PAGE

The 2-D Quant Kit (#80-6453-56; Amersham Biosciences) was used to quantify proteins for 2D-PAGE. The assay quantitatively precipitates proteins whilst leaving the contaminants in solution. This assay is a colorimetric assay and is compatible with CHAPS, urea, thiourea and DTT used in the preparation of protein samples for 2D-PAGE. The assay works by resuspending precipitated proteins in a copper containing solution. The copper ions bind specifically to the proteins and the assay measures any unbound copper. If lots of free copper ions are present, then the solution will become a darker orange colour. Therefore a lighter solution indicates more protein than a darker solution. A graph of optical density vs. amount of protein can be generated by using known amounts of a protein standard.

Six standard protein samples were prepared in 1.5ml microfuge tubes from a stock solution of BSA at 2mg/ml. The standards contained 0µg, 10µg, 20µg, 30µg, 40µg and 50µg of BSA.

Precipitation/Clean up

Five µl of protein sample was transferred into 1.5ml microfuge tubes. Two technical replicates were performed for each sample. Five hundred µl of 2-D Quant Kit Precipitant was added to each tube. Tubes were vortexed briefly to mix and left to stand at room temperature for 2mins. Five hundred µl of 2-D Quant Kit Co-precipitant was added to each tube and solutions were vortexed. The proteins were centrifuged at 10,000xg for 5min and the liquid was discarded. Tubes were centrifuged again for 30sec to enable small amounts of excess liquid to be discarded with a fine pipette. This step was repeated until all the excess liquid had been discarded.

Quantification Assay

One hundred µl of Copper Solution and 400µl of DH_2O was added to each tube. The tubes were thoroughly vortexed for 5min at room temperature to ensure the protein pellet was fully suspended. One ml of Working Colour Reagent (100 parts Colour Reagent 1 with 1 part Colour Reagent b, from the kit) was added and the tubes were vortexed immediately to ensure instantaneous mixing. Samples were incubated at room temperature for 15 min and 200µl of each sample and standard was added to a 96 well plate. The absorbance was read at 492nm on a plate reader. The BSA standard protein results were used to produce an optical density vs. protein amount graph, which was used to determine the protein amounts of the samples (Figure 4.9).

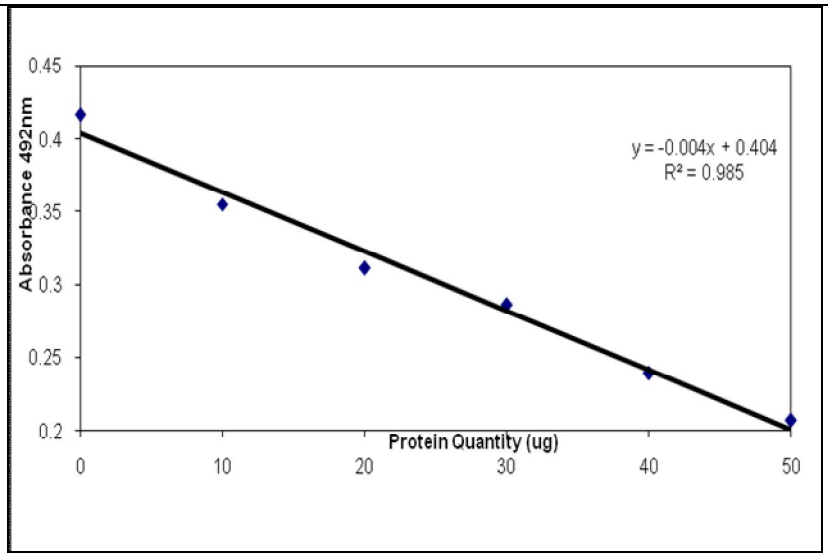


Figure 4.9: Graph of optical density (y-axis) vs. protein amount (x-axis) using BSA protein standards and the 2-D Quant Kit (Amersham)

The equation from this graph can be used to calculate a protein sample's quantity when the optical density is known.

4.6.3 2D-PAGE Sample Preparation and Clean Up

Protein samples were removed from the -80°C freezer and left to thaw on ice. Two hundred µg of protein was used for 2D-PAGE. This amount has been optimised for use with Coomassie Blue protein stain, if more protein was to be used the gels would be too streaky, if less protein was to be used the spots would be too faint. From the data obtained from the 2D quantification (see section 4.6.2), 200µg of sample was placed in a 1.5ml polypropylene microfuge tube and the sample was diluted to 200µl with 2D sample buffer, therefore a minimum of 1mg/ml protein is needed from the extraction. Protein samples were vortexed thoroughly and the ReadyPrep 2-D Cleanup Kit (#163-2130; Bio-Rad) was used to remove contaminants such as salts, lipids, nucleic acids and ionic detergents that may interfere with IEF and produce streaky gels. The kit is a precipitation based clean up, so it can also concentrate dilute samples. The kit is

compatible with 1-500µg of protein per reaction but can only be used with a volume of 100µl (or 200µl if reagents are scaled up as described in the kit protocol). When the protein has been precipitated and washed it can be resuspended in any buffer at any concentration.

Precipitation

For a 200µl sample: Three hundred µl of Precipitation Agent 1 was added to each sample. Tubes were mixed and left on ice for 15mins. Three hundred µl of Precipitation Agent 2 was added and tubes were mixed and centrifuged at 16,000xg for 5mins at room temperature. The liquid was discarded and the tube was centrifuged again to remove trace liquid and leave only a protein pellet.

Washing

Forty µl of Wash Reagent 1 was added to the pellet and tubes were vortexed. Samples were centrifuged as before for 5mins and excess liquid was discarded. Forty µl of DH_2O was added and samples were vortexed for 20secs. One ml of cold Wash Reagent 2 and 5µl of Wash Additive 2 were added and samples were vortexed for 1min. Samples were incubated for 30mins at -20°C and vortexed every 10mins for 30sec. Tubes were centrifuged as before for 5mins. The liquid was discarded and the tubes were centrifuged until the all the excess liquid could be removed with a fine pipette. The pellets were air dried for 4mins or until the pellet appeared translucent.

Resuspending

The samples were resuspended in the appropriate amount of 2D buffer. For 11cm IPG strips, 200µg of protein was resuspended in 210µl of 2D buffer. The samples were mixed with a pipettor and then vortexed for 1min. Tubes were incubated at room temperature for 5mins, vortexed for 1min and centrifuged for 5mins.

If the correct protocol is followed and Wash Reagent 2 is added cold, then the kit should recover over 95% of the protein.

4.6.4 Isoelectric Focusing

An 11cm ReadyStrip IPG strip (#163-2015; Bio-Rad) can absorb 180µl of solution; therefore 180µg of protein will be loaded from a 1mg/ml solution. The Bio-Rad ReadyPrep IPG strips come as 7cm, 11m or 17cm long, the equivalent sized 2D gel must also then be used. For this project 11cm IPG strips were used, this is because 17cm gels are fragile and difficult to handle, and 7cm gels do not separate out the proteins enough. The IPG strip's pH ranges used in this project were 3-10 and 4-7. An 11cm pH3-10 strip does not separate the proteins enough and gels are usually streaky making the spots difficult to excise. Therefore it is best to do an experiment in two sections. Firstly separate and analyse protein in the pH4-7 region, then (sample quantity permitting) analyse proteins in the pH7-10 region. The pH7-10 region does not yield as many proteins as the pH4-7 region and gels are very streaky. Between pH7-9, disulphide groups on the protein are prone to unspecific oxidation causing extra spots and streaking (Figure 4.10).

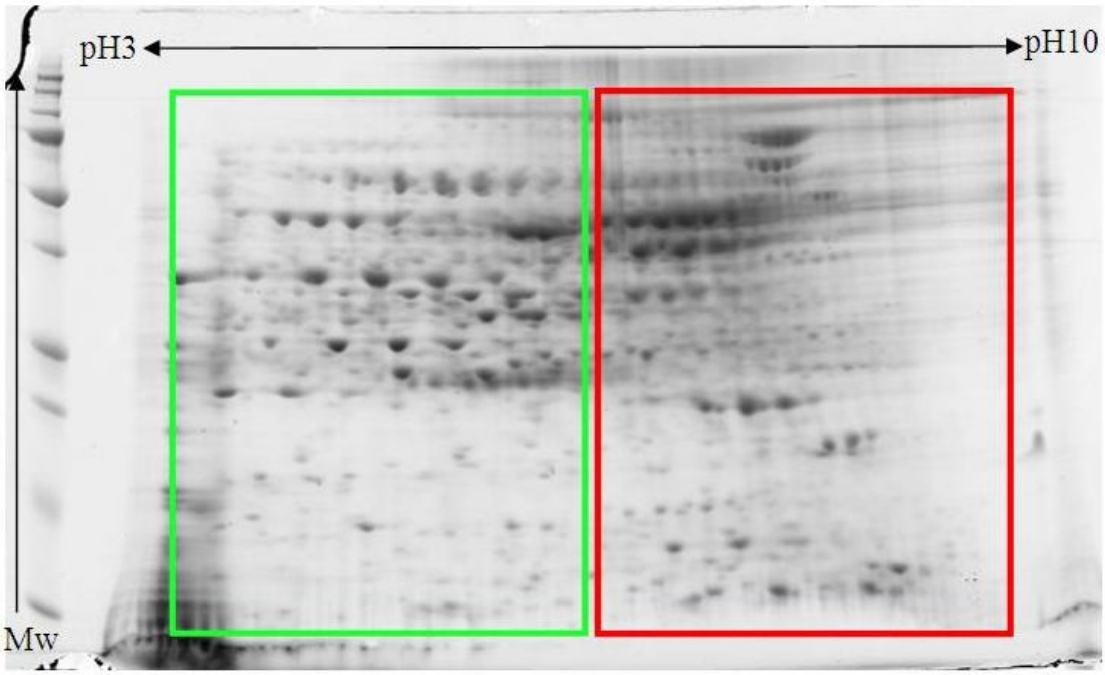


Figure 4.10: 2D Gel of proteins separated on a pH3-10 IPG strip

The picture shows a 2D gel of E.coli run on a pH3-10, 11cm, non-linear ReadyStrip IPG strip (#163-2016; Bio-Rad) (see section 5.5.1). The gel shows how streaky the proteins spots become at a high pH (red) making proteins difficult to excise. The gel also shows more proteins in the lower pH region (green).

Loading the IEF Tray

The protein sample was carefully pipetted along the back edge of a new rehydration tray (#165-4025; Bio-Rad). Care was taken to avoid getting the sample on the sides of the tray. When all the protein had been loaded the coversheet was peeled off a thawed 11cm IPG strip. The strip was placed gel side down in the channel containing the protein. Care was taken to avoid getting any air bubbles trapped between the protein sample and the IPG strip, and that all the protein solution was in contact with the strip. Three ml of mineral oil (#163-2129; Bio-Rad) was layered onto each strip to prevent evaporation and the strips drying out. The samples tray was covered over and the IPG strips were left to absorb the protein for 16hrs at room temperature.

IEF Focusing

Electrode wicks were placed over the electrodes in a clean, dry IEF tray (#165-4020; Bio-Rad). Eight µl of DH_2O was added onto each wick. The IPG strips were taken out of the rehydration tray with forceps and held vertically for 7secs over blotting paper and blotted to remove any excess oil and unabsorbed protein which can lead to streaky gels. The strips were placed in the IEF tray gel side down and ensuring that the positive side of the strip corresponded with positive end of the tray. Three ml of mineral oil was added to each strip to prevent the strips from drying. The lid was placed on the tray and the tray was placed in the Protean IEF cell (Bio-Rad). The cell was programmed with the method suggested for an 11cm IPG strip (Table 4.3). The completed method takes approximately 5.5hrs. The strips are first subjected to 250V for 20mins on a linear ramp, then 8000V for 2.5hrs and then 8000V on rapid ramp for 20,000 Volt Hours (V hrs). Finally the strips are held at 500V.

Table 4.3: Method for the Protean IEF Cell for an 11cm IPG strip

Step	Voltage (V)	Ramp	Time (hrs:mins & V hrs)
1	250	Linear	00:20
2	8000	Linear	02:30
3	8000	Rapid	20,000 V hrs
4	500	Linear	Hold

The strips were removed from the IEF tray and held vertically for 7 sec over blotting paper and blotted to remove excess liquid. The strips were then placed in a rehydration tray gel side up. The tray was wrapped in saran wrap and stored at -80°C. The IPG

strips can be stored for up to two months without any effect on the second dimension separation.

4.6.5 Equilibration

Stock equilibration buffer (see Appendix D) was made up and stored in 40ml aliquots at -20°C. Equilibration buffers 1 and 2 were prepared fresh (see Appendix D). Equilibration buffer 2 is light sensitive and was kept in the dark. The protein loaded IPG strips were taken from the freezer and thawed in laminar flow hood at room temperature until translucent. The strips were not left to thaw longer than 15mins. They were then transferred to a clean, dry rehydration tray gel side up.

Reduction

Four ml of equilibration buffer 1 (containing DTT) was added to each IPG strip in the tray and the tray was placed on an orbital rocker at room temperature for 10mins. Equilibration buffer 1 was discarded.

Alkylation

Four ml of equilibration buffer 2 (containing IAA) was added to each strip. The tray was placed on an orbital rocker for 10mins at room temperature in the dark and equilibration buffer 2 was discarded.

4.6.6 SDS-PAGE

Pre-cast 13.3 x 8.7cm, 8-16% Criterion Tris-HCl gels (#345-0105; Bio-Rad) were removed from their packaging and the comb was removed. The wells were rinsed three times in DH_2O and the excess water was blotted off using filter paper. The IPG strips were washed in running buffer (taking care not to introduce any air bubbles on the surface of the strip) and placed on the back of the gel, gel side up. Molten agarose solution (see Appendix D) was pipetted in the IPG strip well, and the IPG strip was gently pushed into the well ensuring contact with the gel, and making sure no air bubbles were present. The gel was left for 5mins to let the agarose solution set. The gel was then placed into a Criterion Cell (Bio-Rad), which was subsequently filled with running buffer (upper buffer chambers filled with ~60ml and lower chamber filled with ~800ml). The Criterion Cell could run one or two Criterion pre-cast gels simultaneously. Ten µl of Precision Plus Dual Colour Molecular Protein Standard (#161-0374; Bio-Rad) was added to the molecular weight standard well. The lid was placed on the tank and electrophoresis was performed at a constant 200V for 65mins. The gel was taken out of the plastic cassette and placed in a Nalgene staining box. Gels were washed for 5mins with approximately 300ml of DH_2O three times. The water was discarded and the gels were stained as detailed in section 4.8.

4.7 One Dimensional Gel Electrophoresis

Proteins were separated as described in section 4.5.3; however the Cruz Marker™ was not required. After the gel had run it was removed from the plastic casing, transferred to a clean Nalgene staining box and washed for 5mins in approximately 100ml of DH_2O three times. It was important to keep western blot staining boxes and coomassie blue

staining boxes clean and separate, as the blocking solution used for westerns binds to the sides of the boxes and will be stained by a protein stain. The water was discarded and the gels were stained as detailed in section 4.8.

4.8 Protein Staining

Stains that have been used and compared for this project are Bio-Safe™ Coomassie stain (#161-0787; Bio-Rad), SYPRO® Ruby protein gel stain (#s-12000; Invitrogen), Krypton protein stain (#46629; Pierce), Flamingo™ fluorescent gel stain (#161-0492; Bio-Rad) and Pro-Q® Diamond phosphoprotein gel stain (#p-33300; Invitrogen). The fluorescent stains and the fluorescent stained gels were handled in the dark throughout the protocols.

4.8.1 Coomassie Protein Stain

After the gels were washed with DH_2O, enough Bio-Safe™ Coomassie stain was added to the Nalgene staining box to cover the gel (approximately 50ml for a 1D gel and 150ml for a 2D gel). The staining box was placed on an orbital rocker for 1hr at room temperature. The staining solution was discarded and the gels were placed in DH_2O (approximately 100ml for a 1D gel and 250ml for a 2D gel) for 16hrs to destain. Gels were scanned with a GS-800 laser densitometer (Bio-Rad) into the Quantity One software (Bio-Rad).

4.8.2 SYPRO Ruby Protein Stain

For a 1D gel, 100ml of fix solution (see Appendix E) was added to the gel in a Nalgene staining box, which was agitated on an orbital rocker for 30mins. This step was repeated with fresh fix solution. Sixty ml of SYPRO® Ruby protein gel stain was added to the gel, which was left for 16hrs at room temperature on an orbital rocker in the dark. The gel was transferred to a clean staining box and 100ml of wash solution (see Appendix E) was added and left on for 30mins on an orbital rocker in the dark. The gel was washed in DH_2O before being scanned into Quantity One with a PharosFX Molecular Imager System (Bio-Rad) fitted with a 532nM laser and a 605nM bandpass filter.

4.8.3 Krypton Protein Stain

For a 1D gel 100ml of fix solution (see Appendix E) was added to the gel in a Nalgene staining box and agitated on an orbital rocker for 30mins. The fix solution was discarded and the procedure repeated with fresh fix solution. The gel was washed with DH_2O on an orbital rocker for 5mins. The water was discarded and 5ml of Krypton stain was diluted in 45ml DH_2O and was added to the gel. The gel was left for 16hrs at room temperature on an orbital rocker in the dark. The stain was discarded and the gel was incubated in 50ml destaining solution (see Appendix E) on an orbital rocker in the dark for 5mins. The gel was transferred to a clean staining box and 100ml of DH_2O was added which was left on for 15mins on an orbital rocker in the dark. The wash was repeated before the gel was scanned into Quantity One with a PharosFX Molecular Imager System fitted with a 532nM laser and a 605nM bandpass filter.

4.8.4 Flamingo Protein Stain

For a 1D gel 100ml of fix solution (see Appendix E) was added to the gel in a Nalgene staining box and agitated on an orbital rocker for 16hrs. Five ml of Flamingo™ fluorescent gel stain was diluted in 45ml DH_2O and was added to the gel, which was left for 3hrs at room temperature on an orbital rocker in the dark. The gel was transferred to a clean staining box and 100ml of DH_2O was added which was left on for 10mins on an orbital rocker in the dark. The gel was washed in again in DH_2O before being scanned into Quantity One with a PharosFX Molecular Imager System fitted with a 532nM laser and a 605nM bandpass filter.

4.8.5 Pro-Q Diamond Phosphoprotein Gel Stain

For a 1D gel 100ml of fix solution (see Appendix E) was added to the gel in a Nalgene staining box and agitated on an orbital rocker for 30mins. The fix solution was discarded and the procedure repeated with fresh fix solution for 16hrs. The gel was washed with 100ml DH_2O on an orbital rocker for 15mins. The wash step was repeated a further two times. The water was discarded and 60ml of Pro-Q® Diamond phosphoprotein gel stain was added to the gel, which was left for 90mins at room temperature on an orbital rocker in the dark. The stain was discarded and the gel was incubated in 100ml destaining solution (see Appendix E) on an orbital rocker in the dark for 30mins. The destaining step was repeated a further two times. The gel was transferred to a clean staining box and 100ml of DH_2O was added which was left on for 5mins on an orbital rocker in the dark. The wash was repeated before the gel was scanned into Quantity One with a PharosFX Molecular Imager System fitted with a 532nM laser and a 555nm longpass filter. To re-stain the proteins with a total protein

stain, the gel was washed in DH_2O twice for 5mins and the total protein stain was added (see sections 4.8.1 and 4.8.3) without a fixing step.

4.9 Data Analysis

Two software packages, which identify differentially expressed spots in 2D gels, were compared for use in the project. The software packages were PDQuest 2-D Analysis software (Bio-Rad) and Progenesis SameSpots (Nonlinear Dynamics). The two software packages were compared for;

- Reliability
- Ease of use
- Accuracy of results
- Time for analysis

Based on the finding PDQuest 2-D analysis software was used to analyse gels (see section 5.6).

4.9.1 PDQuest 2-D Analysis Software

Three replicate gels were run for the test sample and the control sample. The six gels were uploaded from the Quantity One scanned images (in .1sc format) to PDQuest 2-D analysis software which creates the experimental file (in .ms format). Version 8.0 was used in this project which includes a wizard guide to setting up the experiment. The software asks the user to define a small spot and a faint spot. Spots should be selected

which are not too faint or too small to be excised. The software runs automatic detection and matching based on the spot information given and de-speckles the gels to discard non-specific spots. The software picks the gel with the most spots and creates a master gel from it. The master gel contains all the spots from all the gels which have been detected and matched. Each spot is given a corresponding letter (A-Z) so that it is easy to recognise individual spots on each gel. When the software has analysed the gels it then opens up the results box.

However, it is necessary to manually edit the analysed gels as the software is unable to give 100% accuracy. This requires removing false spots (which maybe water bubbles from scanning) and clustered spots/streaks (which may be impossible to excise and take up analysis time), adding in missing spots, and identifying incorrect matching (Figure 4.11 and Figure 4.12). This procedure usually takes up to five days if done thoroughly. Only if time and dedication is spent manually editing the automatically analysed gels will the results be true, and this will reduce false positive Western blot results after MS identification. The Dual Colour protein standards must also be added and assigned a molecular weight.

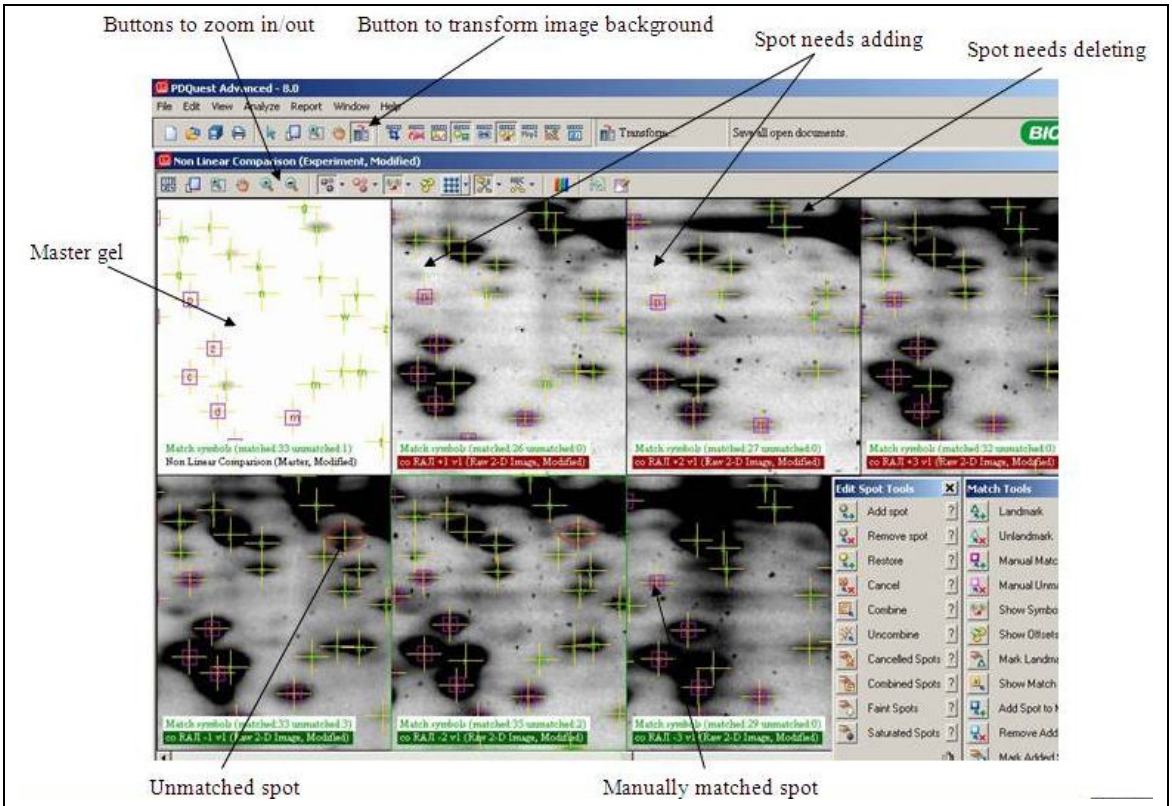


Figure 4.11: Screenshot of PDQuest Editing

The picture shows in a zoomed in area of the gel where editing is needed. Spots which have a red circle around them have not been automatically matched and added to the master gel and need manually matching. When the spots have been manually matched it will show a purple square on it. There are also spots in this area which need adding because the automatic detection has not picked them up, and spots which need deleting because they are in streaks and clusters and are un-excisable. To visualise the spots better a transform tool increases or decrease the contrast of the picture, this does not affect the results.

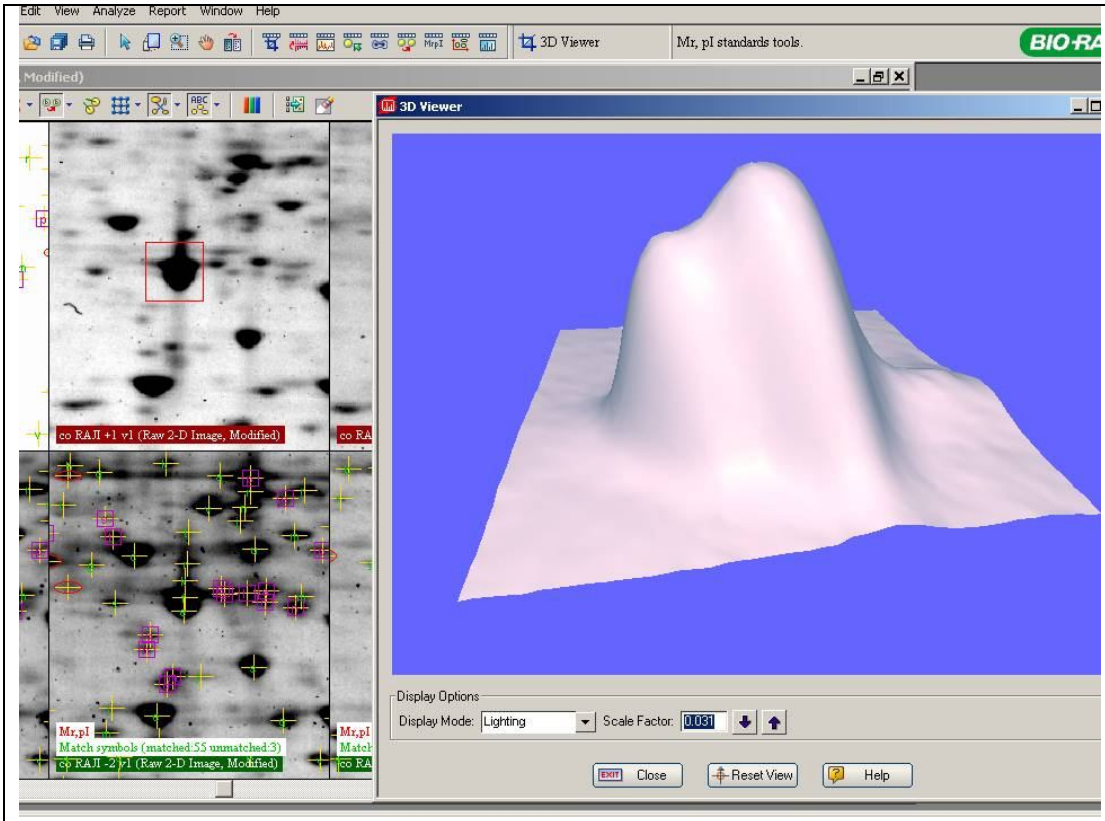


Figure 4.12: PDQuest 3D Viewer

A 3D viewer allows the gel to be seen geographically, showing the proteins like hills on the gel. The picture shows a very large, dark spot highlighted in the red box. The 3D viewer clearly shows that this is two proteins overlapping.

When the editing is complete, the gels can be analysed. Only protein spots present in all 3 replicates are included in the analysis therefore it is important to make sure that if a spot is present in one gel, it is highlighted in all the replicates or it will not be counted in the results. The "total quantity in valid spot" normalisation method is used, which divides the raw quantity of each spot in the member set by the total quantity of all spots in the gel, which have been included in master gel (this is recommended when little is known about sample variation). A Boolean analysis set is created by taking into account a quantitative 2-fold analysis set and a statistical Students *t*-test ($P<0.05$) analysis set (Figure 4.13). Proteins which are differentially expressed between the two groups are

identified by intersecting the two analysis sets. Histograms of the replicates are shown in the results to allow the analyst to discard any ambiguous results (Figure 4.14).

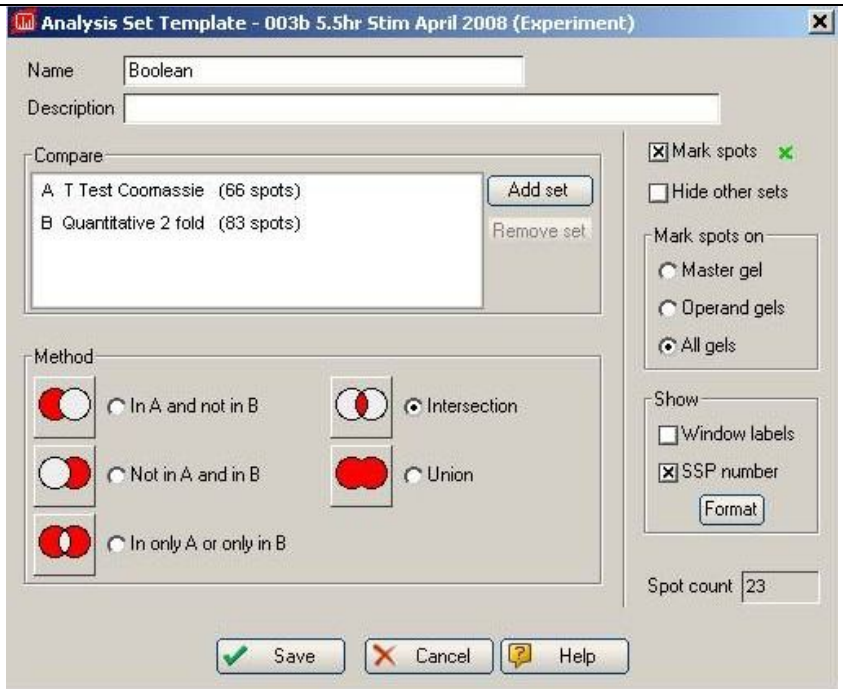


Figure 4.13: PDQuest Analysis Sets

The picture shows a Boolean analysis set. From the data shown above, 66 spots were found to have $p<0.05$ and 83 spots had a 2 fold quantitative change. Of these 149 spots, 23 of them had both $p<0.05$ and a 2 fold quantitative change.

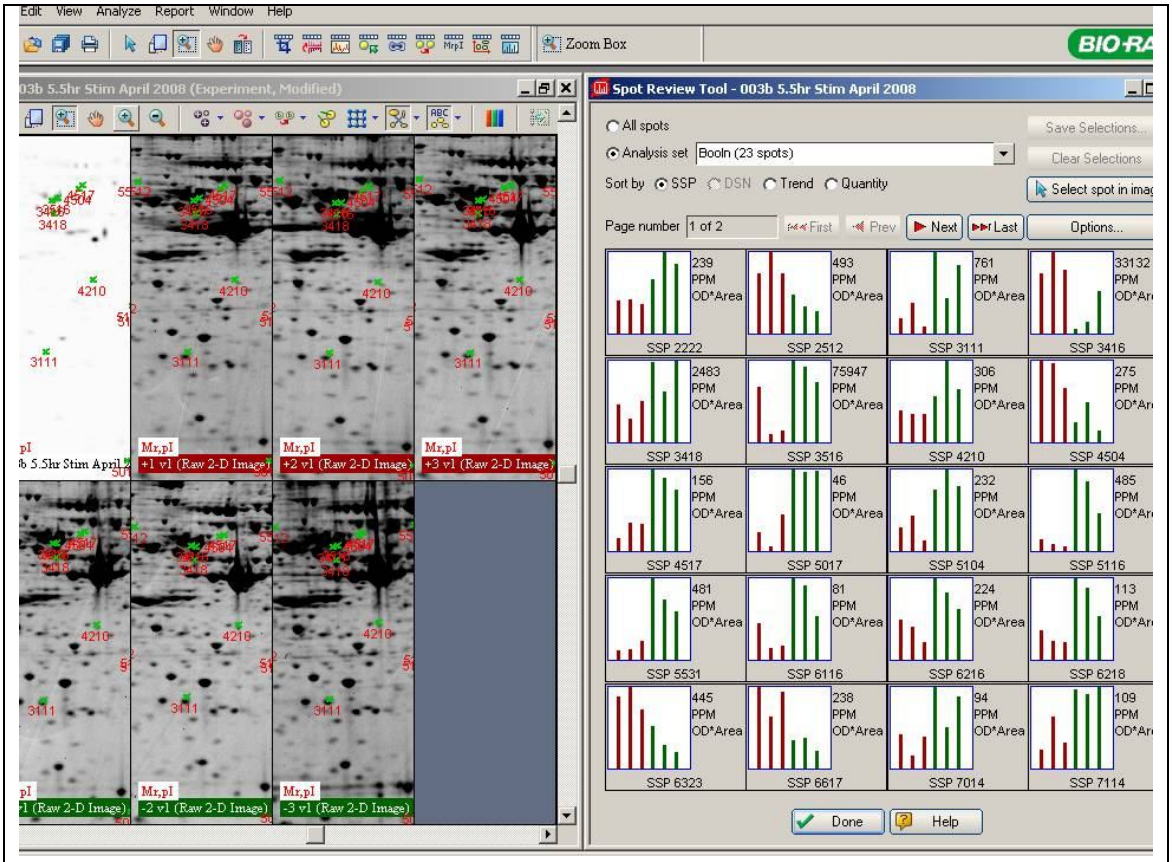


Figure 4.14: PDQuest Spot Review Results

Spots which are shown to be differentially expressed by the Boolean analysis set are shown in the spot review tool box, complete with histograms of the replicate gels. A print out of the gel with the differentially expressed spots highlighted is then used to manually excise spots from the gels.

A print out of the gel highlighting the differentially expressed spots to be excised, the pI and the molecular weights is used to excise and to subsequently help with MS identification. For manual excision of the spots, it is helpful to have printouts of zoomed-in images of the gel and spots.

4.9.2 Progenesis SameSpots

Gels were uploaded into the software (in .tif format) and categorised into their groups. The software asks the user to pick a gel which could represent the experiment (usually

the one with the most spots on). The software works by aligning the pictures of the gels to the representative gel (Figure 4.15). The gels (coloured green) are placed on top of the representative gel (coloured pink) one at a time, and are aligned by the addition of vectors. The analyst first adds vectors manually until they are confident that the software has been trained sufficiently to add vectors automatically (Figure 4.16). Vectors need to be added in all regions of the gel if the automatic vectors are to be placed correctly. It is important that time is spent at this stage in the analysis if well aligned gels pictures are to be obtained. When enough vectors have been placed manually, and the software has correctly added automatic vectors, the gel images can be aligned (Figure 4.17). When this stage has been done for each replicate gel the software can analyse the gels to identify any differentially expressed spots (Figure 4.18).

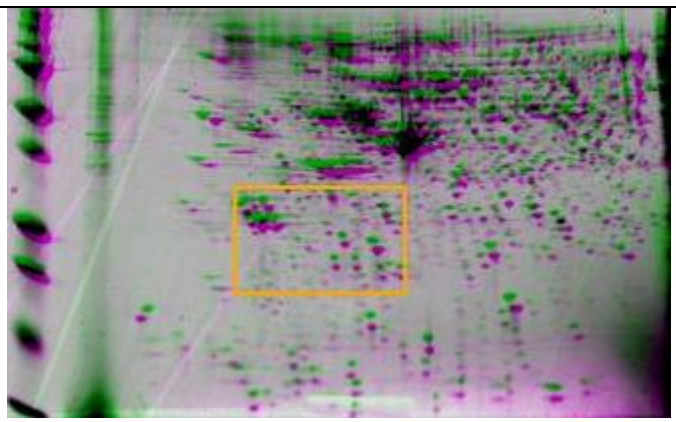


Figure 4.15: Unaligned gels from Progenesis SameSpots

The representative gel (coloured pink) is layered on one of the replicate gels (coloured green). It is possible to see from the picture the similarities between the gels. The gels need to be aligned for analysis. To do this vectors are placed on the picture to show where the gels need to be moved to. The spots are good landmarks for placing the vectors.

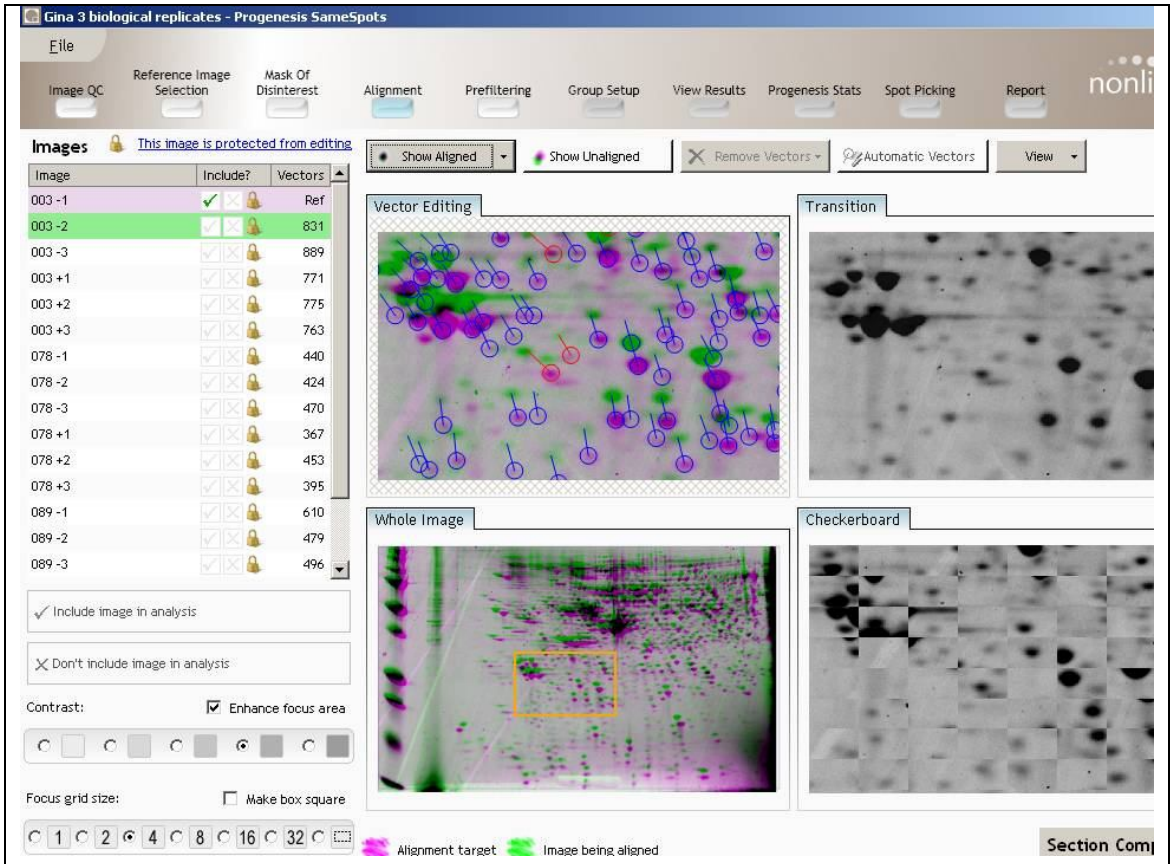


Figure 4.16: Unaligned gels with vectors from Progenesis SameSpots

Vectors are added to train the software where to pull the gel image to. The manually added vectors are shown in red. When enough vectors have been added by the analyst the software can then add vectors in automatically, these vectors are shown in blue. The screen shot shows four gel images. Top left is a zoomed in area of the gel showing vectors. Bottom left is the full gel image. Top right pictures alternates between the representative gel and one of the replicate gels. Bottom right is the checkerboard effect from overlying the two images. The contrast and size of zoomed area can be altered.

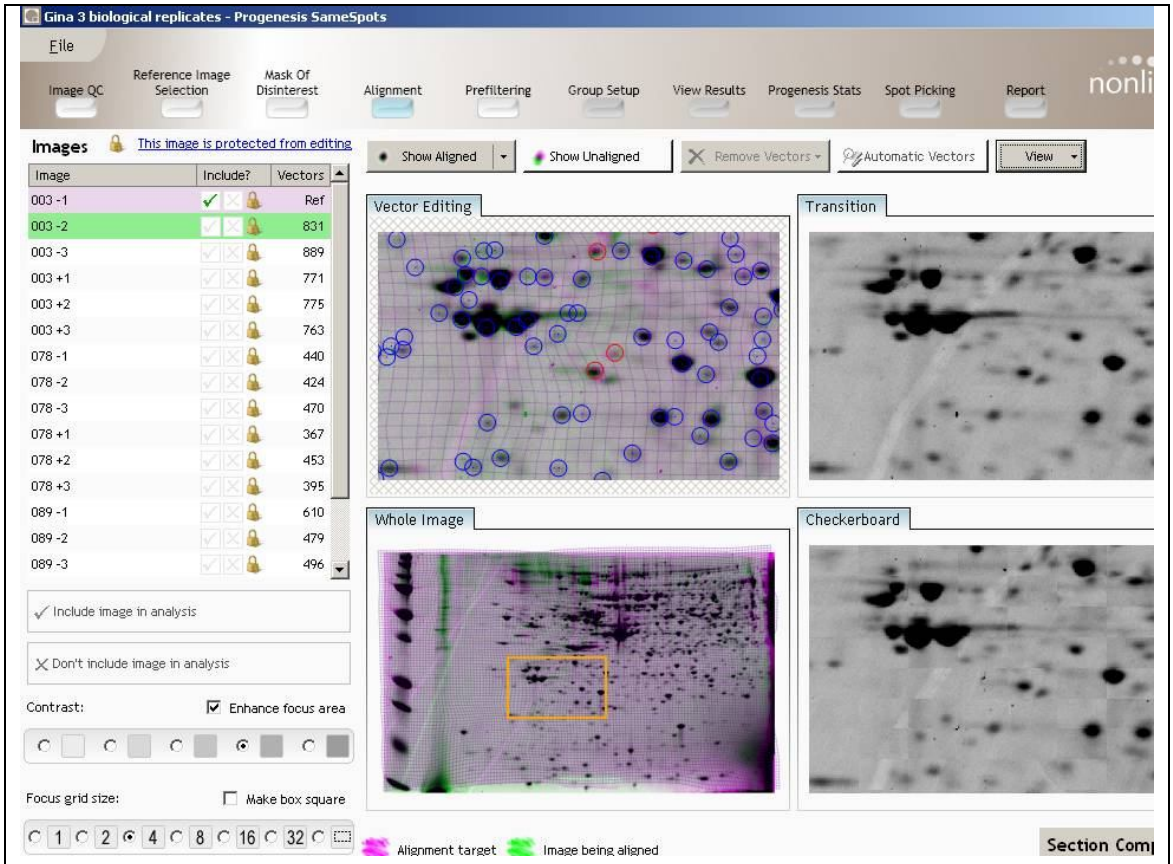


Figure 4.17: Aligned gels from Progenesis SameSpots

When all the vectors have been added, the images can be aligned. The checkerboard image (bottom right) is a good indication of a successful alignment. The image at the top right of the screen shot will still alternate between the representative gel and one of the replicate gels but changes should be less noticeable. The picture top left shows the manually added vectors (red) and the automatic vectors (blue).

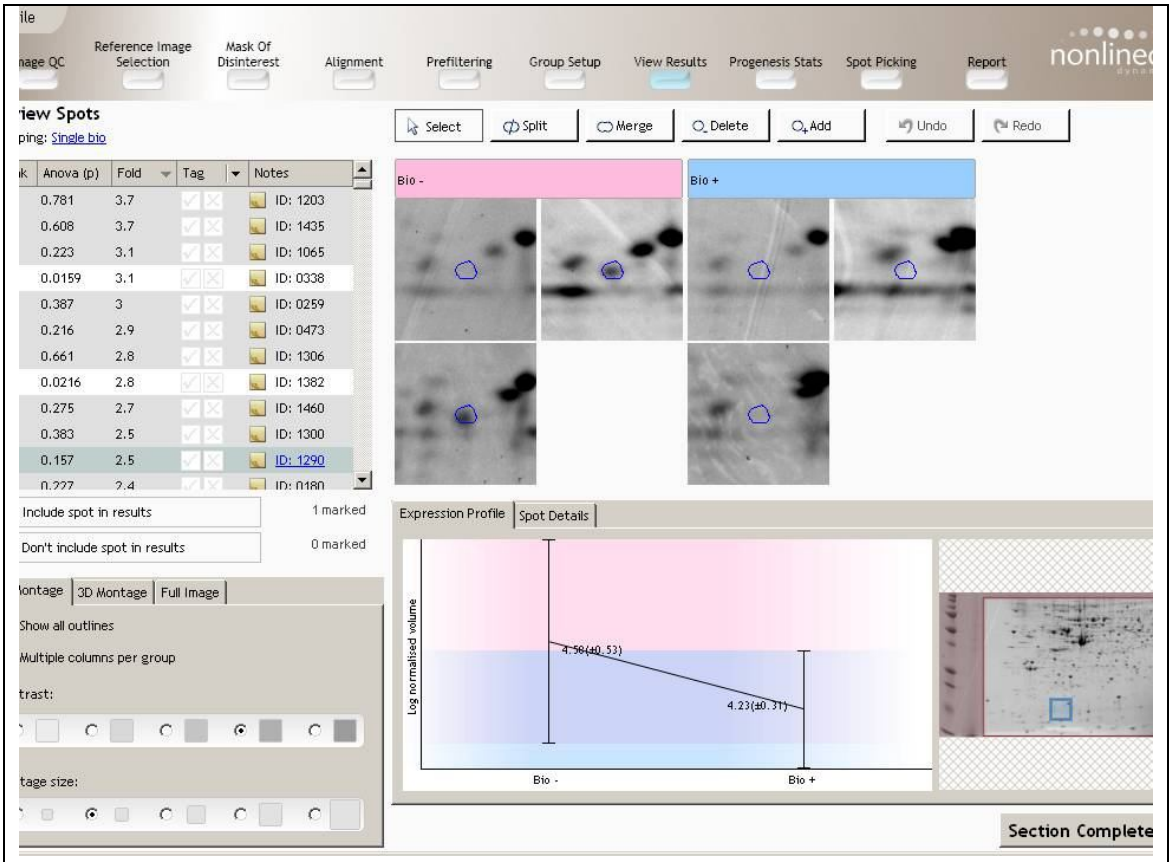


Figure 4.18: Results from Progenesis SameSpots

The software creates a table of all spots detected in the gels, the fold change between groups and the Anova p value. It is possible at this point in the software to edit the automatic spot detection. The spot list is analysed by the user and spots are either included or discounted from the final report. A print out of the gel with the differentially expressed spots highlighted is then used to manually excise spots from the gels.

4.10 In-Gel Digestion

4.10.1 Excision and Washing of Gel Pieces

Stained 2D gels (see section 4.8) were washed with distilled water and transferred to gel cutting sheets (#165-7018; Bio-Rad). Protein spots of interest were excised with a scalpel using a magnifying glass and print outs of the gels with the differentially expressed spots highlighted. The gel spots were cut into small pieces to increase the

surface area for trypsin digestion. Each spot was pooled from all of the replicate gels into a labelled EZ polypropylene 1.5ml microfuge tube (#223-9480; Bio-Rad) containing 500µl DH_2O. By using polypropylene tubes, the risk of peptides sticking to the surface and of polymer contaminants is greatly reduced. The gel pieces were centrifuge at 16000xg for 30sec.

4.10.2 Reduction and Alkylation

The following procedures were carried out in a laminar flow hood. A 0.1M ammonium bicarbonate NH_4HCO_3 (AMBIC) solution was prepared fresh. Acetonitrile (ACN) was filtered into a clean, sterile bottle before use.

The DH_2O was removed with a fine pipette and 200µl of ACN was added to dehydrate the gel pieces. The tubes were vortexed and left to stand at room temperature for 15mins. The samples were centrifuged at 16000xg for 30sec and the ACN was removed with a fine pipette. The open tubes were placed in a vacuum-centrifuge (Uniequip) to dry for 30mins. Sixty µl of DTT/0.1M AMBIC solution (see Appendix D) was added to each tube and the tubes were vortexed and placed in an oven for 30mins at 56°C to reduce any disulphide bonds. The samples were checked every 10mins to ensure they were fully covered by the DTT/0.1M AMBIC solution and to ensure the gel pieces were not sitting in an air bubble at the bottom of the tube. The tubes were centrifuge for 30sec at 16,000xg and the DTT/0.1M AMBIC solution was removed with a fine pipette. Two hundred µl of ACN was added and the samples were vortexed and left to stand at room temperature for 15mins to dehydrate. The ACN was removed with a fine pipette after centrifuging the samples at 16000xg for 30secs. Sixty µl of IAA/0.1M AMBIC

solution was added to each tube and the samples were stored in the dark at room temperature for 20mins to alkylate. Samples were centrifuged at 16000xg for 30sec and the IAA/0.1M AMBIC solution was removed with a fine pipette. Two hundred µl of 0.1M AMBIC was added to the gel pieces. Tubes were vortexed for 15mins. Samples were centrifuged at 16000xg for 30sec and the 0.1M AMBIC solution was removed with a fine pipette. Two hundred µl of ACN was added to dehydrate the gel. Samples were vortex for 15mins. Samples were centrifuged at 16000xg for 30sec and the ACN solution was removed with a fine pipette. The gel pieces were vacuum centrifuged for 30mins.

4.10.3 Washing and Destaining of Gel Pieces

One hundred and fifty µl of 0.1M AMBIC solution was added. Tubes were vortexed for 15mins. One hundred and fifty µl of ACN was added to the samples in the 0.1M AMBIC solution. Samples were vortexed for 15mins, centrifuged at 16000xg for 30sec and the ACN/0.1M AMBIC solution was removed. A further 200µl of ACN was added and the samples were vortex for 15mins centrifuged at 16000xg for 30sec. The ACN was removed with a fine pipette. The gel pieces were dried in a vacuum centrifuge for 30mins.

If the gel pieces were blue at this point (i.e. still had coomassie stain in them), the washing step was repeated until the gel pieces were transparent.

4.10.4 Trypsin Digestion

Trypsin Gold, Mass Spectrometry Grade (#V5280; Promega) was reconstituted in 50mM of acetic acid to 1µg/µl. The reconstituted trypsin was aliquoted and stored at -80°C. Digest buffer 1 (which contained reconstituted trypsin) and digest buffer 2 were prepared fresh prior to digestion (see Appendix D).

When all the stain had been washed out of the gel pieces 30µl of digest buffer 1 (containing trypsin) was added to each tube and they were incubated at room temperature for 1hr. During this time the gel pieces would absorb the solution. Larger gel pieces would absorb more trypsin than the smaller ones. The excess of trypsin digest buffer 1 was removed with a pipette after 1hr. Trypsin is a protein and if in excess will digest itself causing contaminating trypsin peptides. By removing the excess solution and only allowing the gel pieces 1hr to absorb the solution, the risk of trypsin autolysis is minimised.

When the excess of digest buffer 1 was removed 50µl of digest buffer 2 was added to the samples. Digest buffer 2 keeps the gel pieces hydrated during the digestion and also acts as a suitable volatile solvent for the peptides to diffuse into. The samples were incubated in an oven at 37°C for 16hrs.

4.10.5 First Peptide Extraction

The following procedures were carried out in a laminar flow hood. A 0.025M AMBIC solution (see Appendix D) was prepared fresh. ACN was filtered before use. During the

peptide extraction, lids on the microfuge tubes were sealed with parafilm prior to all vortexing and shaking steps.

Fifteen µl of 0.025M AMBIC solution was added to the tubes. Samples were shaken for 15mins at 37°C. Fifty µl of ACN was added to the tubes and samples were shaken again for 15mins at 37°C. The gel pieces were centrifuged at 16000xg for 30sec and the supernatant (containing peptides) was transferred to new 1.5ml polypropylene microfuge tubes. Care was taken not to transfer any gel pieces with the supernatant. The gel pieces were retained in the original microfuge tubes for the second peptide extraction step on the gel pieces.

Second Peptide Extraction Step

A second peptide extraction step was necessary to retrieve a maximal yield of peptides for MS. Lids on the microfuge tubes were sealed with parafilm prior to vortexing and shaking.

Fifty µl of 5% formic acid was added to the gel pieces which were vortexed for 15mins at 37°C. Tubes were centrifuged for 30sec at 16000xg and 50µl of ACN was added to the gel pieces in 5% formic acid. Samples were shaken for 15mins at 37°C. The tubes were centrifuged for 30sec at 16000xg and the supernatant was combined with the previous extraction. The gel pieces were discarded.

4.10.6 Preparation for Mass Spectrometry

The combined extracts from section 4.6.1 were dried down in a vacuum centrifuge until approximately 1-2µl of solvent was remaining (approximately 40min). It was important not to dry the solution completely as the peptides may stick to the tube and become difficult to get back into solution. Fifteen to twenty µl of ACN/DH_2O (50:50) with 1% formic acid was added to the tubes which were vortexed and sonicated for 5mins at room temperature to ensure all peptides have been resuspended. It is necessary to resuspend the peptides in a minimum volume of 15µl to ensure the solvent mixes with the peptides in the vortexing step.

An AnchorChip™ MALDI Target (#209515; Bruker Daltronics) (Figure 3.3) was cleaned by sonicating in methanol:DH_2O (50:50). A saturated solution of DHB (#85707; Sigma-Aldrich) in ACN:DH_2O (50:50) with 1% formic acid was prepared fresh.

Two µl of sample was mixed with 1µl of DHB saturated solution in a polypropylene 0.5ml microfuge tube by gentle pipetting. The remaining peptide extracts could be stored at -20°C for up to 5 days or dried down in a vacuum centrifuge and stored at -20°C for long term storage. Three µl of sample/matrix solution was loaded onto the MALDI sample plate, with a fine, gel loading pipette tip. Each sample was loaded onto a separate sample spot on the plate and the coordinates of each sample was recorded. The samples were left to air dry. It was sometimes necessary to re-spot certain samples if they had not dried well. For example, if the room temperature was high, the solvent would evaporate away too quickly, leaving the matrix/peptide crystals clumped and too

thick. The crystals need to be spread out enough to be thin enough for the MS laser to be able to penetrate through. This is only a cause for concern when using DHB matrix.

4.11 Matrix Assisted Laser Desorption/Ionisation, Time of Flight Mass Spectrometry

A Bruker Daltronics Reflex IV MALDI-TOF MS fitted with a 337nm nitrogen laser in reflectron mode was used to acquire PMFs. Ions were accelerated through a potential of 20Kv into the TOF mass analyser and ions within m/z 950-2500 were detected. Laser power was set between 19 and 35%. A minimum of 200 laser shots were used to acquire the averaged spectra and 20 shots were fired in a single acquisition. Analysis was performed using the Bruker software suite and acquisition of spectra was obtained with Flex Control. Spectra were processed using Flex Analysis and Biotools. Peaks were edited in Flex Analysis (small undetected peaks were added and multiple isotopic peaks were deleted); however keratin contaminants were kept in at this point.

PMFs were searched against the NCBInr database using MASCOT search engine (www.matrixscience.com). Taxonomy was restricted to human. Fixed carbamidomethyl modifications and variable methione and oxidation modifications were selected. The search allowed for two missed cleavage and a +/- 0.1% mass tolerance for monoisotopic peak. The MOWSE scoring was used by MASCOT to determine whether protein identifications were significant ($P<0.05$).

The spectra were first submitted to the database search without taking out any keratin peaks. Any results which matched the molecular weight and the pI of the protein spot of

interest were investigated. If a significant score had not been achieved the first set of contaminant peaks (see section 5.7.2 and Appendix H) were taken out.

Prior to starting the project a list of contaminants was obtained from the internet which included keratin contaminants and trypsin autolysis peaks. A blank piece of polyacrylamide gel was digested to obtain background peaks. Multiple digests of standard, commercially available proteins were analysed on the MALDI-TOF-MS and the spectra was compared to reference spectra. This allows the analyst to obtain their own personal contaminants list. Therefore it is vital that the analyst conducts their own work throughout (see section 5.7.2).

It is important not to take all contaminants out at once as some peaks may overlap with real peptide peaks. Therefore four sets of contaminant peaks (see Appendix H) were taken out sequentially if a protein match (significant score and correct pI and MW) was not shown. These sets have been derived by myself over the past four years and range from the first set containing peaks which have a high intensity and are present in over 90% of experiments, to the fourth set of peaks which have lower intensities and are present in less than 60% of experiments.

If a significant score had not been achieved the first set of contaminant peaks were taken out and the search was submitted again. If a significant protein still did not show then the second set of contaminant peaks were taken out, and so on. After the fourth set of contaminant peaks were taken out and there was still no protein of interest then that protein spot was listed as unidentified. A protein can be unidentified for many reasons, such as:

- Protein not listed in database
- Too many keratin contaminants masking small peptides peaks
- Not enough protein present in gel spot (spot too faint/small)
- Contaminated with neighbouring spot on gel during excision
- Small number of peptides present in digest, which requires all peptide peaks to be seen in spectra
- Large number of peptides present in digest, which results in a large number of protein matches

4.12 The Sigma-Aldrich Panorama® Antibody Microarray – XPRESS Profiler725 Kit

The Panorama® Antibody Microarray – XPRESS Profiler725 (#XP725; Sigma-Aldrich) contains 725 different antibodies each spotted in duplicate on a nitrocellulose-coated glass slide. By labelling the protein extracts with fluorescent dyes, the expression of a protein can be detected when it binds to its corresponding antibody spotted on the slide. The fluorescent signal intensity for each sample is recorded individually at the wavelength corresponding to the dye label of the sample and control. The protein expression for the two samples can then be compared.

4.12.1 Protein Extraction for Antibody Microarray

Approximately $1.2x10^8$ cells from clinical sample 003 were stimulated (see section 4.4) for 5.5hrs, and approximately $1.2x10^8$ cells were exposed to an isotype control for 5.5hrs (see section 4.4). Cells were washed twice in cold PBS and 20% were lysed in

Laemmli buffer (see section 4.5) for Western blot confirmations, and 80% were lysed in 1ml of Buffer A from the XPRESS Profiler725 kit. The antibody microarray samples were vortexed for 30secs and incubated on ice for 5mins. Samples were centrifuged for 10secs at 10,000xg, and the supernatant was transferred to a new 1.5ml microfuge tube.

4.12.2 Protein Quantification for Antibody Microarray

The Bradford Assay was used to determine the protein quantification as recommended by Sigma-Aldrich. At least 1ml of protein at 1mg/ml was needed for the experiment. The Bradford assay is a colorimetric assay based on the formation of a complex between the dye (Coomassie Brilliant Blue G) and the proteins in solution. When the dye (originally coomassie red) binds to the protein, it stabilises to coomassie blue. The assay uses the shift in the absorbance from 465nm (red) to 595nm (blue) to measure protein. The amount of absorption is proportional to the protein present.

Three technical replicates of differing dilutions were used for the stimulated extract and isotype control extract in the assay. An undiluted sample and dilutions of 1:2.5 and 1:5 were used.

One hundred µl of samples were place into a 96 well plate along with 100µl of four BSA standards of 0.1, 0.5, 1.0 and 1.4mg/ml. The linear range of the assay is 0.1-1.4mg/ml.

Three ml of Bradford Reagent (#B6916; Sigma-Aldrich) was added to each of the wells and the plate was vortexed gently. Samples were incubated at room temperature for 15mins and the absorbance was read at 595nm on a Multiscan MS plate reader

(Thermoelectron, UK). A plot of absorbance vs. standard concentrations was used to determine the concentration of the samples.

4.12.3 Protein Labelling

The following procedures were carried out in the dark.

Extracts were diluted to 1mg/ml with Buffer A from the XPRESS Profiler725 kit. One ml of protein extracted from stimulated cells was added to the Cy5 dye (#PA25001; GE Healthcare) and 1ml of protein extracted from unstimulated cells was added to the Cy3 dye (#PA23001; GE Healthcare). The dyes were supplied in a lyophilised form. The Cy5 dye was a blue powder and the Cy3 dye was a pink powder.

The dye vials were mixed thoroughly by briefly vortexing. The samples were incubated at room temperature for 30mins with vortexing briefly every 10mins. The unlabeled dyes were removed by using a Sigma-AldrichSpin column (provided with the XPRESS Profiler725 kit). The Sigma-AldrichSpin column was first placed in a collection tube and centrifuged at 750xg for 2mins to remove any excess buffer. The eluate was discarded and the column was placed in a new collection tube. One hundred and fifty µl of dye labelled protein was then carefully pipetted directly onto the centre of the Sigma-AldrichSpin column. The remaining labelled protein was stored at -80°C. The Sigma-AldrichSpin column and collection tube were centrifuged for 4mins at 750xg. The Sigma-AldrichSpin column was discarded and the eluate was retained.

The protein quantification was determined using the Bradford assay (see section 4.12.2) but this time samples did not need diluting. This was to ensure there was still 1mg/ml protein. The labelled protein could then be stored short term at 2-8°C.

4.12.4 Determination of Dye to Protein Molar Ratio

The following procedures were carried out in the dark.

In accordance with the XPRESS Profiler725 kit the dye to protein ratio (D/P) should be above 2. A lower ratio may affect the array and produce a higher background. The D/P was calculated by the following equation:

$$\text{D/P ratio} = \frac{\text{Dye Concentration (µM)}}{\text{Protein Concentration (µM)}}$$

The protein concentration (µM) is calculated by the following equation:

$$\text{Protein Concentration (µM)} = (\text{Protein Concentration (mg/ml)}/60{,}000)\ X10^{6}$$

The dye concentration is calculated by the following equations:

$$\text{Cy3 concentration (µM)} = A_{552}/0.15$$

$$(CY3: \varepsilon^{\mu M}_{(552nm)} = 0.15 \mu M^{-1} cm^{-1})$$

$$\text{Cy5 Concentration (µM)} = A_{650}/0.25$$

$$(CY5: \varepsilon^{\mu M}_{(552nm)} = 0.25 \mu M^{-1} cm^{-1})$$

Therefore the absorbance of the samples was read at 552nm for Cy3 and 650nm for Cy5. A Libra S11 Spectrophotometer (Biochrom) was used to measure absorbance. A 1:10 dilution of each labelled sample was used and 100µl of sample was added to the cuvette. The cuvette was thoroughly washed with DH_2O in between each reading and the spectrophotometer was blanked with Buffer A from the XPRESS Profiler725 kit.

4.12.5 Sample Incubation on the Array

The following procedures were carried out in the dark.

The slide was washed by dipping briefly in PBS in a 50ml tube. Using the protein quantification results, 50ug of each of Cy3 and Cy5 labelled samples (at equal concentrations) were mixed with 5ml of Array Incubation Buffer from the kit. The samples were mixed well by inverting the tubes several times. The mixture was added to well 1 of the Incubation Tray supplied with the kit. The slide was immersed into well 1 and was incubated at room temperature for 30mins with gentle agitation on an orbital rocker. Five ml of Wash Buffer was added to wells 2, 3, and 4 in the Incubation Tray. The slide was carefully taken out of well 1 and placed into well 2 and incubated at room temperature for 5mins with gentle agitation on an orbital rocker. This step was repeated by transferring into wells 3 and 4. The liquid was decanted from well 4 and 5ml of DH_2O was added. The slide was incubated for 2mins on the bench and then allowed to air dry for 40mins on an incline to allow excess liquid to drain. The slide was not covered over so it was essential to let it dry in a dust and debris free environment. It was essential that the slide was absolutely dry before scanning to avoid water marks.

4.12.6 Scanning the Antibody Microarray

The slide was kept in the dark at all times.

The microarray slide was scanned using a GenePix Personal 4100A Microarray scanner (Axon Instruments) using a 532nm laser for the excitation of Cy3, and a 635nm laser for the excitation of Cy5. Analysis was performed using the GenePix Pro software package 4.1 (Axon Instruments).

The microarray was scanned at a resolution of 5µm/pixel. The laser photomultiplier tube (PMT) gains were manually set for each dye to ensure a normal count ratio of 1 (±0.1) across the whole array (Figure 4.19).

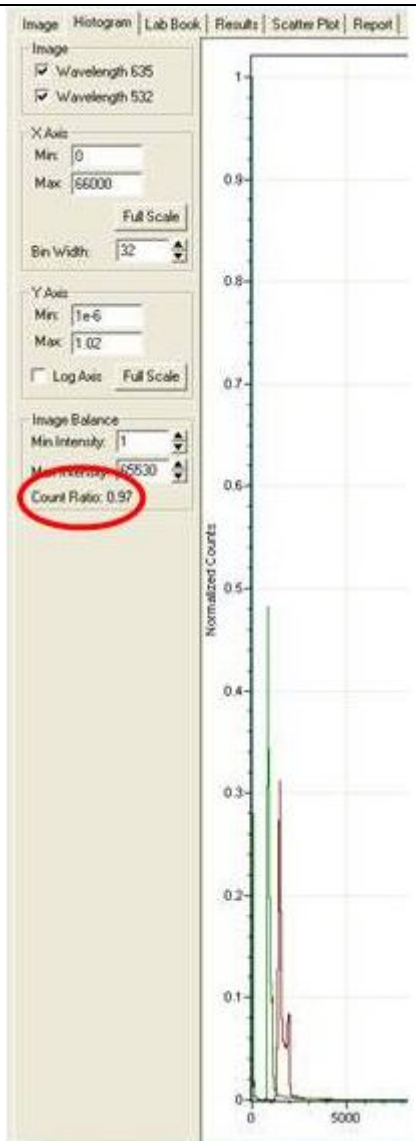


Figure 4.19: Histogram from an antibody microarray scan

The picture shows the count ratio of 0.97. The PMT gains should be adjusted until this value is as close to 1 as possible.

Scanned microarray images (in .tif format) were set in a grid (Figure 4.20) and linked to the protein print array list included in the kit so that each spot could be linked back to a specific protein. Any features which were not correctly aligned or sized were altered manually using the manipulation tool. The result files were imported into Acuity 4.0 (Axon Instruments) and differentially expressed proteins were highlighted. The antibody microarray was normalised using the Lowess method. Unreliable data was

removed from the analysis. Only spots with less than 3% of saturated pixels, spots that were not flagged bad or absent, spots with relatively uniform intensity and uniform background and spots that are detectable above background were included in the analysis. Proteins showing a ≥2 change in expression were classed as significant.

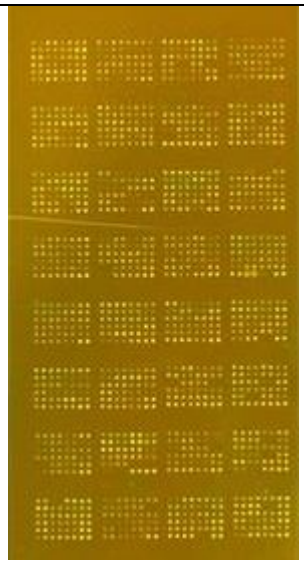


Figure 4.20: Scanned image of the Sigma-Aldrich Panorama® Antibody Microarray – XPRESS Profiler725 slide

The image shows the scanned slide containing 725 antibodies and bound, dye labelled protein samples. The mixture of the two labelled protein extracts (test and control) will appear orange if they are in the same concentration, but if a protein is over expressed in one of the samples, the spot will appear more green or red accordingly.

Chapter 5 - Establishment and Optimisation of the Western Blot Signaling Assay and 2D-PAGE/MS to Analyse BCR Signaling

Aims:

Establish and Optimise:

- A method to artificially stimulate the BCR in RAJI B-cells to induce kinase pathways
- A western blot based BCR signaling assay
- A 2D-PAGE/MS method for the analysis of a protein extract

Establishment and Optimisation of the Western Blot Signaling Assay and 2D-PAGE

5.1 Optimisation of the BCR Stimulation with a Surrogate Antigen

B-cell lines and clinical samples were artificially stimulated through the BCR as described in section 4.4. The number of cells, the concentration of antibody fragment and the incubation time needed to be optimised, so initial experiments were set up on RAJI, DAUDI and RAMOS-EHRB B-cell lines to find the optimum stimulation conditions.

5.1.1 Optimisation of Stimulation Incubation Time

After cell counting (section 4.3) RAJI B-cells were suspended in a concentration of $5x10^5$cells/ml (Allsup *et al.* 2005). Cells were incubated for 5, 25, 45 and 60mins with 10μg/ml of AffiniPure $F(ab')_2$ fragment goat anti-human IgM, $Fc_{5\mu}$ fragment specific (see section 4.4) (Allsup *et al.* 2005). Cells incubated with 100nM PMA for 5mins were included as a positive control and cells incubated with 10μg/ml of ChromPure Goat IgG $F(ab')_2$ fragment for 60mins were included as an isotype control (see section 4.4) (Allsup *et al.* 2005). Incubations were performed at 37°C with 5% CO_2.

Protein was extracted as described in section 4.5.1 and western blots were performed as described section 4.5. P-ERK was used as a primary antibody and GAPDH was used as a loading control (see Table 4.1 and Table 4.2).

The western blot showed that 5mins incubation with 10μg/ml of the anti-human IgM antibody was sufficient to activate the BCR and induce the MAPK pathway (Figure 5.1).

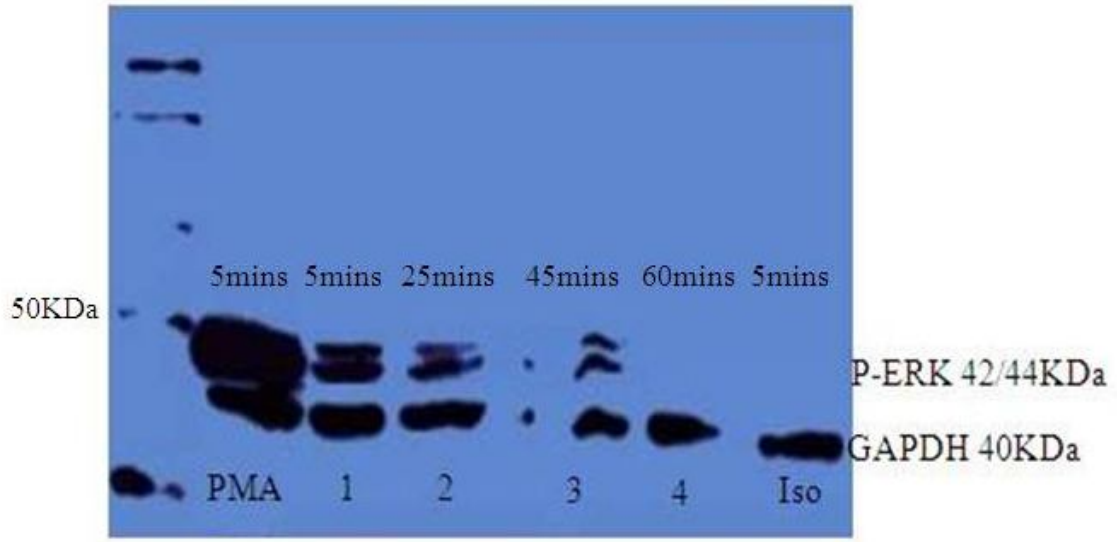


Figure 5.1: Western blot showing ERK phosphorylation over different B-cell stimulation times

The western blot showed that a 5mins incubation with 10μg/ml of AffiniPure F(ab')$_2$ fragment goat anti-human IgM, Fc$_{5\mu}$ fragment specific antibody is sufficient to activate the BCR and induce the MAPK pathway. The isotype control shows that after 5mins the cells were negative for P-ERK, providing evidence that P-ERK levels were purely induced by the anti-human IgM antibody. The PMA positive control shows that after 5mins the cells are viable and have reacted to stimulation.

5.1.2 Optimisation of Stimulant Concentration

After cell counting (see section 4.3) RAJI B-cells were suspended in a concentration of 5x10^{5}cells/ml (Allsup *et al.* 2005). Cells were incubated with 1.0, 10, 50 and 100 μg/ml of AffiniPure F(ab')$_2$ fragment goat anti-human IgM, Fc$_{5\mu}$ fragment specific for 5mins

(see section 4.4). Cells incubated with 100nM PMA for 5mins were included as a positive control and cells incubated with 100µg/ml of ChromPure Goat IgG $F(ab')_2$ fragment for 5mins were included as an isotype control (see section 4.4). Incubations were performed at 37°C with 5% CO_2.

Protein was extracted as described in section 4.5.1 and Western blots were performed as described section 4.5. P-ERK was used as a primary antibody and GAPDH was used as a loading control (see Table 4.1 and Table 4.2).

The concentration to be used throughout the project was chosen as 10µg/ml as this concentration corresponded with the literature (Allsup *et al.* 2005; Petlickovski *et al.* 2005) and has been shown in western blots to be effective (Figure 5.2).

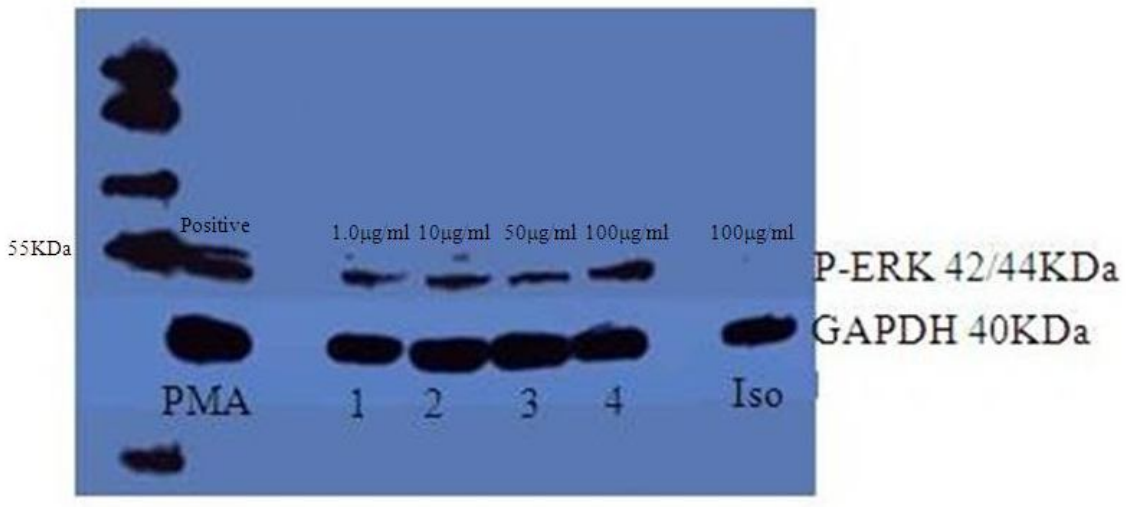


Figure 5.2: Western blot showing ERK phosphorylation over different concentrations of the B-cell stimulant

The western blot shows that incubation with all of the chosen concentrations of AffiniPure F(ab')$_2$ fragment goat anti-human IgM, Fc$_{5\mu}$ fragment specific antibody activated the BCR and induced the MAPK pathway as indicted by phosphorylation of the ERK protein. The isotype control shows that at concentration of 100µg/ml cells were negative for P-ERK, providing evidence ERK phosphorylation was induced by the anti-human IgM antibody. The PMA positive control shows that after 5mins the cells are viable and have reacted to stimulation.

5.1.3 Stimulant Concentration and Incubation Time Optimisation Summary

For the western blot signaling assay, B-cell lines were incubated in 10µg/ml F(ab')$_2$ fragment goat anti-human IgM, Fc$_{5\mu}$ fragment specific antibody for 5mins to induce BCR signaling as shown in Figure 5.1 and Figure 5.2.

5.2 Western Blot Gel Optimisation

There are many pre-cast 1D gels on the market. To decide which gels to use for the assay gels from the two market leaders, Bio-Rad and Invitrogen were compared.

12% Tris-HCl Ready Gels (#161-1120; Bio-Rad) were compared to homogenous Novex® 12% Tris-Glycine gel (1.0mm, 8x8cm, 10 well) (#EC6005box; Invitrogen). Samples were randomly selected from stimulated and unstimulated CLL clinical samples. Twenty micrograms of protein extract were run on the two gels simultaneously. Gels were probed with a P-ERK antibody (see Table 4.1 and Table 4.2). Samples should show varying levels of P-ERK depending on their response to stimulation. The experiment was performed in duplicate on separate days with different samples.

The Invitrogen gels were a much better quality then the Bio-Rad Gels and were consistently reliable producing good quality data, whereas the Bio-Rad gels had batch variability and produced unreliable results. Therefore the Homogenous 12% NuPAGE Tris-Glycine gels (Invitrogen) were used for western blots and 1D gels throughout this project.

5.3 Optimisation of Stripping Buffers for Western Blot on a Nitrocellulose Membrane

Western blots were used to determine if a CLL clinical sample was a responder to BCR stimulation. PBMCs were extracted from blood as described in section 4.2.2. Protein was extracted from stimulated PBMCs as described in sections 4.4 and 4.5.1 Protein Extraction and quantified as described in section 4.5.2. Western blots were performed as described in section 4.5.

Expression of P-ERK was used to assess BCR activation. If a BCR is activated successfully then the MAPK pathway will be triggered, causing the ERK protein to become phosphorylated (see section 2.4). The increase in phosphorylation of ERK can be used to determine the BCR responsiveness. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. GAPDH is an enzyme used to break down glucose. It is stable, and constitutively expressed at high levels in most cells therefore is considered a housekeeping gene, making it an ideal loading control for western blots. Dilutions of the P-ERK and GAPDH antibodies were optimised prior to assay (see Table 4.1 and Table 4.2).

It is sometimes necessary to strip a primary antibody off a membrane in order to re-probe with another primary antibody of a similar molecular weight. Western blots of randomly selected clinical samples were used for the stripping optimisation. The blots were probed for P-ERK raised in a mouse (see Table 4.1 and Table 4.2). Membranes were stripped using two different types of stripping buffer, a homemade stripping buffer and a commercially available stripping buffer (Restore PLUS Western Blot Stripping Buffer #46430; Pierce). The two buffers were compared to see which would give the best results. The membranes were stripped as described in section 4.5.8.

Homemade Western Blot Stripping Buffer

The homemade stripping buffer comprised of 2% SDS, 100mM β-mercaptoethanol and 50nM Tris pH 6.8. A western blot of P-ERK (see 4.5) on stimulated RAJI B-cell lysates (see section 4.4) was run and developed (see Figure 5.3 (A)). The nitrocellulose membrane was then incubated in 15ml of buffer with gentle agitation for 5mins,

15mins, 30mins, 45mins and 60mins, at room temperature, 50°C and 70°C. The membrane was then incubated for 1hr with a secondary mouse HRP conjugated antibody and the blot was developed to show any unstripped primary antibody (see Figure 5.3 (B)). The primary antibody was only successfully removed by incubating at 70°C for 60mins. The membrane was probed again for P-ERK to compare the amount of protein with the original blot. There was a significant loss in signal intensity (see Figure 5.3 (C)). The stripping buffer had successfully removed the primary antibody, but had also removed protein sample. Therefore, although this buffer was efficient at removing the antibody, the method was too harsh to be used in this project.

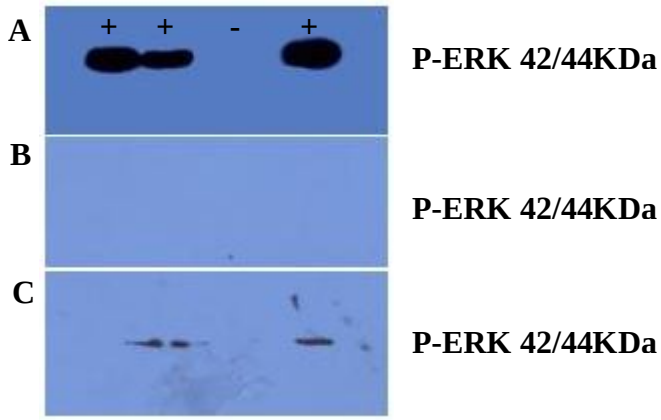


Figure 5.3: Western blot which has been incubated with a homemade stripping buffer for 60mins at 70°C

The Western blot was probed for P-ERK with a mouse antibody (A). After stripping the membrane in 15ml of homemade stripping buffer for 60mins at 70C it was incubated with a secondary mouse HRP conjugated antibody and the blot was developed to show any unstripped primary antibody (B). The membrane was re-probed for P-ERK as before and developed to compare the signal intensity with the original blot (C). The blots show that the stripping buffer had successfully removed the primary antibody (B) but had also removed some protein sample (C).

Pierce Restore Western Blot Stripping Buffer

The Restore PLUS Western Blot Stripping Buffer (#46430; Pierce) is advertised as an effective buffer that is not as harsh as a homemade stripping buffer and so less protein is removed.

Two western blots of P-ERK (see 4.5) on stimulated RAJI B-cell lysates (see section 4.4) were run and developed (Figure 5.4 (A)). The nitrocellulose membranes were incubated in 15ml of Restore PLUS Stripping Buffer. One membrane was incubated for 5mins and the other for 10mins at room temperature with gentle agitation (The protocol given by the manufacturer suggests an incubation time of 5-15mins). The membranes were incubated in a secondary mouse HRP conjugated antibody for 1hr and developed.

The results show that an incubation time of 5mins was not sufficient, and an incubation time of 10mins was necessary to completely remove the primary antibody (Figure 5.4 (B)). The membrane which had been incubated in Restore PLUS Stripping Buffer for 10mins was then re-probed for P-ERK to determine if protein sample had also been stripped off the membrane. The western blot shows that little protein sample had been lost (Figure 5.4 (C)).

To see if this membrane could be stripped repeatedly, it was incubated again for a further 10mins in the Restore PLUS Stripping Buffer, and as before all the primary antibody was removed (Figure 5.4 (D)). The membrane was re-probed for P-ERK as before, but the signal intensity showed that protein had been lost on the second stripping (Figure 5.4 (E)). The loss of protein appeared to be less of a problem when compared

with the amount lost when using the homemade stripping buffer only once (Figure 5.3 (C)).

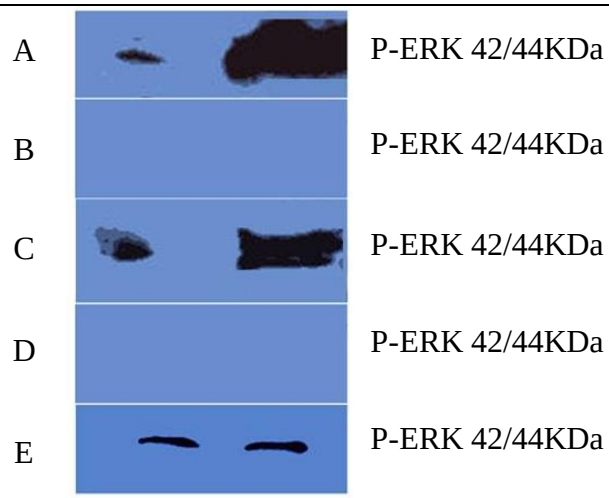


Figure 5.4: Western blot which has been repeatedly incubated with Restore PLUS Stripping Buffer (#46430 Pierce) for 10mins at room temperature

The Western blot was probed for P-ERK with a mouse antibody (A). After stripping the membrane in 15ml of Pierce Restore PLUS Stripping Buffer for 10mins at room temperature it was incubated with a secondary mouse HRP conjugated antibody and the blot was developed to show any unstripped primary antibody (B). The membrane was re-probed for P-ERK as before and developed to compare the signal intensity with the original blot (C). The membrane was stripped in 15ml of Pierce Restore PLUS Stripping Buffer for 10mins at room temperature it was incubated with a secondary mouse HRP conjugated antibody and the blot was developed to show any unstripped primary antibody (D). The membrane was re-probed for P-ERK as before and developed to compare the signal intensity with the original blot (E). The blots show that a 10mins incubation period with Restore Plus stripping buffer is sufficient to remove the primary antibody (B and D). Little protein is lost upon the first stripping (C) but repeated stripping results in a loss (E).

Because of its gentle stripping properties and ease of use, the Pierce Restore Stripping Buffer was used to strip all membranes in the project. Anti-P-ERK is a mouse antibody and was successfully removed under the condition described. A membrane was only stripped once to avoid loss of which may lead to inaccurate GAPDH protein loading readings.

Antibodies raised in rabbits such as Anti-GAPDH and Anti-ERK proved to be more difficult to remove and required heating to 37°C and incubating for 15-30mins, which resulted in significant loss of protein sample. Therefore a strategy was adopted whereby antibodies raised in mouse were applied first and then stripped before the application of a rabbit antibody. This meant that the stripping of antibodies raised in rabbits was not necessary.

5.4 Optimisation of Protein Stains for 2D-PAGE

Coomassie blue visible stain and three fluorescent stains were compared for ease of use and sensitivity. The stains that were used and compared for this project were Bio-Safe™ Coomassie total protein stain (#161-0787; Bio-Rad), SYPRO® Ruby total protein gel stain (#s-12000; Invitrogen), Krypton total protein stain (#46629; Pierce), Flamingo™ fluorescent total protein gel stain (#161-0492; Bio-Rad) and Pro-Q® Diamond phosphoprotein gel stain (#p-33300; Invitrogen).

The total protein stains were investigated first. A 1D gel of different cytochrome C concentrations was run in quadruplicate according to the protocol described in section 4.7. Eight concentrations of cytochrome C were used at 10µg, 2µg, 400ng, 80ng, 16ng, 3.2ng, 0.64ng and 0.25ng. Each gel was stained with a different total protein stain (Figure 5.5) and scanned as described in section 4.8.

Krypton protein stain was the most sensitive fluorescent stain and gave the cleanest gel. Coomassie is not as sensitive as Krypton stain however the advantage of coomassie is

that it is visible without the use of a fluorescent scanner. Krypton fluorescent protein stain and Coomassie visible stain were chosen to be used in the project.

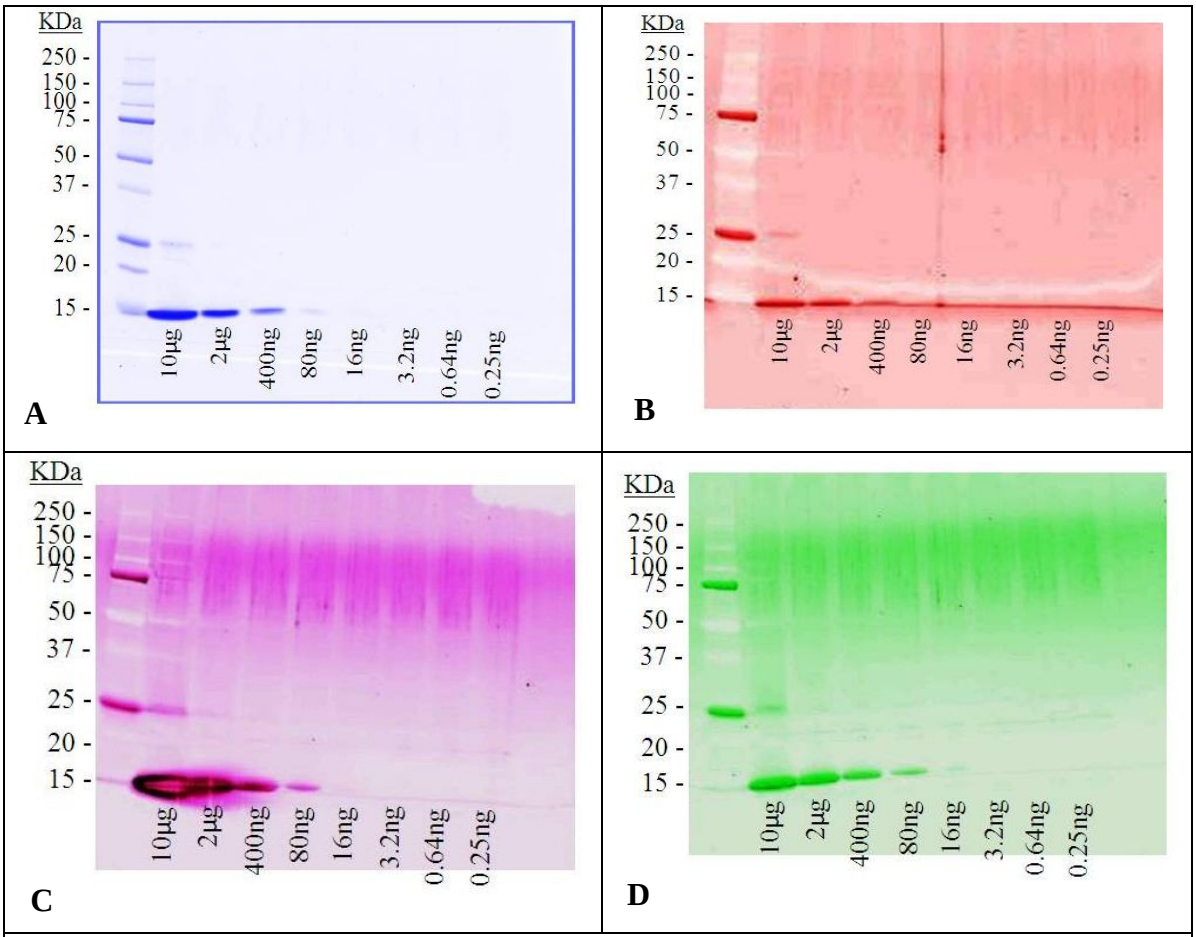


Figure 5.5: 1D gels of varying concentration of cytochrome C stained with Bio-Safe Coomassie stain, SYPRO Ruby protein gel stain, Flamingo fluorescent gel stain and Krypton protein stain

Gel stained with Coomassie (A). Gel stained with Sypro (B). Gel stained with Flamingo (C). Gel stained with Krypton (D). The gels show that Krypton protein stain is the most sensitive and gave the cleanest gel, the stain detected 16ng of protein. SYPRO Ruby stain streaked across the dye front making it difficult to distinguish individual bands. Flamingo protein stain saturated bands at higher concentrations. Coomassie blue stain was the only visible stain compared and detected 80ng of protein.

Krypton is a fluorescent total protein stain so it needed to be compatible with Pro-Q Diamond phosphoprotein fluorescent gel stain if phosphoproteins were to be visualised. The literature on Pro-Q Diamond stain specified that it was only compatible with

SYPRO ruby stain. A 1D gel was run to determine if Krypton stain was compatible with Pro-Q Diamond stain.

PeppermintStick phosphoprotein molecular weight standard (#P33350; Invitrogen) is a mixture of phosphorylated and non-phosphorylated proteins ranging from 14.4 – 116.25KDa. A 1D gel of varying PeppermintStick concentrations was run according to the protocol described in section 4.7. Separation by polyacrylamide gel electrophoresis resolves the standard mixture into two phosphorylated protein bands and four non-phosphorylated protein bands (Figure 5.6).

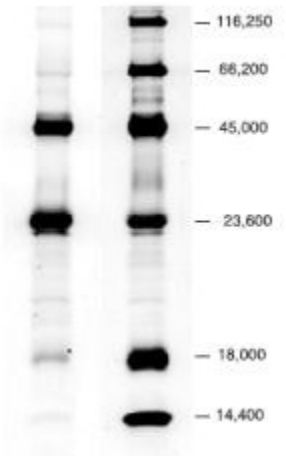


Figure 5.6 Peppermint Stick Molecular Weight Marker from Invitrogen

The picture shows a scanned image of the PeppermintStick molecular weight marker. The bands on the left show phosphorylated proteins. The bands on the right show unphosphorylated proteins. Proteins are as follows: β-galactosidase 116.3KDa, BSA 66.2KDa, ovalbumin (phosphorylated) 45KDa, β-casein (phosphorylated) 23.6KDa, avidin 18KDa, and lysozyme 14.4KDa.

Nine different concentration of PeppermintStick were loaded onto a gel, they were 500ng, 250ng, 125ng, 62.5ng, 31.3ng, 15.6ng, 7.8ng, 3.9ng and 1.95ng. The gel was then stained with Pro-Q Diamond and scanned as described in section 4.8.5. The

Phosphoprotein stain was then washed off and the gel was stained with Krypton protein stain and scanned as described in section 4.8.3. Results show that Krypton total protein stain is compatible with Pro-Q Diamond phosphoprotein stain which can detect protein at 15.6ng (Figure 5.7).

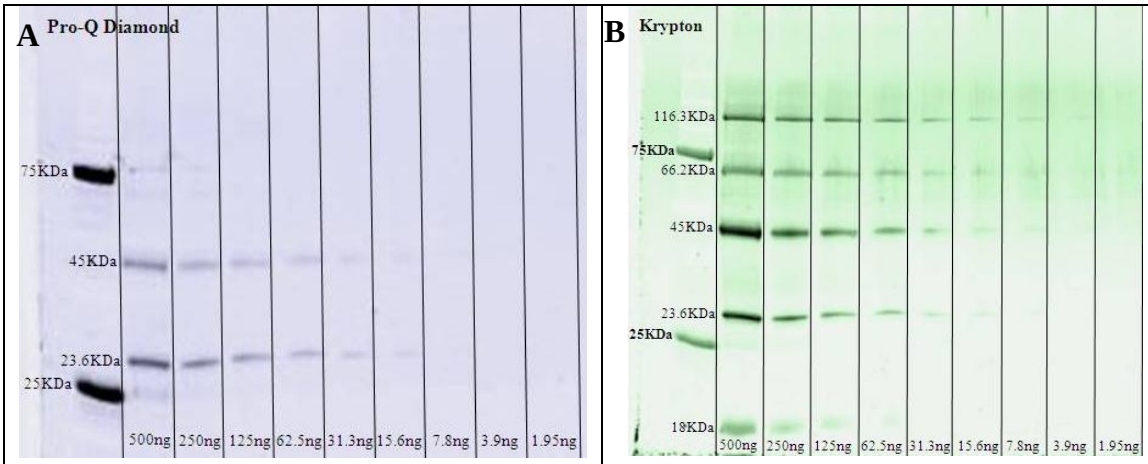


Figure 5.7: 1D gel stained with Pro-Q® Diamond Phosphoprotein Gel and then stained with Krypton Total Protein Stain

The picture shows a 1D gel of nine concentrations of PeppermintStick Phosphoprotein Molecular Weight standard (#P33350; Invitrogen). Ten µl of Precision Plus Dual Colour Marker (#161-0374; Bio-Rad) was placed in lane 1 as a visible marker. The gel was stained first with Pro-Q Diamond Phosphoprotein Stain (A), and then stained with Krypton Total Protein Stain (B). The gels show that the two stains are is compatible. The Pro-Q Diamond stained image (A) shows the two phosphoprotein bands in the PeppermintStick Standard. The Krypton stained image (B) shows all protein bands in the PeppermintStick Standard with the exception of the 14.4KDa band which has run off the gel during electrophoresis. Proteins can be visualised by Pro-Q diamond at 15.6ng, Krypton total protein fluorescent stain can detect high molecular weight proteins down to 7.8ng stain.

5.5 Optimisation of 2D-PAGE

5.5.1 2D-PAGE of *E.coli* Protein Standard

To gain experience in running a 2D gel and to check the optimised running conditions the ReadyPrep 2-D starter kit (#163-2105; Bio-Rad) was used. The kit contained a prepared *E.coli* protein sample (#163-2110; Bio-Rad) and allowed the analyst to focus on the technique rather than the sample and reagent preparations. The kit included step by step instructions and tips for first time users.

The protein extract from *E. coli* from the kit was separated using the methods described in sections 4.6.4, 4.6.5 and 4.6.6. ReadyStrip IPG strips of 11cm, pH 3-10 (#163-2016; Bio-Rad) were used. The gel was comparable to that of example shown in the Bio-Rad starter kit. Spots would have been better defined if the protein extract had been separated across a smaller pH range such as pH4-7 and pH7-10 (Figure 5.8).

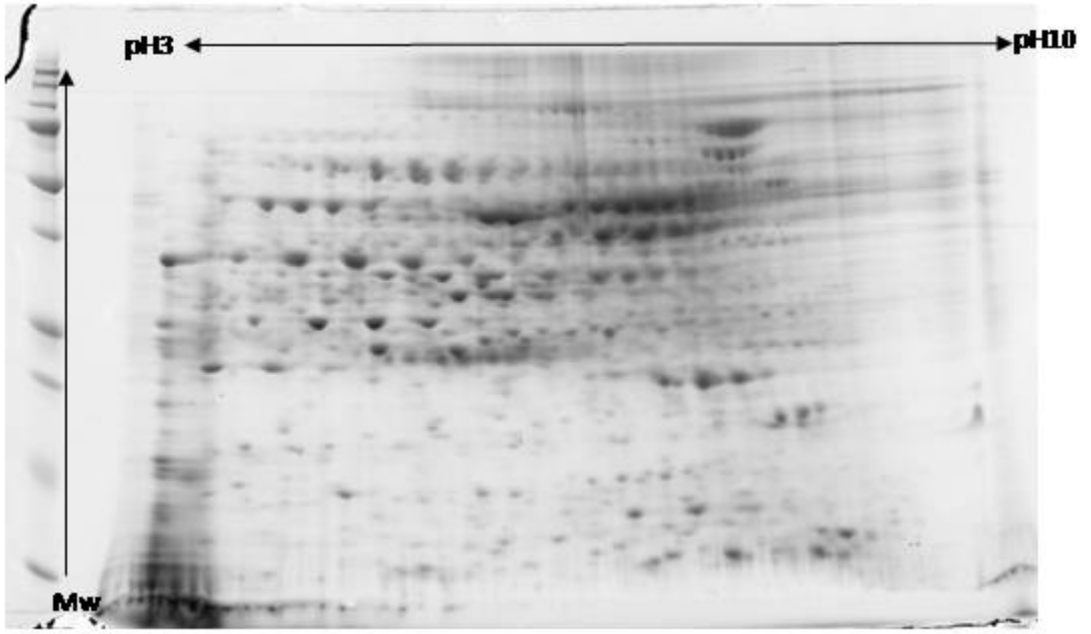


Figure 5.8: 2D gel of an *E.coli* protein extract from the ReadyPrep 2-D starter kit (Bio-Rad)

The gel was comparable to that of reference image in the Bio-Rad starter kit. The proteins were separated by pH on an 11cm, pH3-10 ReadyStrip IPG strip (Bio-Rad) and then separated by molecular weight on a Criterion 8-16% gel (Bio-Rad). The gel is cleaner at a lower pH because the disulphide groups on the protein at a higher pH are prone to unspecific oxidation causing extra spots and streaking. The separation of spots in the middle of the gel is quite poor; this is because the protein was separated across a large pH range.

5.6 2D Gel Analysis Software

Two software packages, which identify differentially expressed spots in 2D gels, were compared for use in the project. The software packages were PDQuest 2-D Analysis software V8.0 (Bio-Rad) and Progenesis SameSpots V1.01 (Nonlinear Dynamics). The two software packages were compared for;

- Reliability
- Ease of use
- Accuracy of results
- Time for analysis

PDQuest had been used for previous work at our laboratory but is time consuming (takes one week to analyse an experiment) and relies greatly on human intervention which may lead to biased results. Progenesis SameSpots is advertised as giving reliable data as human intervention is not required, and takes only one day to complete the analysis.

Protein extracts from stimulated and unstimulated RAJI B-cells (see sections 4, 4.4 and 4.5.1) were separated by 2D-PAGE in triplicate as described in section 4.6 and stained with Bio-Safe™ Coomassie stain (Bio-Rad). The gels were then analysed by PDQuest V8.0 and Progenesis SameSpots V1.01.

The first problem highlighted was that Progenesis SameSpots had no manual as it was comparatively new software so the manufacturer's technical support department guided us through the programme. PDQuest did have a manual and wizard guide to allow for ease of use. As with most new pieces of software, other problems followed with Progenesis SameSpots such as crashes and freezes in the software which were reported to technical support. But ultimately the decision for which analysis to use was based on reliability of results and speed.

PDQuest requires the analyst to intervene and manually match and edit spots (see section 4.9.1) which takes up to one week to complete, however it became apparent that Progenesis SameSpots also requires this intervention, but because the manufacturers say that no intervention is needed, the editing tools in the software are inadequate. Figure 5.9 shows the poor spot detection of Progenesis SameSpots and how manual intervention is needed to split the spots.

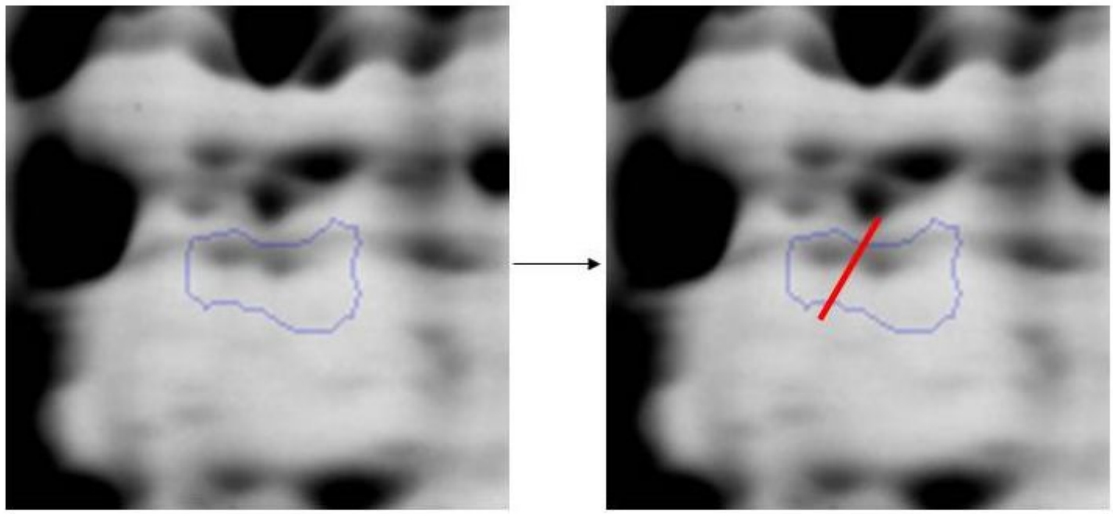
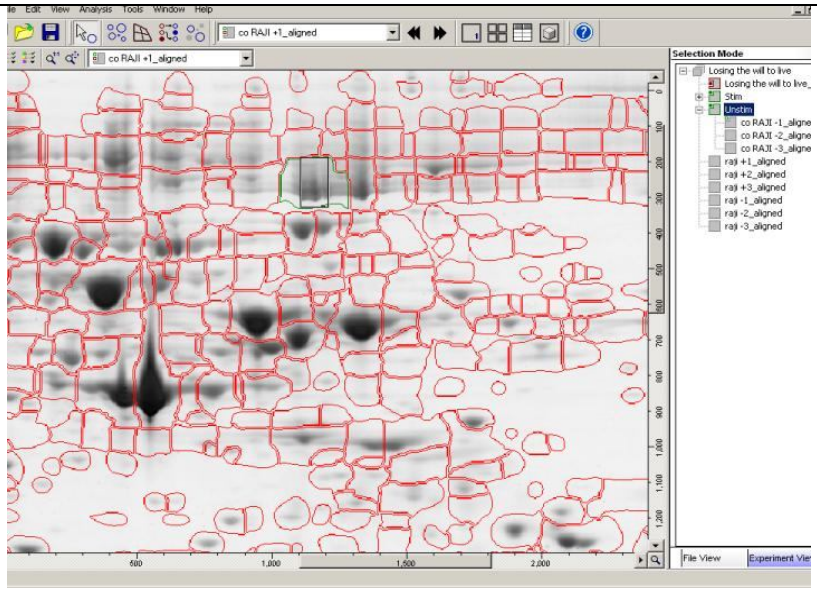


Figure 5.9: Picture to show poor spot splitting in Progenesis SameSpots

The picture shows an example of spot detection done automatically using Progenesis SameSpots V1.01 (Nonlinear Dynamics). The area highlighted in blue (left) is clearly two spots which require splitting (right).

The spot editing problem was seen throughout the gels (Figure 5.10). It took longer than one week to manually edit all the gels due to the inadequate editing tools and because not all the gel was accessible for editing. As all the 6 gels are overlaid in this software, only one gel needed editing, but accurate results relied greatly on the gels being correctly aligned. The manufacturers of the software do not recommend editing the gels as state that it will affect the reliability of the results.

A



B

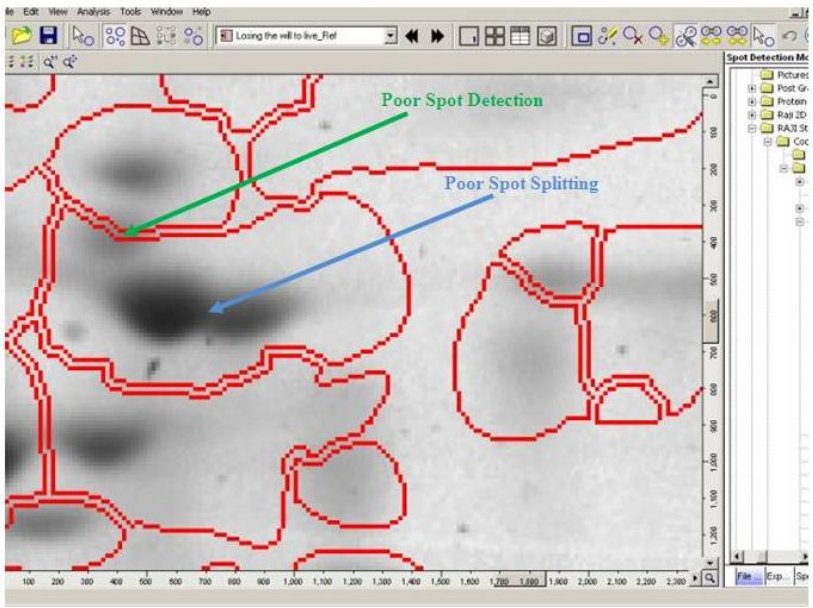


Figure 5.10 Pictures to show spot detection by Progenesis SameSpots

A: An example of spot detection on a gel done automatically by Progenesis SameSpots V1.01 (Nonlinear Dynamics).
B: An enlarged area from picture A. It clearly shows the poor spot splitting and also the poor spot detection.

PDQuest did require manual intervention which took one week (Figure 5.11), however, the editing tools were easy to use and the programme did not suffer from freezes and

crashes. PDQuest also uses crosshairs to mark the centre of the spot making editing easier than trying to select the area of the spot.

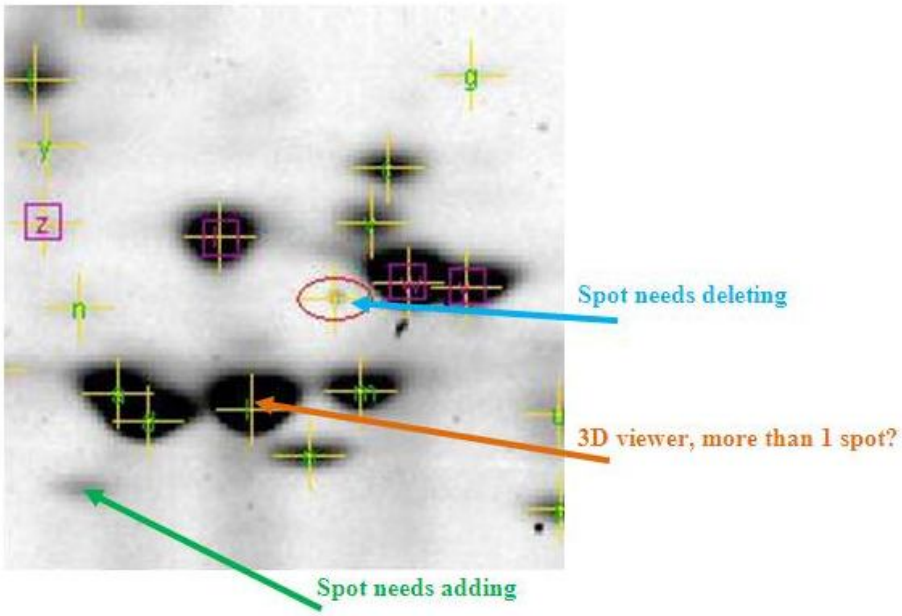


Figure 5.11: Picture to show spot editing using PDQuest

The picture shows in a zoomed in area of the gel where editing is needed. There is a spot which needs adding because the automatic detection has not picked it up, a spot that needs deleting because it is a speckle on the gel, and a large spot which need to be checked with the 3D viewer to see how many spots are overlapping.

Previous 2D-PAGE studies in the laboratory, which have used PDQuest, have confirmed the fold changes (by immunoblotting) of proteins highlighted by PDQuest (Smith *et al*. 2007); however, studies which have used Progenesis SameSpots have not confirmed the fold changes of the proteins (Little. 2006).

Because of its ease of use, accuracy and reliability of data, PDQuest V8.0 was chosen to analyse gels in this project, despite the lengthy time for analysis.

5.7 Optimisation of the Analysis of Protein Digests by MALDI-TOF MS

Commercially available purified proteins were digested to gain experience in the methods and to obtain a MALDI-TOF-MS contaminants list (see section 4.11).

5.7.1 Protein Digests

Four purified proteins comprising of two phosphorylated proteins and two unphosphorylated proteins (PeppermintStick phosphoprotein molecular weight standards (#P33350; Invitrogen)), were run on a 1D gel and stained with Bio-Safe™ Coomassie stain (Bio-Rad) (see section 4.8.1) (Figure 5.12).

Two hundred and fifty ng of PeppermintStick phosphoprotein molecular weight standards (#P33350; Invitrogen) (see section 5.4) was run on a 1D gel (see section 4.7). Previous experiments show that the MALDI-TOF-MS system available in the department can analyse down to the sensitivity of coomassie stain, which, on a 1D gel is a band of 80ng of protein (see section 5.4). A 1D gel separation of 250ng of PeppermintStick marker results in all of the six proteins being visible when stained with coomassie (see Figure 5.12). It was important not to mask the contaminants with large amounts of protein sample; therefore the minimum amount of sample necessary to obtain spectra was used. Four of the six proteins were excised, these were:

- Beta galactosidase from *E.coli* (116KDa)
- Bovine serum albumin (66KDa)
- Phosphorylated ovalbumin from chicken egg (45KDa)
- Phosphorylated beta casein from bovine milk (18KDa)

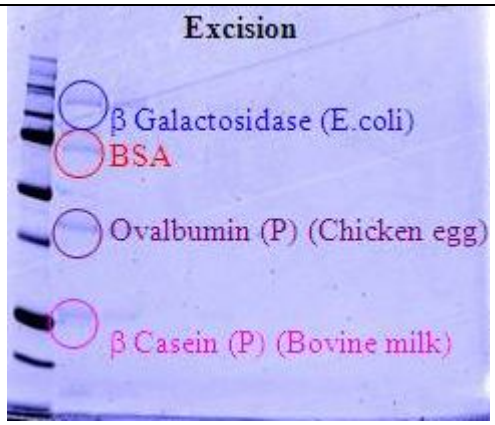


Figure 5.12: 1D gel of proteins standards showing bands excised for MALDI-TOF-MS optimisation

The picture shows a 1D gel of four protein bands excised for MALDTI-TOF-MS analysis. Bands are of two phosphorylated proteins (P) and two unphosphorylated proteins which have been stained with coomassie stain.

Proteins were excised and digested (see section 4.10) and peptides were analysed by MALDI-TOF-MS (see section 4.11). A blank piece of gel was also digested to obtain background contaminations. Because the proteins were derived from different species the MASCOT search was conducted under all taxonomy.

Spectra were obtained for all four proteins; however, significant identifications could only be obtained for the two unphosphorylated proteins. Thus proving that modified proteins are difficult to identify by MALDI-TOF-MS. Bovine serum albumin was identified with a Mowse score of 74 (where >70 was significant) and a 30% sequence

coverage. Beta galactosidase (Figure 5.13) was identified with a score of 115 (where >60 significant) and a 38% peptide sequence coverage (see Appendix H: Mascot Search Results – Beta Galactosidase (LacZ Protein)).

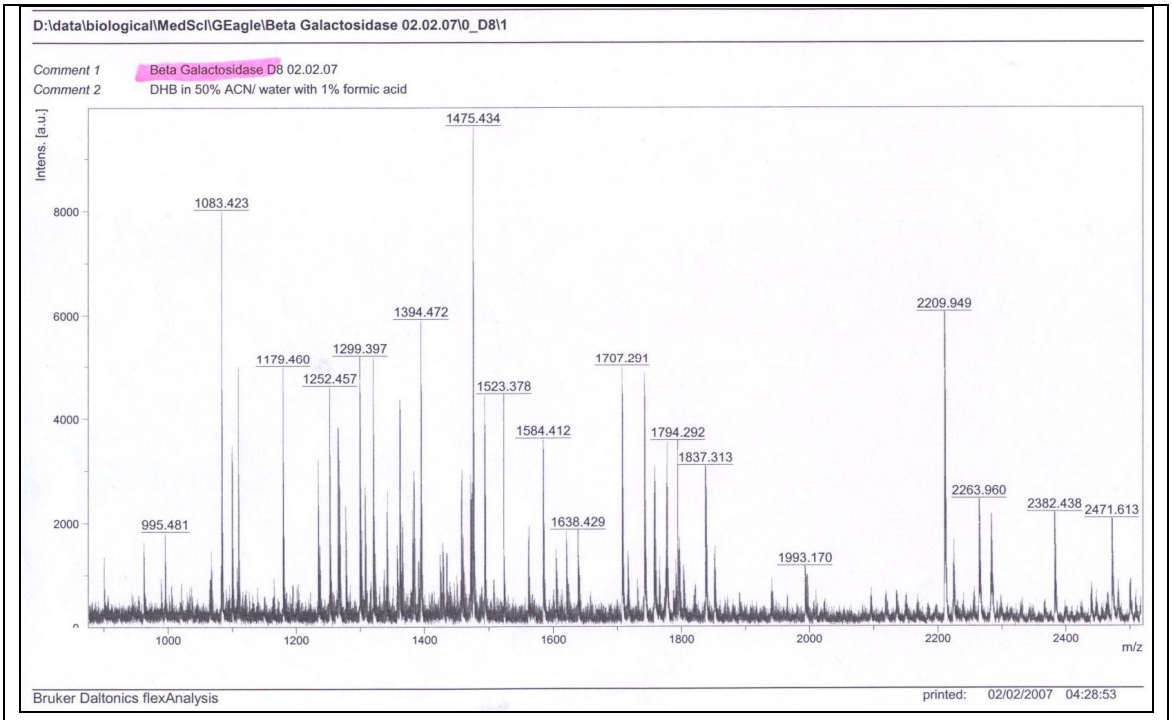


Figure 5.13: MALDI-TOF mass spectra of beta galactosidase

Peptide mass fingerprint of beta galactosidase from *E.coli*.

5.7.2 Keratin Contaminations

Spectra generated from previous proteomic projects were combined with spectra from this project to comprise a list of MALDI-TOF-MS protein contaminants, which could be later excluded from 2D-PAGE work. The same methods were applied to all experiments (see sections 4.7, 4.8.1, 4.10, 4.11).

Spectra acquired from previous proteomic projects included digests of myoglobin (7.3µg), cytochrome C (11.3µg), BSA (16.67µg) and transferrin (16µg). These proteins

were separated on a 1D gel (see section 4.7) (Figure 5.14). The bands were excised and digested (see section 4.10) and analysed by MALDI-TOF-MS (see section 4.11).

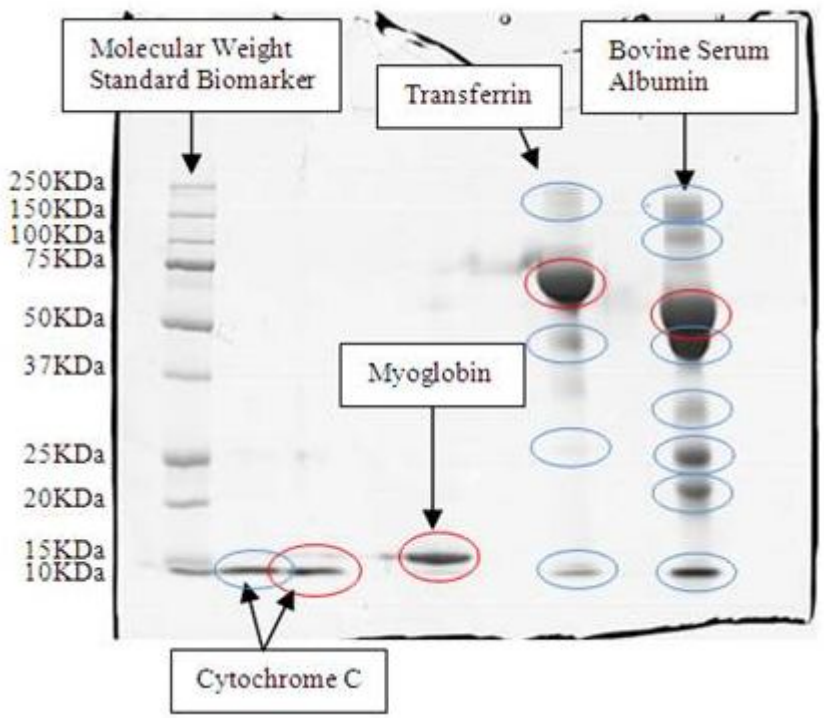


Figure 5.14: 1D gel of pure proteins standards

The 1D gel shows bands (red) excised for tryptic digestion from 11.3µg of cytochrome C (12KDa), 7.3µg of myoglobin (16.7KDa), 16µg of transferrin (77KDa) and 16.67µg of BSA (70KDa). The blue bands were also excised for tryptic digestion.

Initially 20 spectra from 8 standard proteins were compared (Figure 5.12 and Figure 5.14), but as more experiments were processed, more spectra were added. Peaks which appeared in more than one spectrum were cross checked with a contaminants list (Smith. 2005) and with the peaks from a blank piece of 2D gel (Figure 5.15). Contaminants were put into four groups:

1. Peaks present in over 90% of spectra (Table 5.1)
2. Peaks present in between 70-90% of spectra (Table 5.2)

3. Peaks present in over 60-70% of spectra (Table 5.3)
4. Peaks present in 10-60% of spectra (see Appendix H)

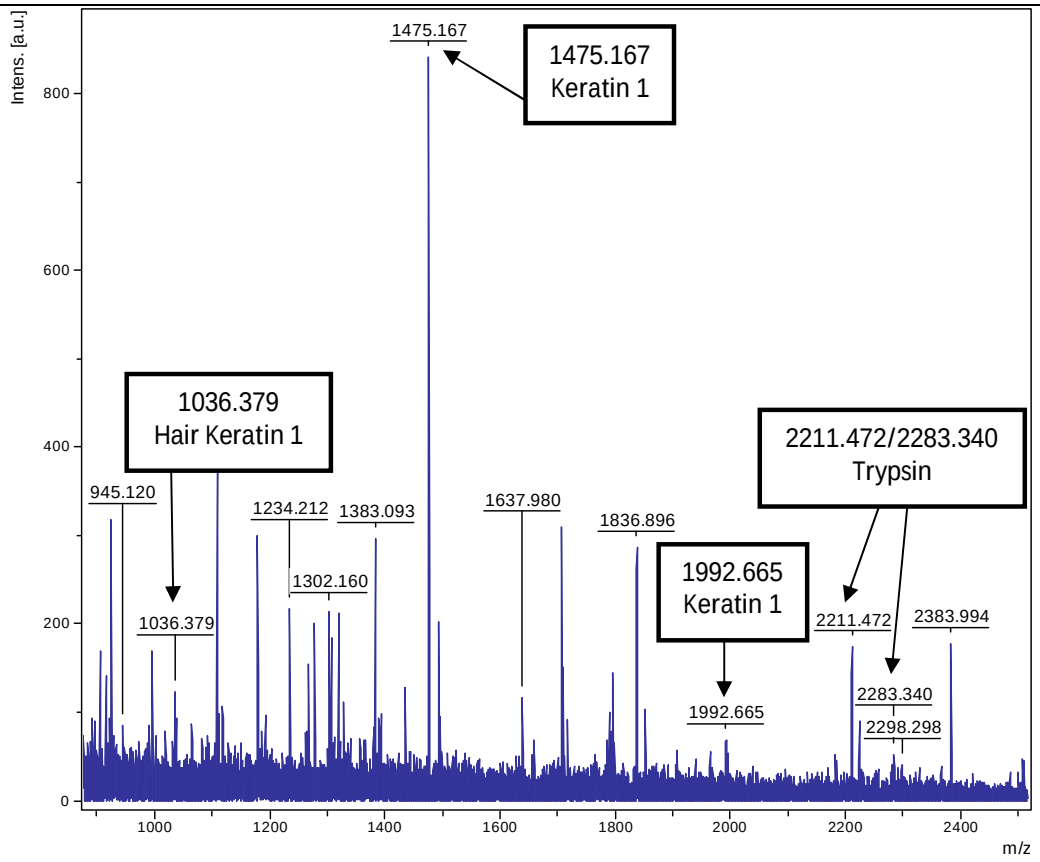


Figure 5.15: MALDI mass spectrum of a piece of 2D gel subjected to tryptic digestion

A blank piece of 2D gel was subjected to tryptic digestion to obtain a peak list of keratin and trypsin contaminants. The spectra shows common contaminants such as keratin 1 (m/z 1036, 1475, 1993) and trypsin autolysis peaks (m/z 2211, 2283).

In future 2D-PAGE experiments, if a significant score had not been achieved the first set of contaminant peaks (Keller *et al.* 2008) were taken out and the search was submitted again (see section 4.11). If a significant protein still did not show then the second set of contaminant peaks were take out, and so on. After the fourth set of contaminant peaks are taken out and there was still no protein of interest then that

protein spot was listed as unidentified. However, it is worth noting that slight variations can occur between each experiment, e.g. batch variations of consumables, environment variation etc, and so for every 2D-PAGE experiment, spectra were gathered from all the samples first, then interrogated in this way to account for new contaminant peaks.

Table 5.1: Table showing group 1 MALDI-TOF-MS peptide contaminants

M/Z	Contaminant	Modification	Unmodified Peak M/Z
993.5	Keratin 10	N/A	N/A
1036.5421	Type 1 Hair Keratin 1	N/A	N/A
1066.4986	Keratin 9	N/A	N/A
1179.6004	Keratin 1	N/A	N/A
1277.6332	Keratin 1	N/A	N/A
1475.7489	Keratin 1	N/A	N/A
1638.8598	Keratin 1	N/A	N/A
1837.9654	Keratin 9	N/A	N/A
1851.9269	Keratin 9	N/A	N/A
1993.9766	Keratin 1	N/A	N/A
2211.104	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
2225.3	Background Peak	N/A	N/A
2283.1801	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
2367.2626	Keratin 10	N/A	N/A
2383.9518	Keratin 1	N/A	N/A

Table 5.2: Table showing group 2 MALDI-TOF-MS peptide contaminants

M/Z	Contaminant	Modification	Unmodified Peak M/Z
1107.8	Background Peak	N/A	N/A
1109.49	Keratin 10	N/A	N/A
2055.962	Type 1 Hair Keratin 1	CAM	1998.941
2342.984	Keratin 10	CAM	2285.9628

Table 5.3: Table showing group 3 MALDI-TOF-MS peptide contaminants

M/Z	Contaminant	Modification	Unmodified Peak M/Z
1090.531	Keratin 10	N/A	N/A
1141.5194	Keratin 1	N/A	N/A
1165.5848	Keratin 10	N/A	N/A
1234.679	Keratin 10	N/A	N/A

	1262.5971	Keratin 10	N/A	N/A	
	1265.6372	Keratin 1	N/A	N/A	
	1307.6776	Keratin 9	N/A	N/A	
	1320.1	Background Peak	N/A	N/A	
	1357.7182	Keratin 10	N/A	N/A	
	1434.7699	Keratin 10	N/A	N/A	
	1707.7503	Keratin 9	CAM	1650.7289	
	1716.8511	Keratin 1	N/A	N/A	
	1794.9	Background Peak	N/A	N/A	
	1941.9876	Keratin 1	N/A	N/A	
	2021.9886	Keratin 1	N/A	N/A	
	2082	Background Peak	N/A	N/A	
	2095.0376	Keratin 9	CAM	2038.0161	
	2171.0246	Keratin 9	N/A	N/A	
	2292.2081	Keratin 1	N/A	N/A	

5.8 Summary

A method to artificially stimulate the BCR in RAJI B-cells was established and a western blot BCR signaling assay was used to validate stimulation by probing for P-ERK. A 2D-PAGE/MS method for the analysis of proteins was optimised. Krypton fluorescent total protein stain, coomassie visible total protein stain and Pro-Q Diamond fluorescent phosphoprotein stain were chosen to stain protein extracts separated in gels. PDQuest was chosen to identify differentially expressed proteins and peptide contaminants were identified by digesting pure protein samples and analysing with MALDI-TOF/MS.

Chapter 6 - The Preliminary Phosphoproteomic Analysis of RAJI B-cell Line

Aims:

- To develop a 2D-PAGE/MS experiment based on methods optimised in sections 5.4, 5.5, 5.6 and 5.7 to identify changes in phosphoprotein expression in artificially stimulated and unstimulated RAJI B-cells

Publications and Presentations:

- G. L. Eagle, D. Allsup, L. Cawkwell. Phosphoproteomic Analysis of B-Cell Receptor Signaling in Chronic Lymphocytic Leukaemia. The 4th Hull York Medical School Research Conference, York, UK *(March 2007)*. Poster Presentation.

The Preliminary Phosphoproteomic Analysis of RAJI B-Cell Line

Preliminary investigations were performed to try and identify differentially expressed phosphoproteins in stimulated and unstimulated RAJI B-cells, and thus pinpoint kinase cascade pathways activated by the BCR.

6.1 Stimulation of RAJI B-Cells

RAJI B-cells were cultured as described in 4. Cells were stimulated or exposed to an isotype control for 5mins (see section 4.4). Three biological replicates were produced by repeating the procedure on three consecutive days. Protein was extracted for western blot and for 2D-PAGE (see sections 4.4 and 4.5.1). A western blot of P-ERK was used to confirm stimulation; a PMA positive control was included to assess viability of cells (Figure 6.1).

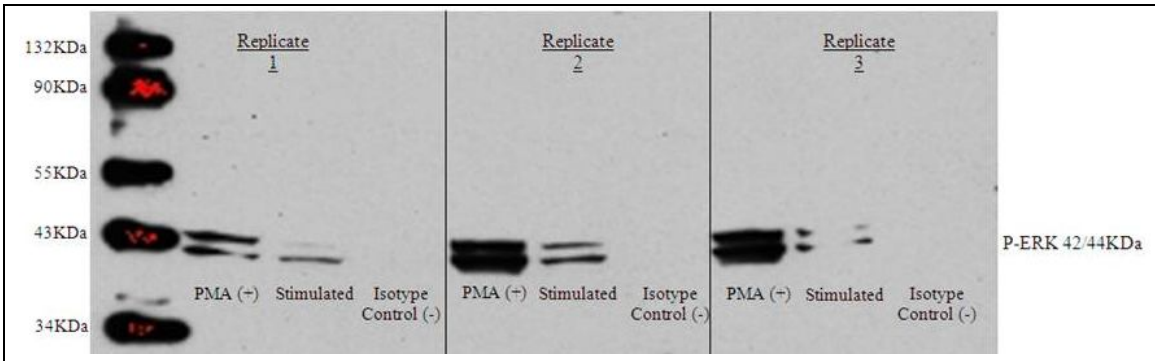


Figure 6.1: Western blot of P-ERK to confirm stimulation of the BCR in RAJI B-cells

The picture shows a western blot of P-ERK for three biological replicates of RAJI B-cells. Replicates were stimulated or exposed to an isotype control for 5mins on three consecutive days. A PMA positive control was included to assess viability of cells. The blots confirm that the stimulation has been successful in all replicates. The stimulated cells are expressing P-ERK and the isotype controls are negative.

6.2 2D-PAGE of RAJI B-Cells and Expression of Phosphoproteins

When the stimulation had been confirmed, 80µg (as recommended by stain manufacturer) of protein extract from stimulated and unstimulated cells was prepared in triplicate, and compared by 2D-PAGE as described in section 4.6. IPG strips of pH3-10 were used to obtain the widest range of proteins. Gels were then stained with Pro-Q Diamond fluorescent phosphoprotein stain and scanned (see section 4.8.5).

The gels showed that expression of phosphoproteins in the cells greatly increased upon BCR stimulation (Figure 6.2 and Figure 6.3). PDQuest counted 60% more phosphoprotein spots on the stimulated gels compared to the isotype control gels.

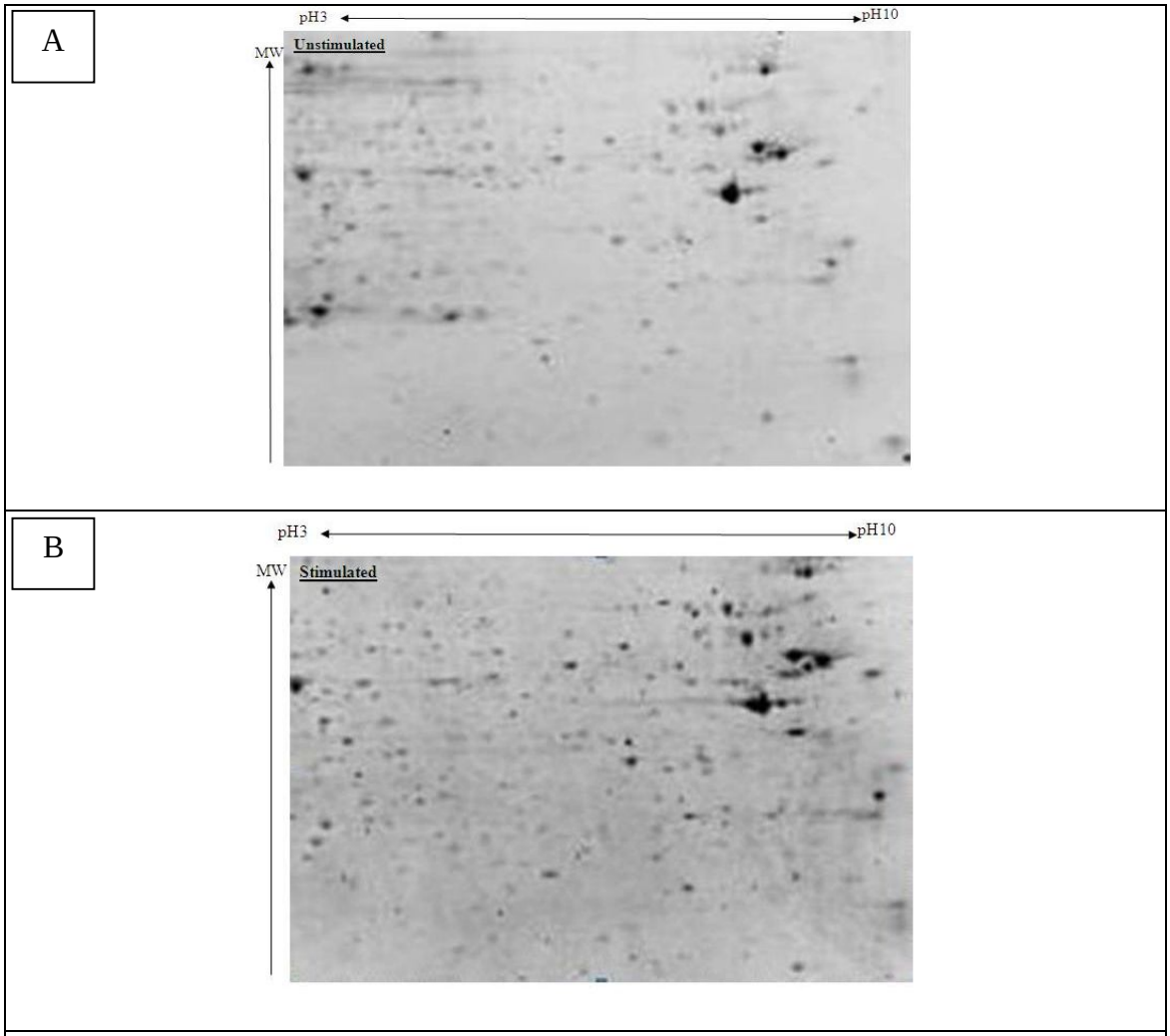


Figure 6.2: 2D gel images of stimulated and unstimulated RAJI B-cells showing expression of phosphoproteins

RAJI B-cells were stimulated for 5mins and protein was extracted from stimulated and isotype control cells. Eighty micrograms of protein was separated on non-linear pH3-10 IPG strips and then separated by molecular weight onto a 2D gel. Gels were stained Pro-Q Diamond Phosphoprotein Gel Stain. The gels show the same area on the gel. A large increase in phosphoproteins can be seen in the protein extract from stimulated cells (B) compared to the protein extract from unstimulated cells (A), suggesting that kinase pathways are being activated and proteins are being phosphorylated as a result of BCR stimulation.

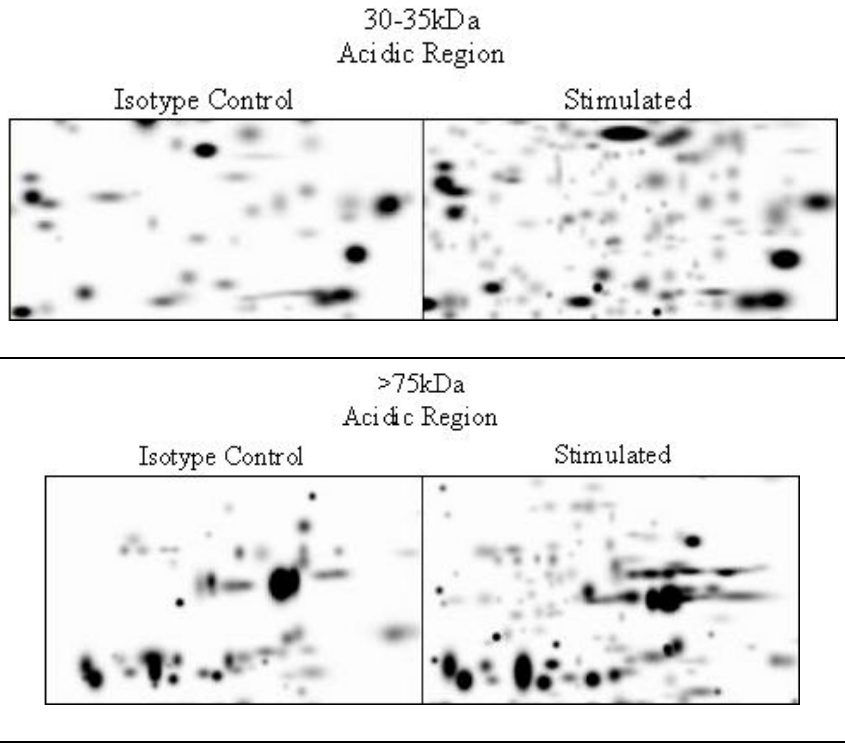


Figure 6.3: Areas on 2D gels from stimulated and unstimulated RAJI B-cells showing expression of phosphoproteins

The pictures show two areas of the gels shown in Figure 6.2, the image has been edited to improve background. A large increase in phosphoproteins can be seen in stimulated cells in both regions, suggesting that cell signaling pathways are being activated and proteins are being phosphorylated as a result of BCR stimulation.

6.3 Expression of Total Protein

The gels were stained with Krypton total protein stain after being stained with Pro-Q Diamond to confirm that a similar amount of total protein had been loaded onto each gel (see section 4.8.3). The gels showed that protein loads were comparable between stimulated and unstimulated gels (Figure 6.4), confirming that the increase in phosphoprotein expression in the stimulated sample is due to the activation of signaling pathways and not due to incorrect protein loadings.

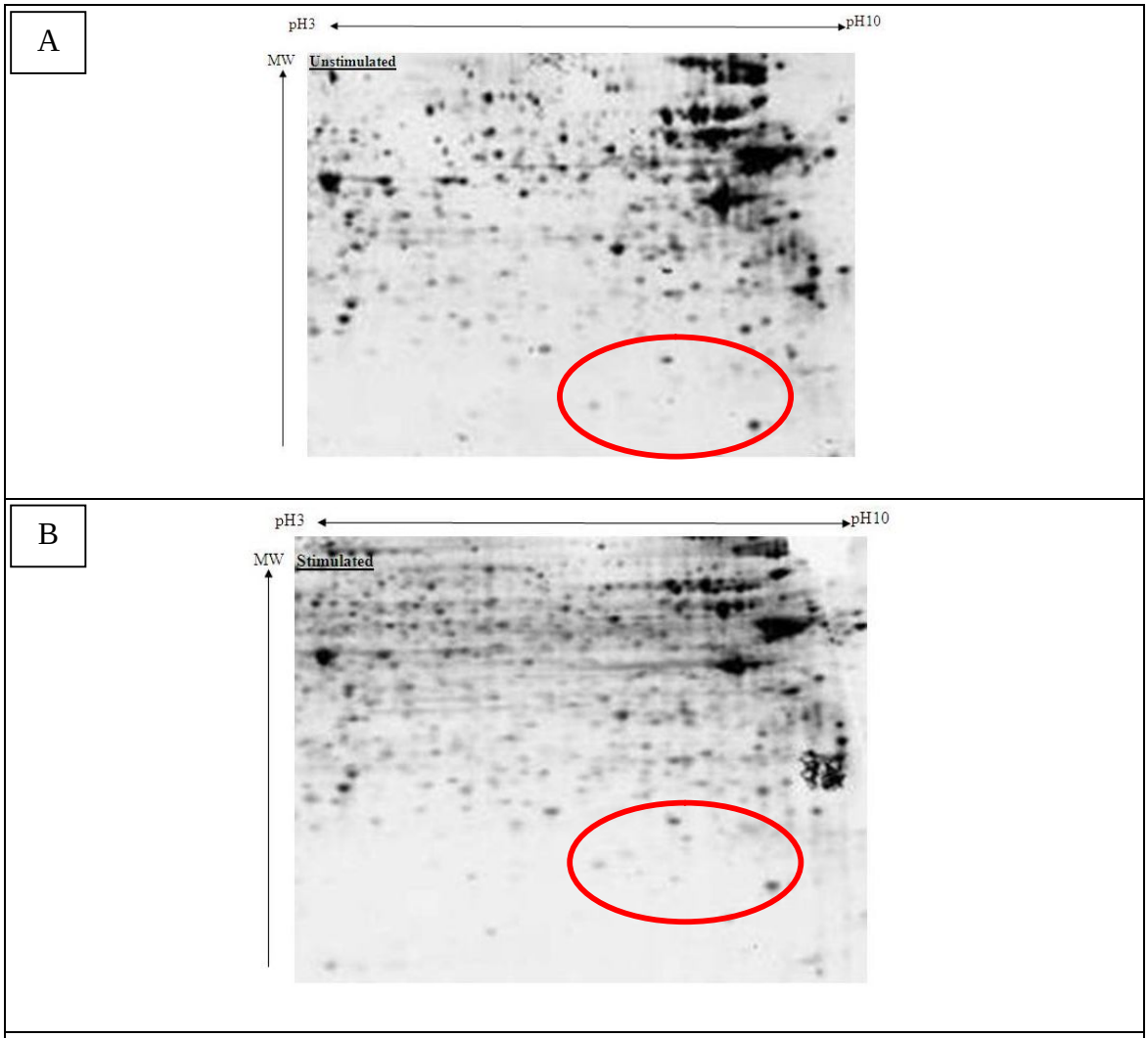


Figure 6.4: 2D gels of stimulated and unstimulated RAJI B-cells stained with Krypton fluorescent total protein stain

The gels have been stained with Pro-Q Diamond phosphoprotein stain (Figure 6.2). To visually check protein loading they were then stained with Krypton total protein stain. The pictures shown are from one representative gel from each set of triplicate gels. They show that protein loads are similar between stimulated (B) and unstimulated gels (A), confirming that the increase in phosphoprotein expression in the stimulated sample is due to the activation of signaling pathways and not due to incorrect protein loadings.

6.4 Summary

Unfortunately due to problems identifying phosphoproteins with the MALDI-TOF-MS (see section 5.7.1) and with problems excising spots stained with a fluorescent stain, differentially expressed spots could not be identified. Excision was attempted by exciting the fluorescent dye with a UV transilluminator, however, the stains were light sensitive and so UV rays bleached them within seconds. Excision was also attempted by overlaying the gel onto a picture of the scanned image, but this was not accurate enough to pick out the smaller fainter spots, thus making it impossible to excise the spots without a robotic spot picker.

To attempt to solve the excision problems with fluorescently stained proteins gels were stained with Pro-Q Diamond, to identify differentially expressed phosphoproteins, and then stained coomassie visible total protein stain to excise. However, the amount of protein required for coomassie staining was too much for fluorescent staining and fluorescent phosphoprotein spots were streaky and undefined.

It was therefore decided that gels would be stained with coomassie visible total protein stain alone, and differentially expressed unmodified proteins would be analysed. This would solve problems with excision and with MALDI-TOF-MS identification (see section 5.7.1). Signaling pathways activated by BCR stimulation could still be traced from proteins identified.

Chapter 7 – The 2D-PAGE/MS Analysis of RAJI B-Cell Line Following BCR Stimulation

Aims:

- To optimise a method to artificially stimulate the BCR in RAJI B-cells to produce changes in protein expression
- To develop a 2D-PAGE/MS experiment based on methods optimised in sections 5.4, 5.5, 5.6 and 5.7 to identify differentially expressed proteins in artificially stimulated and unstimulated RAJI B-cells

2D-PAGE/MS Analysis of RAJI B-Cell Line Following BCR Stimulation

Because of the difficulties in excising fluorescent stains (see Chapter 6) and the problems identifying modified proteins with MALDI-TOF-MS (see section 5.7), it was decided that gels would be stained with a visible total protein stain.

7.1 2D-PAGE of RAJI B-Cell Line Following Stimulation of the BCR for 5mins

7.1.1 Experimental

RAJI B-cells were cultured as described in section 4. Cells were stimulated in triplicate (three consecutive days) for 5mins (see section 4.4) and protein was extracted for western blot and for 2D-PAGE (see sections 4.4 and 4.5.1). A western blot of P-ERK was used to confirm stimulation; a PMA positive control was included to assess viability of cells (Figure 6.1).

When the stimulation had been confirmed, 200µg of protein was prepared and analysed by 2D-PAGE as described in section 4.6. IPG strips of pH4-7 were used. Gels were then stained with Bio-Safe™ Coomassie stain and scanned (4.8.1). PDQuest was used to highlight any differentially expressed proteins (4.9.1).

7.1.2 Results

Only one protein was found to be differentially expressed between stimulated and unstimulated gels (Figure 7.1). The protein was 24.6KDa with a pI >5.0 and was up-regulated in stimulated cells. However, the protein was not identifiable by MALDI-TOF-MS.

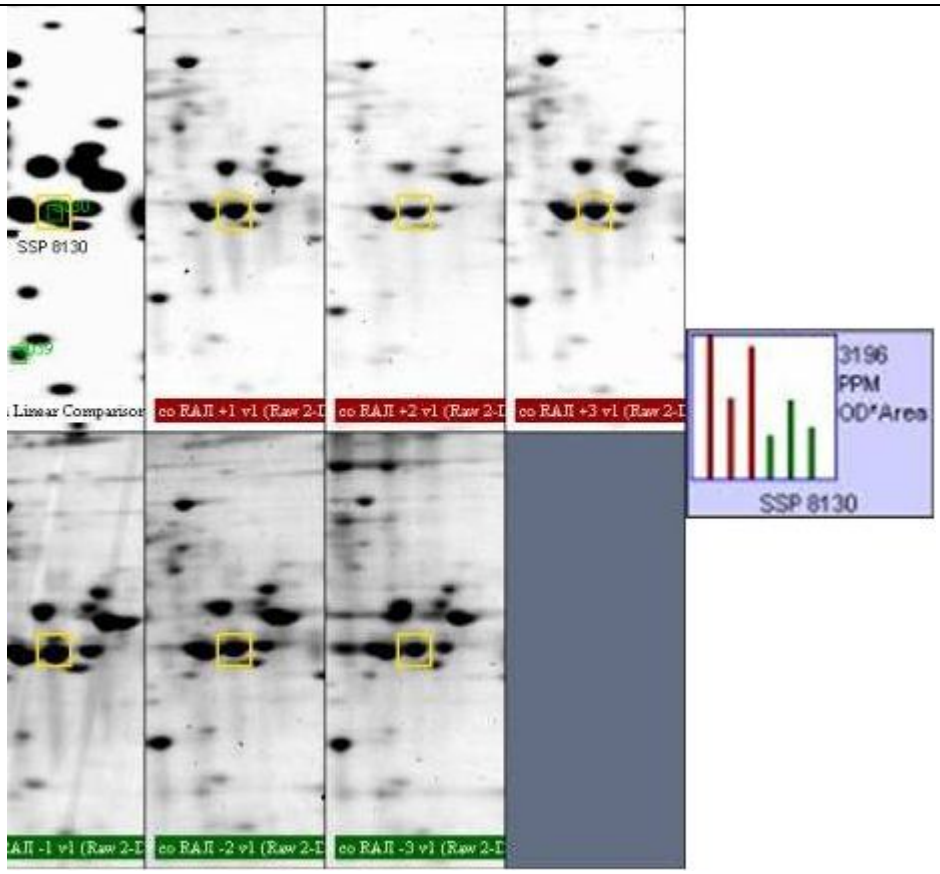


Figure 7.1: Histogram and picture of differentially expressed protein spot from stimulated vs. unstimulated cells (5mins stimulation)

RAJI B-cells were stimulated in triplicate for 5mins and protein was extracted from stimulated and unstimulated cells. Two hundred µg of protein from each replicate was separated by 2D-PAGE. IPG strips of pH range 4-7 were used. Gels were then stained with Bio-Safe™ Coomassie stain and scanned. PDQuest was used to highlight any differentially expressed proteins. Only one protein was found to be differentially expressed between stimulated and unstimulated gels (highlighted in yellow). The figure shows the master gel (top left) and the gels from stimulated cells (top, red), and unstimulated cells (bottom, green). The histogram shows the three replicate groups from stimulated cells (red) and unstimulated cells (green). The protein is 24.6KDa with a pI >5.0. This protein could not be identified by MALDI-TOF-MS.

The RAJI B-cells had been stimulated for 5mins originally to investigate phosphoproteins; however, proteins are phosphorylated very early in signaling pathways and only a short stimulation time is required to see changes in

phosphoproteins (see section 6.2). Results from this 2D-PAGE experiment suggest that a 5min stimulation time was not long enough to produce differences in the total protein in the cells. Therefore it was decided to increase the stimulation time in order to see if this result in a greater number of protein expression changes.

7.2 Optimisation of Increased Stimulation Time for RAJI B-Cells

The stimulation time needed to be increased to produce more protein expression changes, so a suitable stimulation time had to be found before proceeding with 2D-PAGE. Unfortunately there was a lack of literature available on artificial B-cell stimulation times that were longer than 15mins, therefore a suitable time point had to be optimised. Western blots were used to monitor the cellular activity during different time points.

7.2.1 Experimental

The test was performed on stimulated and unstimulated cells suspended in a concentration of $5x10^5$cells/ml over a period of time. Cells were monitored for media exhaustion and stress. This was done to confirm that the stimulation at the time point chosen was due to artificial activation of the BCR and not due to cell signaling due to stress. The time point chosen for stimulation in the 2D-PAGE experiment would be the maximum time that the cells could remain stable and stress free.

RAJI B-cells were stimulated as described in section 4.4 for 4hrs, 8hrs, 18hrs, and 24hrs. An isotype control was included for each time point. To observe any negative affects from the cells being exposed to the antibody for longer periods, RAJI B-cells

that had been stimulated for 5mins, washed in tissue culture media and then rested for 4hrs, 8hrs, 18hrs, and 24hrs were included in the assay. Flasks were gently agitated after each hour to avoid the suspension of RAJI B-cells sinking and clumping at the bottom of the flask, which would reduce exposure to the stimulant. The colour of the media was recorded, cells were observed under a microscope and cells were stained with trypan blue (see section 4.3) to check cell viability at the end of each time point. Protein was then extracted as described in section 4.5.1. Western blots (see section 4.5) were used to monitor levels of P-ERK, GAPDH, Cyclin D1 and BCL2 in cells. Cyclin D1 and BCL2 were chosen as they are proteins further down the MAPK pathway. P-ERK was used to monitor the MAPK pathway (Kolch. 2000), Cyclin D1 is activated by the ERK/MAPK pathway (Lee *et al*. 1999; Stacey. 2003) and BCL2 is down regulated by the activation of ERK (Tamura *et al*. 2004). GAPDH was used as a loading control

7.2.2 Results

The first observation made from the Western blots was that Cyclin D1 was not expressed in the cells (Figure 7.2). No evidence could be found to confirm or refute that RAJI B-cells express Cyclin D1.

The Western blots shown in Figure 7.2 show that the stimulant needed to be left on for the entire duration, cells which had the stimulant washed off after 5mins were comparable to the negative controls, i.e. no stimulation effects. P-ERK levels dramatically increase at 18hrs in both stimulated and unstimulated cells suggested that this time point is too long and the cells are becoming stressed. The colour of the media at this time point had changed from dark orange to intense yellow suggested a change in

pH and cell viability was poor. BCL2 is downstream of the P-ERK pathway and levels decrease as P-ERK levels increase.

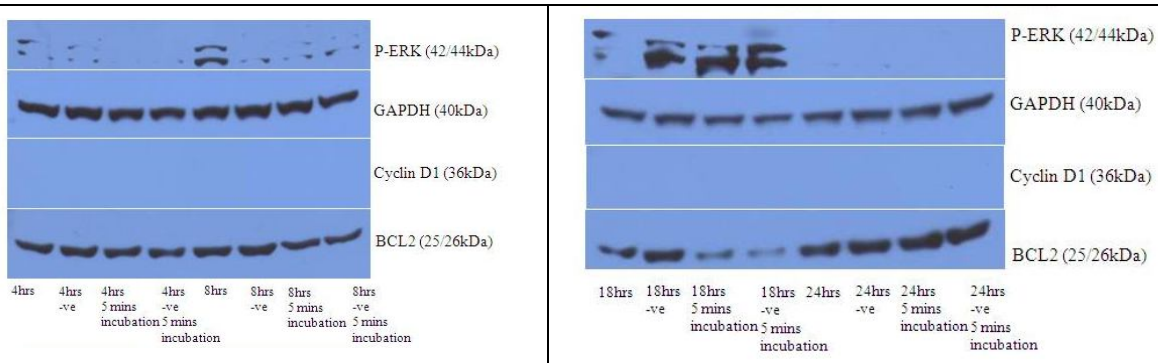


Figure 7.2: Western blots of protein extracts from RAJI B-cells stimulated over different time periods

RAJI B-cells were stimulated for 4hrs, 8hrs, 18hrs, and 24hrs. Isotype controls (-ve) were included for each time point. RAJI B-cells that had been stimulated for 5mins, washed in tissue culture media and rested for the time points specified were also included (labelled 5mins incubation). The Western blots show that the RAJI B-cells are negative for Cyclin D1. Cells which had the stimulant washed off after 5mins were comparable to the respective negative controls suggesting that the cells had to be incubated with the stimulant for the time period. P-ERK levels dramatically increased at 18hrs suggesting that this time point is too long and the cells were becoming stressed. The blots also show that BCL2 levels decreased as P-ERK levels increased.

Figure 7.3 shows a Western blot of just the isotype controls. From this blot it is clear that the maximum time that the cells can be left without interference from signaling due to cells exhausting the media is 8hrs. At this time point the tissue culture media was still orange (i.e. no change in pH), cells looked healthy under a microscope and cell viability was still good. At 18hrs the P-ERK levels suggest that cells have been stimulated for too long and are becoming stressed due to the number of cells to the volume of media.

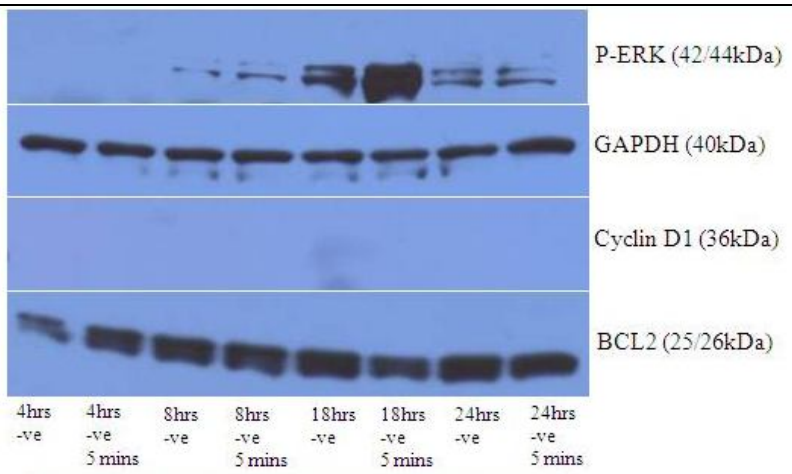


Figure 7.3: Western blots of protein extracts from negative control RAJI B-cells over different time periods

RAJI B-cells were exposed to an isotype control for 4hrs, 8hrs, 18hrs, and 24hrs. RAJI B-cells that had been exposed to an isotype control for 5mins, washed in tissue culture media and rested for the time points specified were also included (labelled 5mins). The Western blot shows that the maximum time that the cells can be left without interference from signaling due to cells exhausting the media is 8hrs. At 18hrs the P-ERK levels suggest that cells have been stimulated for too long and are becoming stressed due to the number of cells to the volume of media.

Further tests could be done to find the absolute optimum time when the cells start to become stressed (6-8hrs). However, it was decided that 8hrs will be sufficient to induce BCR stimulation without causing the cells distress. It is worth noting that proteins found to be differentially expressed may vary with differing stimulation times.

The 8hr stimulation time, chosen from this experiment, is the maximum time that the cells could be artificially stimulated without stress-related cell signaling interferences to allow protein expression changes.

7.3 2D-PAGE of RAJI B-Cell Line Stimulated for 8hrs

7.3.1 Experimental

RAJI B-cells were exposed to the stimulant or the isotype control (see section 4.4) for 8hrs with the flask being gently agitated every hour. Stimulations were performed in triplicate and on consecutive days. Protein was extracted for 2D-PAGE (80%) and Western blot (20%) (see section 4.5.1). The 2D-PAGE protein extract was quantified, cleaned and 200µg from each replicate was separated by 2D-PAGE using pH4-7 IPG strips (see section 4.6). Gels were stained with coomassie protein stain and scanned (see section 4.8.1). PDQuest was used to highlight protein spots differentially expressed between stimulated and unstimulated cells by over 2-fold with $p<0.05$ (see section 4.9.1). Protein spots found to be differentially expressed were excised, digested and analysed by MALDI-TOF-MS (see sections 4.10 and 4.11).

7.3.2 Results

PDQuest highlighted 22 proteins spots which were differentially expressed between stimulated and unstimulated cells by over 2-fold with $p<0.05$ (Figure 7.4). Eighteen protein spots were excisable and were analysed by MALDI-TOF-MS. Eleven of the 18 were identified to significance (score ≥65).

One protein was up-regulated and ten proteins were down-regulated. The protein which was up-regulated in stimulated cells was identified as heat shock 70KDa protein (NCBInr reference gi|16507237) (Figure 7.5). All proteins identified are listed in Table

7.1. The table shows protein spots which were not excised (green), and not identified (red). Proteins highlighted in blue had Mowse scores below the cut off for a significant identification (score ≥65). Increased (↑) or decreased (↓) protein expression changes in stimulated cells are indicated. Also shown are the estimated molecular weights (KDa) and pI according to spots location in the 2D gel and the theoretical values given in the NCBInr database.

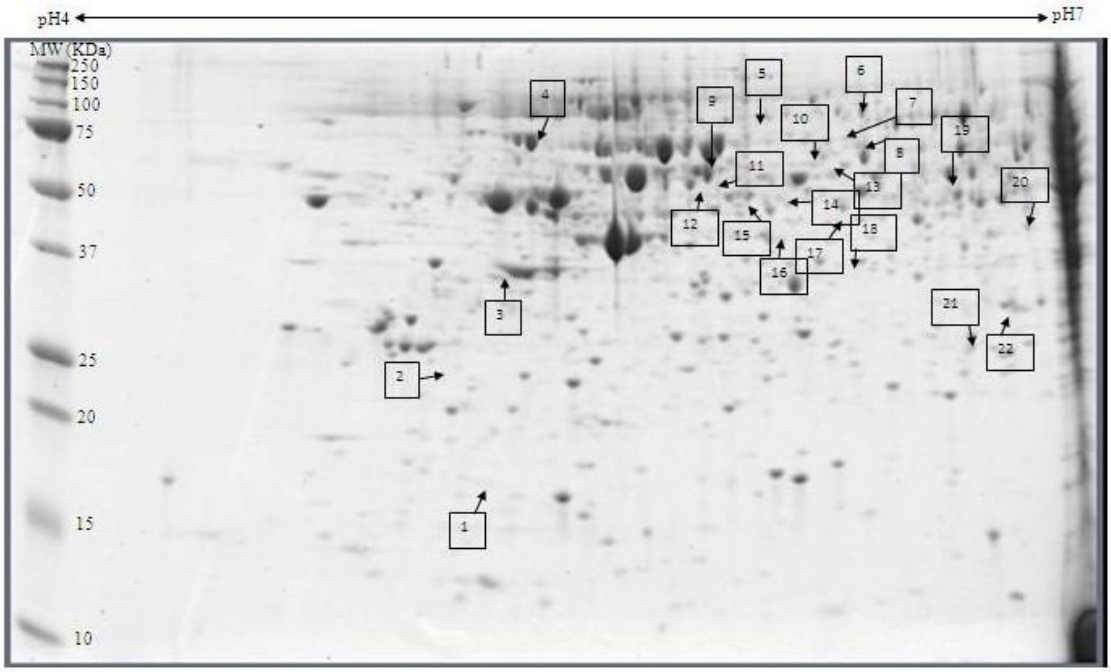


Figure 7.4: 2D gel of protein extracted from RAJI B-cells with spots found to be differentially expressed between cells stimulated for 8hrs and unstimulated cells

The picture shows the 22 proteins spots highlighted by PDQuest, which were differentially expressed between stimulated and unstimulated cells by over 2-fold with $p<0.05$. Four of the protein spots (1, 2, 5 and 20) were not excised as they were either too faint, in a cluster of spots or in a streaky area of the gel.

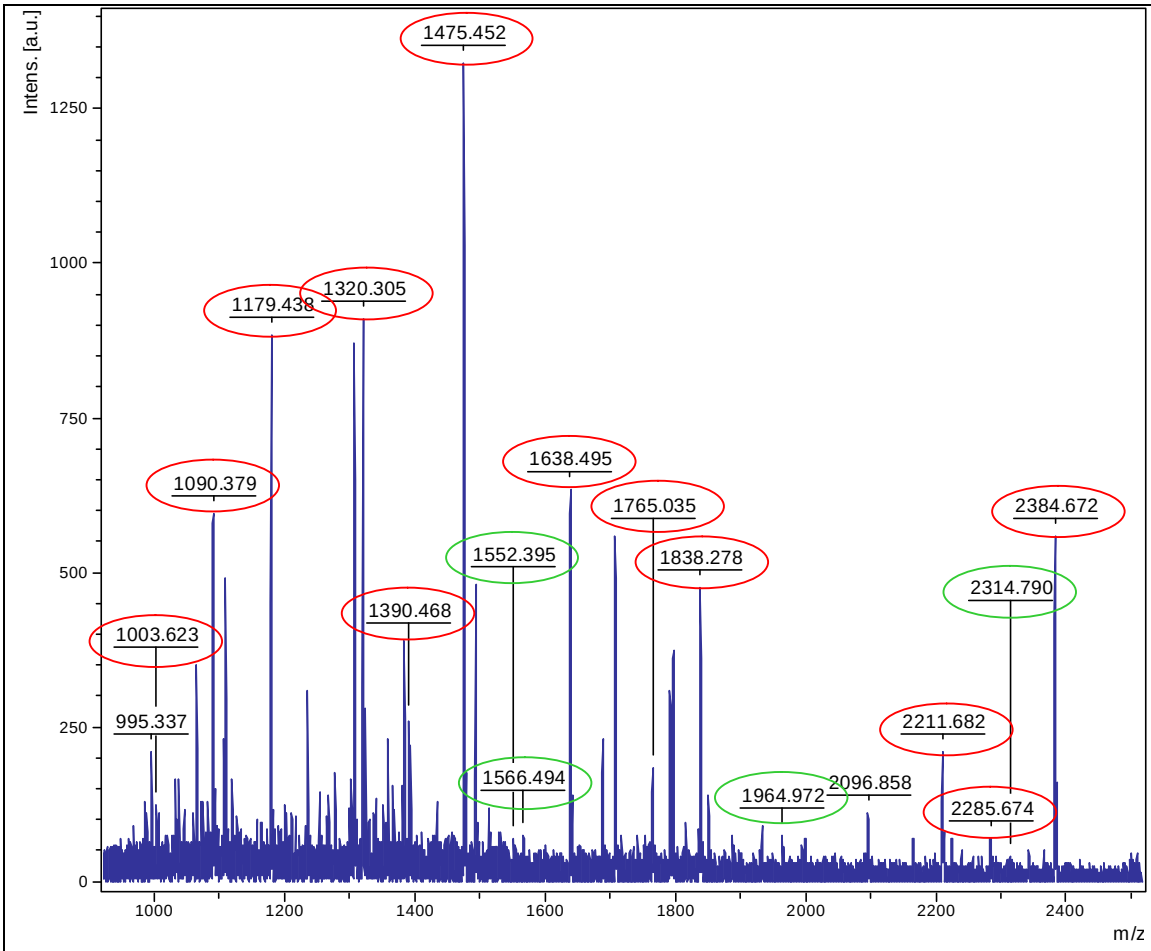


Figure 7.5: Peptide mass fingerprint of a protein identified as heat shock 70KDa protein

MALDI-TOF spectra of spot 4, identified to significance as heat shock 70KDa protein gi|16507237 (72.4KD), which was found to be up-regulated in RAJI B-cells stimulated for 8hrs when compared with unstimulated cells. The peaks highlighted in green correspond to HSP70 peptides, the peaks highlighted in red are contaminant peptides. Many of the low abundant peaks (not shown) correspond to the protein of interest, whereas many of the higher abundant peaks are contaminants.

Table 7.1: Table to show proteins found to be differentially expressed between RAJI B-cells stimulated for 8hrs and unstimulated RAJI B-cells

Spot	PDQuest Number	MW (KDa)	Estimated PI	Stimulated Cells	Protein Identified	NCBInr Reference	Theoretical MW (KDa)	Theoretical PI	Score	Peptides Matched	% Sequence Coverage
1	2018	18	5.0	N/A	Not Excised	N/A	N/A	N/A	N/A	N/A	N/A
2	1112	23	5.0	N/A	Not Excised	N/A	N/A	N/A	N/A	N/A	N/A
3	2314	30	5.0	N/A	Not Identified	N/A	N/A	N/A	N/A	N/A	N/A
4	2715	70	5.5	↑	Heat Shock 70KDa Protein	Gi|16507237	72.402	5.07	111	26	44
5	6716	70	6.0	N/A	Not Excised	N/A	N/A	N/A	N/A	N/A	N/A
6	7821	80	>6.5	↓	Inner Membrane Protein, Mitochondrial Isoform 1	Gi|154354964	84.025	6.08	94	21	37
7	6714	70	>6.5	↓	Glutaminyl-tRNA Synthetase, Isoform CRA-a (QARS Protein)	Gi|119585356	72.548	6.89	80	17	29
8	7701	65	>6.5	↓	SARM 1 Protein	Gi|116063294	68.81	6.13	78	25	41
9	5622	60	6.0	↓	Chaperonin Containing TCP Subunit 8 (Theta)	Gi|48762932	60.153	5.42	66	17	35
10	6605	60	6.5	↓	SARM 1 Protein	Gi|3043572	67.386	6.33	74	13	30
11	5617	50	6.0	↓	Chimerin (Chimaerin) 1	Gi|119631525	50.046	6.45	68	15	43
12	5526	50	6.0	N/A	Not Identified	N/A	N/A	N/A	N/A	N/A	N/A
13	6610	60	>6.5	↓	Chaperonin Containing TCP Subunit 8 (Theta)	Gi|48762932	60.153	5.42	69	13	28
14	6505	55	>6.5	↓	Keratin 14 - Epidermolysis Bullosa Simplex, Dailing-Meara, Koeber	Gi|12803709	51.905	5.09	72	25	54
15	5421	45	6.0	N/A	Not Identified	N/A	N/A	N/A	N/A	N/A	N/A
16	6419	40	6.0	↓	Unknown Protein	Gi|62988628	33.711	5.52	69	12	43
17	6410	40	6.5	N/A	Not Identified	N/A	N/A	N/A	N/A	N/A	N/A
18	7317	30	6.0	N/A	Not Identified	N/A	N/A	N/A	N/A	N/A	N/A

19	8502	45	>6.5	↓	Leukocyte Derived Arginine Aminopeptidase Isoform CRAb (LRAP)	N/A	40.376	6.73	64*	13	34
20	9401	40	>6.5	N/A	Not Excised	N/A	N/A	N/A	N/A	N/A	N/A
21	8115	25	>6.5	N/A	Not Identified	N/A	N/A	N/A	N/A	N/A	N/A
22	8212	28	>6.5	↓	Nuclear Receptor Binding Protein 2	Gi|119602588	31.056	6.87	65	13	47

7.3.3 Summary

A stimulation time of 5mins was not long enough to show differential expression in proteins with only one protein being up-regulated in stimulated cells. However, after an 8hr incubation with the stimulant, 22 proteins were found to be differentially expressed between stimulated and unstimulated cells. Eleven of these proteins were identified by MALDI-TOF-MS. Heat shock 70KDa protein (HSP70) was found to be over expressed in stimulated cells and is of particular interest as recent studies show that over expression indicates poor prognosis in chronic myeloid leukaemia (Nylandsted. 2009; Yeh *et al*. 2009). Chimerin, a protein related to GTPase activity, proliferation and cell cycle arrest, was found to be down regulated in stimulated cells. A decrease in expression of chimerin has also been observed in breast cancer (Yang *et al*. 2005). Western blot confirmation and further analysis is required on these target proteins.

The 8hr stimulation time chosen in this experiment was the maximum time that the cells could be artificially stimulated without cell signaling interferences due to stress. This experiment gives a platform to base further 2D-PAGE experiments on (see section 9.1). It is clear that long stimulation times are needed to produce differential expression in proteins when using a total protein stain. Differentially expressed proteins may vary over different stimulation times. It was decided to stimulate the cells for the maximum time possible in this project to produce protein expression changes.

Chapter 8 - The Categorisation and Selection of Clinical CLL Samples

Aims:

- Optimise western blot signaling assay for use in clinical CLL samples
- To identify three "poor prognosis" clinical CLL samples, to be used as biological replicates in a 2D-PAGE/MS experiment in section 9.3, based on:

i IgV_H mutational status

ii BCR responsiveness

iii B-cell population in sample

iv WBC of patient

The Categorisation and Selection of Clinical CLL Samples

8.1 CLL Sample Collection

Samples were prospectively collected during the project from patients diagnosed with CLL (Binet stages A-C), who had not received chemotherapy treatment in the past 3 months. PBMCs were stored as described in section 4.2. Samples from over 200 patients have been collected and stored over a period of three years.

Cells frozen at -80°C and in liquid nitrogen at a concentration of $1x10^7$cells/ml per cryovial were tested for viability after thawing using trypan blue (see section 4.3). Results showed that over 90% of cells were viable on thawing when stored at this concentration.

8.2 Clinical CLL Sample Classification

Clinical samples were chosen for analysis based on their IgV_H gene mutation status and the BCR responsiveness. All samples were analysed for IgV_H mutational status and samples which showed a poor prognosis (<5% difference in standard germline sequence) were subjected to a BCR signaling assay to determine BCR responsiveness. Samples which were categorised as unmutated and responsive BCR were required for 2D-PAGE/MS.

8.2.1 IgV_H Gene Mutational Status

Samples were analysed at HMDS for IgV_H gene mutational status (see section 4.2.3). Less than 5% difference in standard germline sequence was classified as unmutated IgV_H and more than 5% difference in standard germline sequence was classed as mutated IgV_H (Allsup *et al.* 2005). Approximately half of samples (57 out of 115) have been classed as unmutated samples (see Appendix B)

Figure 8.1 shows a Kaplan Meier plot of mutated vs. unmutated samples from the cohort of 115 patient samples stored. The plot shows the differing prognosis between patients with mutated IgV_H genes and those with less than 2% difference in standard germline sequence. This corresponds with the discussion in section 2.3. A 5% difference in standard germline sequence is generally accepted as a "cut-off point" for determining mutated and unmutated samples (Allsup *et al.* 2005), however the exact point of division between mutated and unmutated samples has not yet been determined. Figure 8.1 shows that in Hull's cohort of samples, a 2% cut off gives visible separation of the two groups, therefore samples with little difference in standard germline sequence were taken forward to 2D-PAGE.

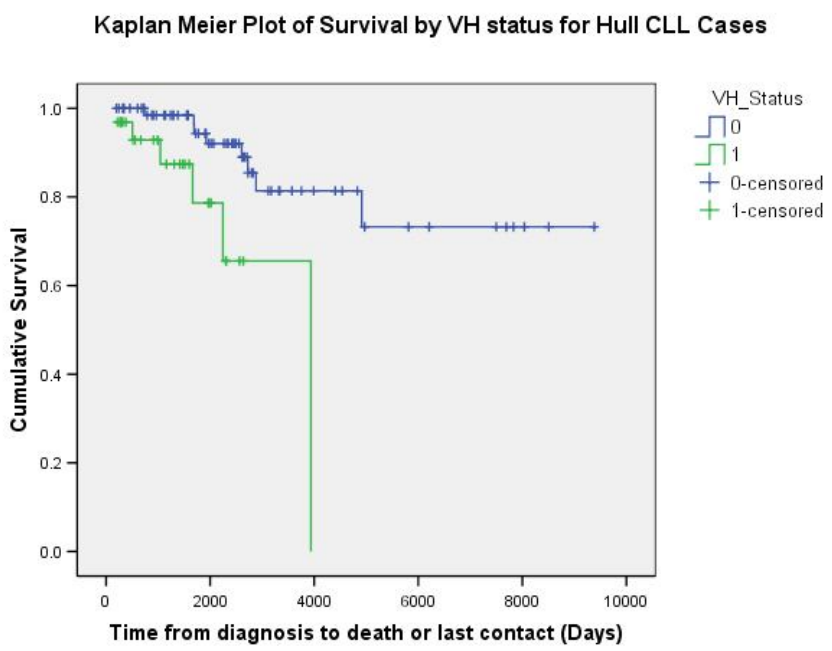


Figure 8.1: Kaplan Meier to show effects of IgV_H mutational status on survival

The plot shows the differing prognosis between the two groups from the cohort of patient samples stored. A <2% difference in standard germline sequence was classified as unmutated IgV_H. Patients with mutated IgV_H genes (n=81) (blue) have a better prognosis than those with unmutated IgV_H genes (n=34) (green). Median survival for patients with unmutated IgV_H genes is 4000 days. For patients with mutated IgV_H genes median survival is not yet reached (P<0.00 Mantel Cox log rank test). *Plot courtesy of J. Bailey 2008.*

8.2.2 BCR Responsiveness

To classify samples as BCR "responders" or "non-responders" (see section 2.4), clinical CLL samples were stimulated as described in section 4.4. Stimulation of the RAJI B-cell line was optimised in section 5.1, however, to ensure that a 5min incubation with 10µg/ml of antibody fragment would be suitable for the clinical samples, the same optimisation experiment was conducted as described in sections 5.1.1 and 5.1.2 using CLL sample 010. This sample has germline concordance of 99% (IgV_H unmutated), and was known to have a responsive BCR from preliminary western blot signaling assays.

8.2.3 Optimisation of Incubation Time

After cell counting (see section 4.3) CLL cells from sample 010 were suspended in a concentration of $5x10^6$cells/ml as recommended (Allsup *et al.* 2005). CLL cells are smaller than RAJI B-cells and so more were required if the amount of stimulant was to be kept the same (RAJI B-cells were stimulated in a concentration of $5x10^5$cells/ml). Cells were incubated for 5, 15, 25 and 45mins with 10μg/ml of AffiniPure $F(ab')_2$ fragment goat anti-human IgM, $Fc_{5\mu}$ fragment specific. Cells incubated with 10μg/ml of ChromPure Goat IgG $F(ab')_2$ fragment for 60mins were included as an isotype control. Incubations were performed at 37°C with 5% CO_2.

Protein was extracted as described in section 4.5.1 and western blots were performed as described in section 4.5. P-ERK was used as a primary antibody and GAPDH was used as a loading control (Table 4.1, Table 4.2).

The western blot showed that 5mins incubation with 10μg/ml of the anti-human IgM antibody was sufficient to activate the BCR and induce the MAPK pathway (Figure 8.2).

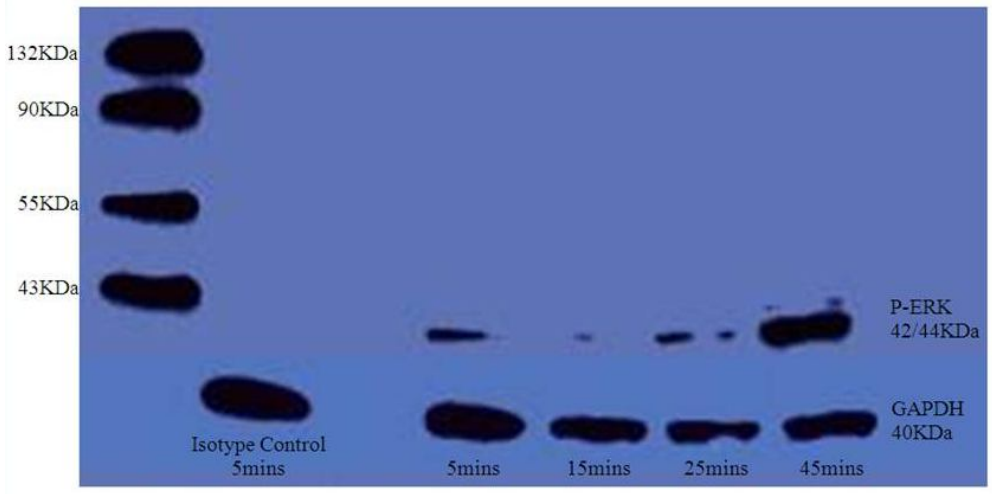


Figure 8.2: Western blot to show optimisation of stimulation time for clinical CLL sample 010

The western blot shows that 5mins incubation with 10μg/ml of the anti-human IgM antibody is enough to activate the BCR and causing ERK to become phosphorylated in the MAPK pathway in a clinical CLL sample. The isotype control showed that after 5mins the cells were negative for P-ERK, providing evidence that P-ERK levels were purely induced by the anti-human IgM antibody.

8.2.4 Optimisation of Stimulant Concentration

After cell counting (see section 4.3) CLL cells from clinical sample 010 were suspended in a concentration of $5x10^6$cells/ml. Cells were incubated with 1.0, 10, 50 and 100 μg/ml of AffiniPure $F(ab')_2$ fragment goat anti-human IgM, $Fc_{5\mu}$ fragment specific for 5mins. Cells incubated with 100nM PMA for 5mins were included as a positive control and cells incubated with 100μg/ml of ChromPure Goat IgG $F(ab')_2$ fragment for 5mins were included as an isotype control. Incubations were performed at 37°C with 5% CO_2.

Protein was extracted as described in section 4.5.1 and western blots were performed as described section 4.5. P-ERK was used as a primary antibody and GAPDH was used as a loading control (Table 4.1 and Table 4.2).

The western blot showed that incubation with all the chosen concentrations of anti-human IgM antibody activated the BCR and induced the MAPK pathway (Figure 8.3).

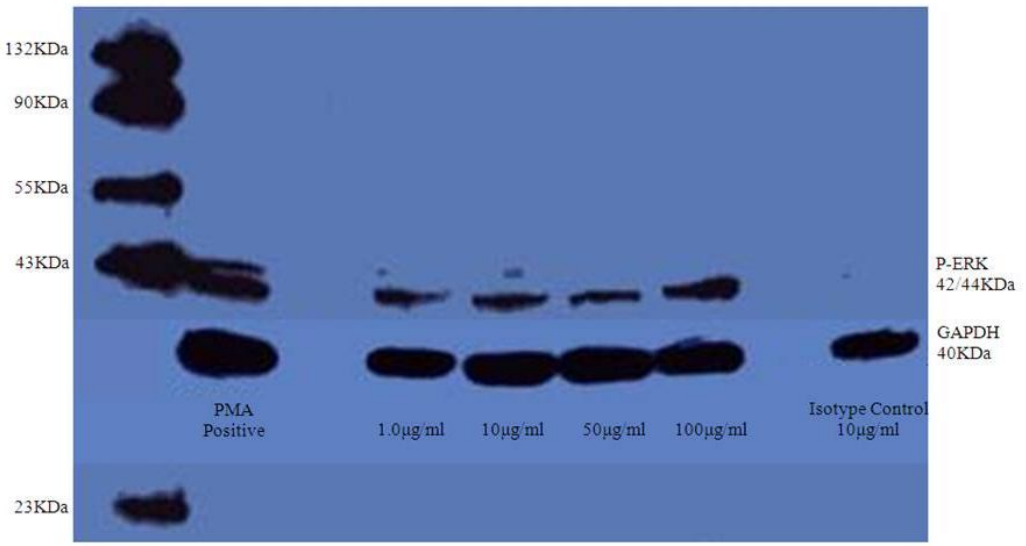


Figure 8.3: Western blot to show optimisation of stimulant concentration for clinical CLL sample 010

The western blot showed that incubation with all the chosen concentrations of anti-human IgM antibody activated the BCR and induced the MAPK pathway. The PMA positive control shows that after 5mins the cells are viable. The isotype control showed that at a concentration of 10µg/ml the cells were negative for P-ERK, providing evidence that P-ERK levels were purely induced by the anti-human IgM antibody.

The incubation time and stimulant concentration used to activate the BCR in CLL samples in this project was chosen as 5mins and 10µg/ml respectively. This time and concentration correspond with the literature (Allsup *et al.* 2005) and is a well established and widely accepted protocol. It also corresponds with the time and concentration optimised to stimulate RAJI B-cells in this project (see sections 5.1.1 and 5.1.2).

8.3 BCR Signaling Assay in Clinical CLL Samples

Clinical CLL samples were collected and stored as described in section 4.2. Samples from patients with a high white blood count (>40x10^9L), with cells classified as IgV_H unmutated (see section 4.2.3) were stimulated as described in section 4.4 to assess BCR responsiveness. Selecting samples from patients a high white blood count was important to be able to extract enough protein for 2D-PAGE from the volume of blood available (50ml blood, see 4.2.1). Also, assuming lack of infections, a high white blood count is an indication of poor prognosis in CLL. Protein was extracted from these samples (see section 4.5.1) and a western blot signaling assay (see section 4.5) was used to determine BCR responsiveness.

To quickly determine from the bank of clinical samples which ones were of interest, multiple samples were stimulated and screened to determine the status of P-ERK (Figure 8.4). Samples demonstrating very high levels would then be assayed again with an isotype control to determine levels of constitutive P-ERK in the cells. It is important to verify that ERK phosphorylation is due to the artificial stimulation of the BCR and not due to intrinsic cell signaling. Samples that had a 2-fold increase in P-ERK when stimulated (Allsup *et al.* 2005) were potential samples that could be used for 2D-PAGE investigations.

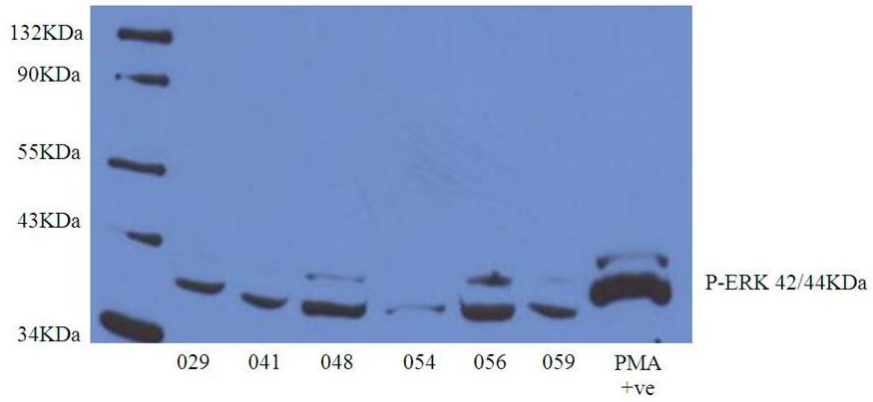


Figure 8.4: Examples of western blot signaling screening assay of clinical CLL samples

To quickly determine from the bank of samples which ones were of interest, multiple samples were stimulated and screened to determine levels of P-ERK. The blots above show six examples of assayed samples, consisting of three IgV_H mutated samples (029, 041 & 059) and three IgV_H unmutated samples (048, 054 & 056). Twenty µg of each protein sample was loaded. Very high P-ERK levels can be seen in clinical samples 048, and 056. These samples are IgV_H unmutated and could be of interest for further testing. They would be assayed again with an isotype control to determine levels of constitutive P-ERK and fold change in P-ERK upon stimulation of the BCR. Sample 054 is also IgV_H unmutated but the P-ERK level is weak upon BCR stimulation suggesting it is not as responsive as samples 048 and 056.

8.4 Clinical CLL Sample Selection

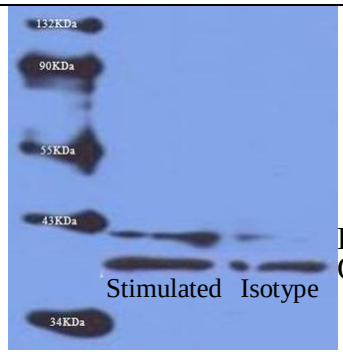
Samples chosen for 2D-PAGE were:

- Samples with a WBC >$50x10^9$L
- Samples with ≤2.5% deviation from the standard germline (IgV_H Unmutated)
- Samples showing >2-fold change in P-ERK expression upon BCR stimulation
- Samples which contain >70% B-cells

From all the samples tested three stood out as being potential biological replicates for a 2D-PAGE experiment, these were 003, 078 and 089.

8.4.1 Sample 003

Patient 003 had a white blood count of 215X10^{9}L. The sample had 0% deviation from the standard germline IgV_H sequence and was classified as unmutated. The sample responded to BCR stimulation and gave a P-ERK fold change of 10.4 in stimulated cells (Figure 8.5). The sample also has a large population of B-cells consisting of 77.6% of the stored PBMCs (Figure 8.6).



	Stimulated	Isotype Control
Optical Density	34.34	3.31
Fold Change	10.3746224	1

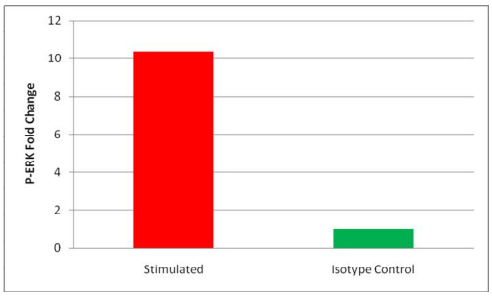


Figure 8.5 Western blot and optical density data on signaling assay of sample 003

Western blot and optical density data to show increase in P-ERK upon stimualtion. GAPDH has been used as a loading control. The results show a 10.4 fold increase in P-ERK upon stimulation of the BCR.

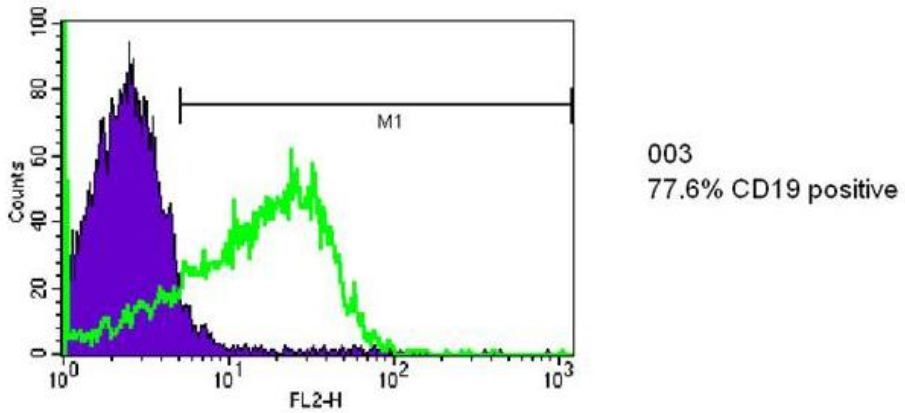


Figure 8.6: FACS plot of CD19 positive cells in sample 003

The picture shows a fluorescence-activated cell sorting (FACS) plot of CD19 postive cells in sample 003. CD19 is a B-cell specific maker. This sample consist of 77.6% B-cells. *Results and plot courtesy of J. Butt.*

8.4.2 Sample 078

Patient 078 had a white blood count of $55X10^9L$. The sample had 0% deviation from the standard germline IgV_H sequence and was classified as unmutated. The sample responded to BCR stimulation and gave a P-ERK fold change of 15.9 in stimulated cells (Figure 8.7). The sample also has a large population of B-cells consisting of 91.39% of the stored PBMCs (Figure 8.8).

	Stimulated	Isotype Control
Optical Density	25.61	1.61
Fold Change	15.9068323	1

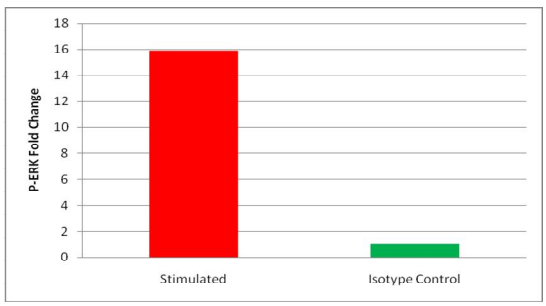


Figure 8.7: Western blot and optical density data on signaling assay of sample 078

Western blot and optical density data to show increase in P-ERK upon stimualtion. GAPDH has been used as a loading control. The blot shows some consitutive P-ERK activity in the isotype control sample, however, the results show a 15.9 fold increase in P-ERK upon stimulation of the BCR.

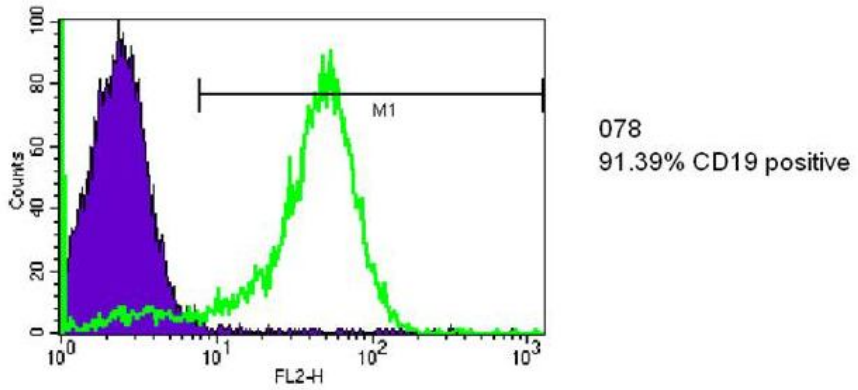
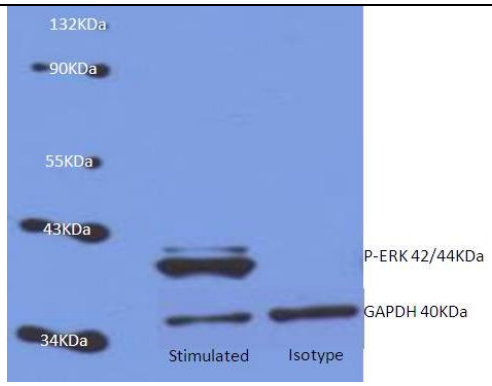


Figure 8.8: FACS plot of CD19 positive cells in sample 078

The picture shows FACS plot of CD19 postive cells in sample 078. CD19 is a B-cell specific maker. This sample consist of 91.39% B-cells. *Results and plot courtesy of J. Butt.*

8.4.3 Sample 089

Patient 089 had a white blood count of $72X10^9L$. The sample had 2.5% deviation from the standard germline IgV_H sequence and was classified as unmutated. The sample responded to BCR stimulation and gave a large change in P-ERK expression with a fold change of 216.1 upon stimulation of the BCR (Figure 8.9). The sample also has a large population of B-cells consisting of 80.11% of the sample (Figure 8.10).



	Stimulated	Isotype Control
Optical Density	749.71	3.47
Fold Change	216.054755	1

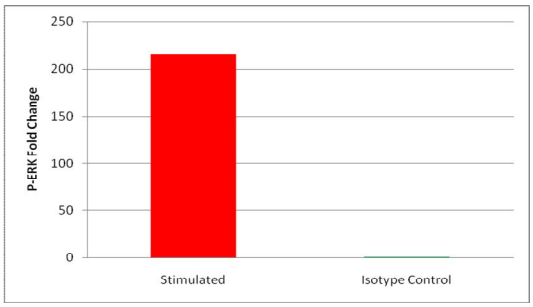


Figure 8.9: Western blot and optical density data on signaling assay of sample 089

Western blot and optical density data to show increase in P-ERK upon stimualtion. GAPDH has been used as a loading control. The results show a 216.1 fold increase in P-ERK upon stimulation of the BCR.

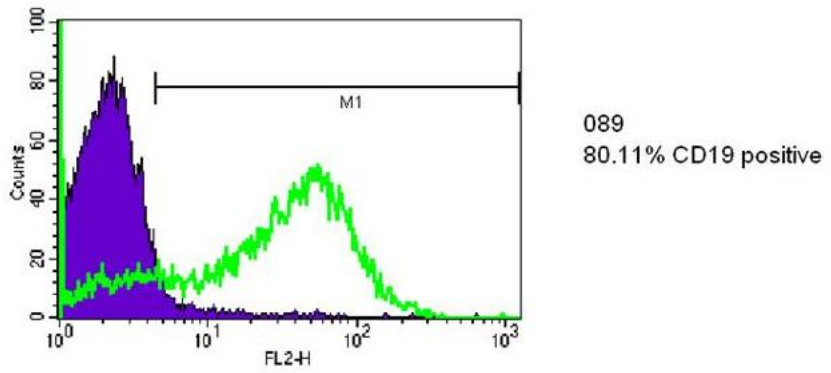


Figure 8.10: FACS plot of CD19 positive cells in sample 089

The picture shows a FACS plot of CD19 postive cells in sample 089. CD19 is a B-cell specific maker. This sample consist of 80.11% B-cells. *Results and plot courtesy of J. Butt.*

8.5 Summary

From all the clinical CLL samples tested, three matched the criteria for potential biological replicates for a 2D-PAGE experiment. The criteria were:

- WBC >50x10^9L
- ≤2.5% deviation from the standard germline (IgV_H Unmutated)
- >2-fold change in P-ERK expression upon BCR stimulation confirmed by western blot and by P-ERK ELISA (data not shown) (Bailey. 2007)
- >70% B-cells

Table 8.1 summarises the three samples selected for further analysis by 2D-PAGE and shows WBC, IgV_H mutational status, P-ERK fold change upon BCR stimulation and B-cell population in each sample.

Table 8.1: Table to show the three clinical CLL samples which matched the criteria set for further analysis by 2D-PAGE

Clinical CLL Sample	WBC	Deviation from Standard Germline IgV_H Sequence	P-ERK Fold Change Upon BCR Stimulation	B-Cells in Sample
003	$215x10^9L$	0%	10.4	77.6%
078	$55x10^9L$	0%	15.9	91.4%
089	$57x10^9L$	2.5%	216.1	80.1%

Chapter 9 - The 2D-PAGE/MS Proteomic Analysis of CLL Clinical Samples

Aims:

- To optimise a method to artificially stimulate the BCR in clinical CLL samples to produce changes in protein expression
- To assess the efficacy of using the 2D-PAGE/MS method developed in section 7.3 for the identification of potential prognostic markers in clinical CLL samples
- To identify changes in protein expression between stimulated and unstimulated cells in the three clinical samples selected in section 8.4
- To validate proteins identified using western blot analysis

Publications and Presentations

- G L Eagle, L Cawkwell, D Allsup. The Proteomic Analysis of B-Cell Signaling in Chronic Lymphocytic Leukaemia. Human Proteome Organisation, 7th World Congress. Amsterdam, The Netherlands *(Aug 2008)*. Poster Presentation.
- G L Eagle, L Cawkwell, D Allsup. A Proteomic Approach to Investigate Targets Associated with B-Cell Receptor Signaling in Chronic Lymphocytic Leukaemia. European Haematology Association, 13th Congress. Copenhagen, Denmark *(June 2008)*. Poster Presentation.
- G L Eagle, D Allsup, L Cawkwell. Proteomics as a Tool to Investigate Signaling Pathways Activated by B-Cell Receptor Stimulation in Chronic Lymphocytic Leukaemia. British Society for Haematology, 48th Annual Scientific Meeting. Glasgow, UK. (April 2008). Poster Presentation.
- G L Eagle, K Welham, D Allsup, L Cawkwell. Proteomics as a tool to Identify Potential Prognostic Biomarkers in Chronic Lymphocytic Leukaemia. US HUPO, 5th Annual Conference. San Diego, CA, USA *(Feb 2009)*. Poster Presentation

The 2D-PAGE/MS Analysis of CLL Clinical Samples

CLL clinical samples were initially stimulated for 8hrs as optimised with RAJI B-cells lines in section 7.2.

9.1 8hr Stimulation of CLL Clinical Sample 003

CLL sample 003 was stimulated as described in section 4.4 for 8hrs (see section 7.2) at a concentration of $5x10^6$cells/ml with a total volume of 10ml. However, clinical CLL samples can not be cultured and after 6hrs in culture the media had turned from orange to yellow (indicating media exhaustion and a change in pH) and cell viability was poor at 8hrs. This was not consistent with the 8hr RAJI B-cell line stimulation as described in section 7.3. Protein was extracted for 2D-PAGE (see section 4.5.1) from these cells however there was not enough protein for an experiment (need >800µg/ml, obtained <1µg/ml) (see section 4.6.2). This was probably because the majority of the cells had died during the long stimulation and were washed out in the washing steps. Therefore it was not practical to use the stimulation time previously optimised for the RAJI B-cell line in section 7.2 for clinical CLL samples. An optimum stimulation time for CLL samples needed to be found.

9.2 Optimisation of Stimulation Time for CLL Clinical Samples

The test was performed on stimulated and unstimulated (isotype control) CLL cells suspended in a concentration of $5x10^6$cells/ml over a period of time. Cells were monitored for media exhaustion and stress. This was done to confirm that the

stimulation at the time point chosen was due to artificial activation of the BCR and not due to cells signaling due to stress. As in section 7.2, the time point chosen for stimulation in the 2D-PAGE experiment would be the maximum time that the cells from the clinical sample could remain stable and when the cells could be artificially stimulated without stress-related cell signaling interfering with the assay.

As the media was exhausted after 6hrs in the 8hr stimulation of clinical sample 003 (see section 9.1), it was decided that the longest stimulation period should be 5.5hrs. CLL cells from clinical sample 063 (good responder to BCR stimulation, known from previous western blot analysis) were stimulated as described in section 4.4 for 1hr, 2hrs, 4hrs, and 5.5hrs. An isotype control was included for each time point. Because the media had been exhausted in the 8hr stimulation of cells from clinical sample 003 (see section 9.1), a sample was included which was stimulated for 5.5hrs but the media and stimulant were replaced after 3hrs. Flasks were gently agitated after each hour to avoid the suspension of CLL cells sinking and clumping at the bottom of the flask, which would reduce exposure to the stimulant. The colour of the media was recorded, cells were observed under a microscope and cells were stained with trypan blue (see section 4.3) to check cell viability at the end of each time point. Protein was then extracted as described in section 4.5.1. Western blots (see section 4.5) were used to monitor levels of BCL2 in cells (see Table 4.1 and Table 4.2). GAPDH was used as a loading control (see Table 4.1 and Table 4.2). BCL2 is down regulated by the activation of ERK (Tamura *et al*. 2004) making it a good marker of downstream MAPK activity.

At 5.5hrs cells viability was good (see section 4.3) and the media had not changed colour. The optical densities of the BCL2 and GAPDH bands were measured (see

section 4.5.8) and BCL2 fold differences were calculated. Figure 9.1 shows changes in expression of BCL2 in stimulated cells compared with the isotype control.

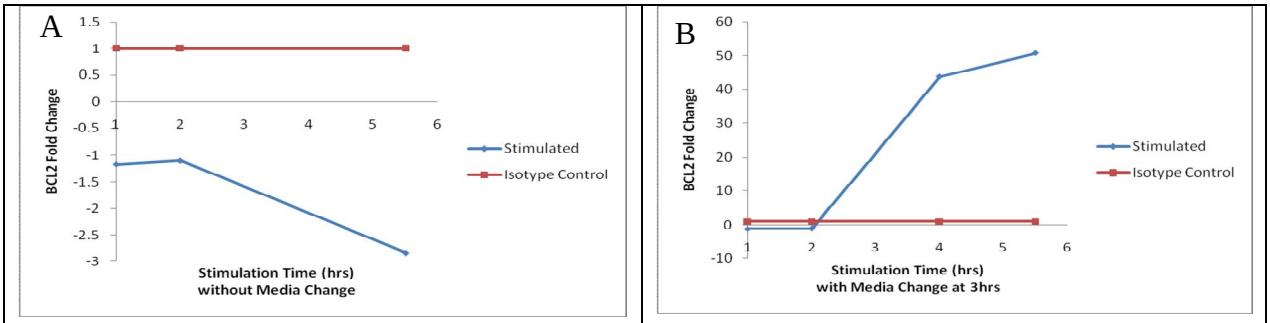


Figure 9.1: Graph to show fold changes of BCL2 in CLL clinical cells stimulated over increasing time periods

CLL cells were stimulated for 1hr, 2hrs, 4hrs and 5.5hrs. One sample was stimulated for 5.5hrs and had fresh media and stimulant added after 3hrs (B). Cells were also exposed to an isotype control for each time point. Western blots were used to monitor expression of BCL2 in cells; GAPDH was used as a loading control. The graphs shows changes in expression of BCL2 in stimulated cells compared with the isotype control. Cells stimulated for 5.5hrs with no media change (A) saw a fold change of >2 with a decrease in BCL2 in stimulated cells, suggesting that ERK had been phosphorylated and the MAPK pathway was activated. Between 1-2hrs BCL2 levels are lower than the isotype control indicating an increase in P-ERK and activation of the MAPK pathway. Changing the media after 3hrs on the 5.5hr stimulation (B) increases levels of BCL2. This suggests that P-ERK levels are down at these time points and that cells have not been stimulated.

Cell viability was good after 5.5hrs. The results showed that changing the media after 3hrs, affects stimulation. A rise in BCL2 levels (and therefore a decrease in P-ERK levels) suggests that changing the media results in vital environmental stimuli being washed out, and thus the MAPK pathway has not been activated. It is worth noting that proteins found to be differentially expressed may vary with differing stimulation times.

9.3 2D-PAGE of CLL Samples

9.3.1 Experimental

One hundred ml of blood was taken from each patient (See section 8.4) and PMBCs were stored at -80°C (see section 4.2.2). CLL cells were thawed (see section 4.1.3) and rested for 3hrs before being resuspended to a concentration of $5x10^6$cells/ml (see section 4.3) and split into six flasks. Three flasks contained the stimulant and three flasks contained the isotype control (see section 4.4). The same volume of cells was placed into each replicate flask. A minimum volume of 15ml of cells in a concentration of $5x10^6$cells/ml was required to extract enough protein for 2D-PAGE. Cells were incubated for 5.5hrs and the flasks were gently agitated every hour. Protein was extracted from each flask for 2D-PAGE (80%) and western blot (20%) (see section 4.5.1). The 2D-PAGE protein extracts were quantified, cleaned and 200µg from each replicate was separated individually by 2D-PAGE using pH4-7 IPG strips (see section 4.6). Gels were stained with coomassie protein stain and scanned (see section 4.8.1). PDQuest was used to highlight protein spots differentially expressed between the three stimulated and three unstimulated extracts. Protein spots found to be differentially expressed (2-fold difference with $p<0.05$ (see section 4.9.1)) were excised, digested and analysed by MALDI-TOF-MS (see sections 4.10 and 4.11).

9.3.2 CLL Sample 003 (see Table 8.1)

PDQuest highlighted 19 proteins spots (Figure 9.2) which were differentially expressed between stimulated and unstimulated cells by over 2-fold with $p<0.05$, two of these

differentially expressed spots are shown in detail in Figure 9.3 and Figure 9.4. All 19 protein spots were excisable and were analysed by MALDI-TOF-MS. Ten were identified with a significant score (score >66). Figure 9.5 shows the peptide mass fingerprint of protein spot 5 (see Figure 9.2) which was identified to significance (score 66, 52% sequence coverage) as interferon alpha 1/13 precursor. Table 9.1 shows 19 protein spots which were excised (see Figure 9.2). Spots which were not identified are shown in red. Proteins highlighted in blue were not identified to a significance (score >66). The increased (↑) or decreased (↓) expression of protein in stimulated cells is indicated. Also shown are the sequence coverage, estimated weights (KDa) and pI from the spot location in the 2D gel, and the theoretical weights and pI according the NCBInr database. Figure 9.6 shows histograms from PDQuest illustrating the expression of proteins identified to significance in the stimulated and unstimulated replicates.

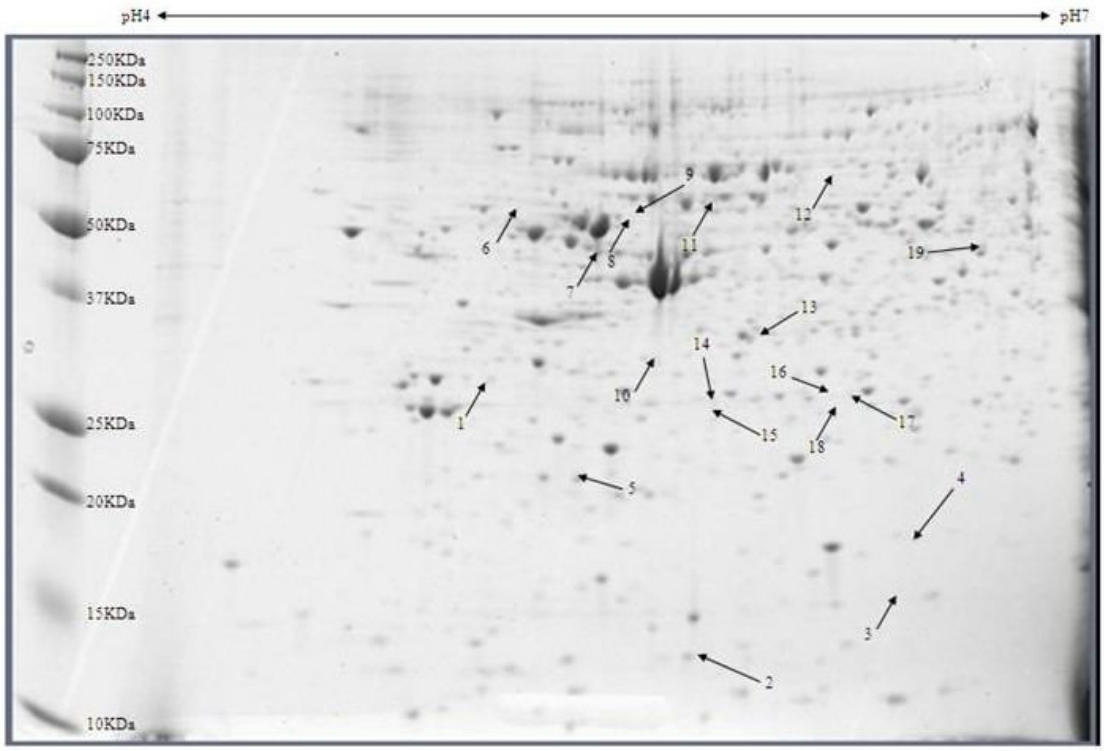


Figure 9.2: 2D gel showing differentially expressed proteins between stimulated and unstimulated cells from CLL clinical sample 003

The picture shows the 19 proteins spots (arrows) which were identified by PDQuest with differential expression between stimulated and unstimulated cells by over 2-fold ($p<0.05$).

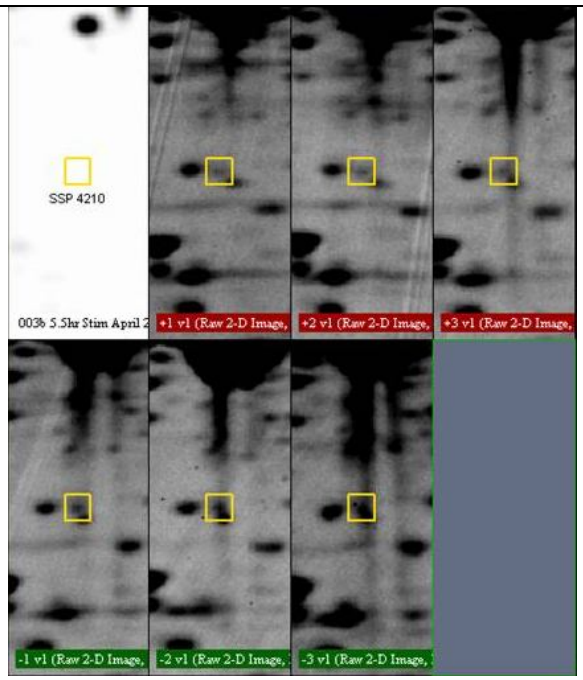


Figure 9.3: Gel pictures of protein spot 10 identified as Glyoxalase domain containing 4, which is down-regulated in stimulated CLL 003 cells

The spots highlighted in yellow on the gel are the spot of interest. The electronically generated master gel is shown top left. The three gels from stimulated cells are shown on the top (red) and the three gels from unstimulated cells are shown on the bottom (green). The protein spot is darker and larger in the three unstimulated gels (bottom). PDQuest identified this spot as having a >2-fold increase ($p<0.05$) in unstimulated cells.

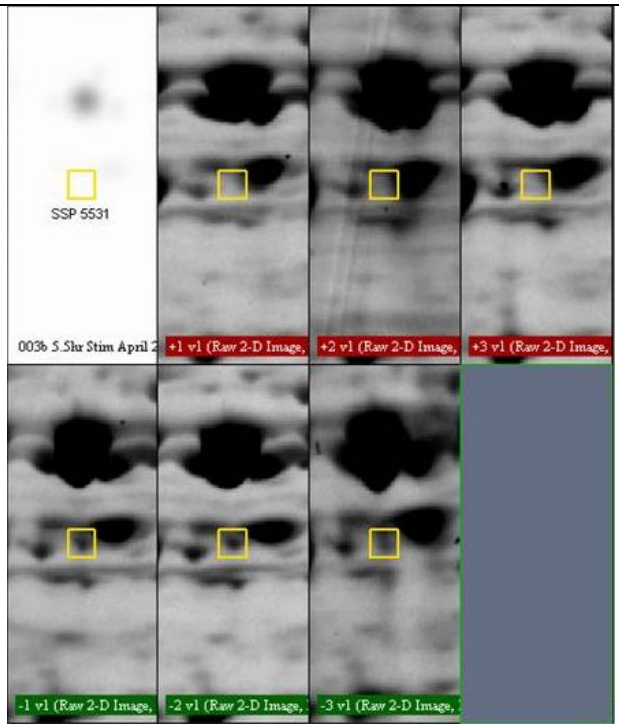


Figure 9.4: Gel pictures of protein spot 11 identified as DARS, which is down-regulated in stimulated CLL 003 cells

The spots highlighted in yellow on the gel are the spot of interest. The electronically generated master gel is shown top left. The three gels from stimulated cells are shown on the top (red) and the three gels from unstimulated cells are shown on the bottom (green). The protein spot is barely visible in the three stimulated gels (top). PDQuest identified this spot as having a >2-fold increase ($p<0.05$) in unstimulated cells.

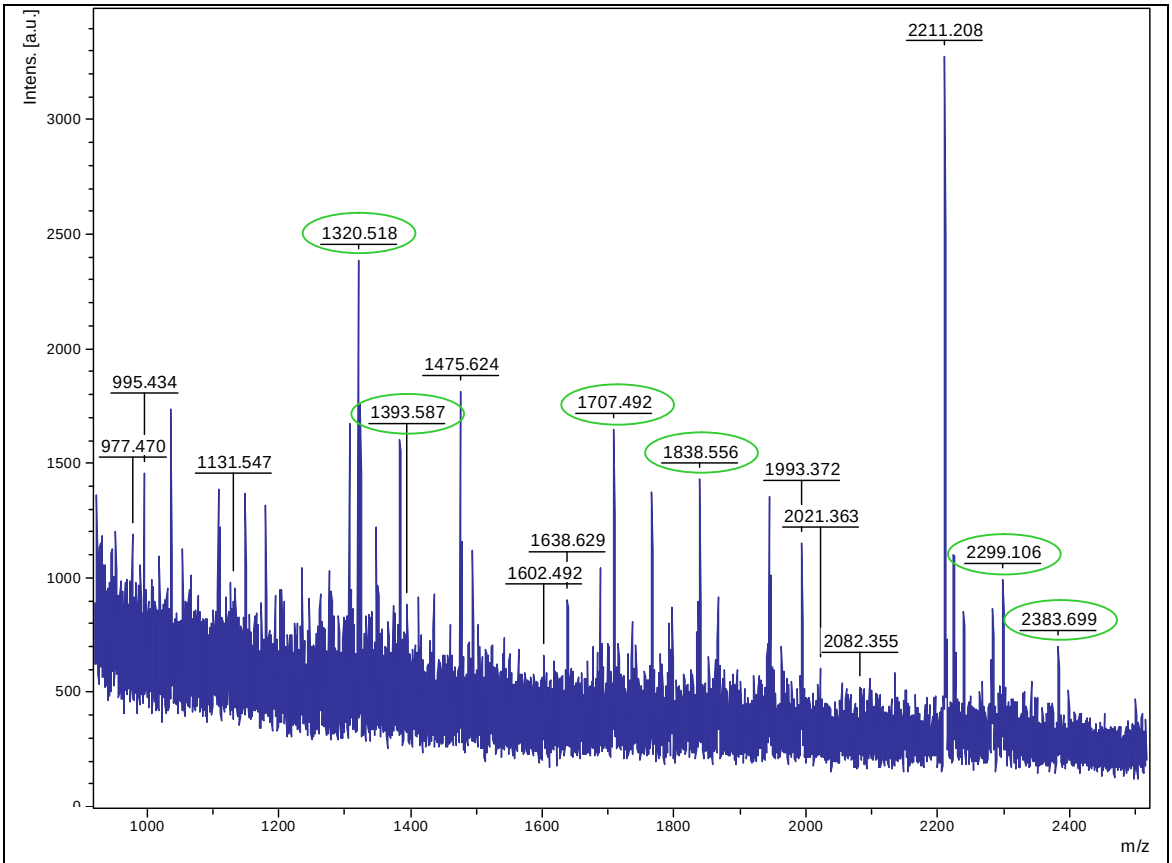
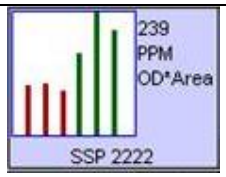


Figure 9.5: Peptide mass fingerprint of protein identified as interferon alpha 1/13 precursor

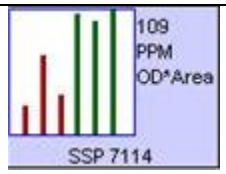
MALDI-TOF spectra of a protein found to be down-regulated in 003 CLL stimulated cells when compared to unstimulated cells. The protein was identified to significance by searching the NCBInr database using the Mascot search engine with a score of 66, as interferon alpha 1/13 precursor (21.7KDa). The peaks highlighted in green correspond to interferon alpha 1/13 precursor peptides. Many of the low abundant peaks (not shown) correspond to the protein of interest, whereas many of the higher abundant peaks are contaminants.

Table 9.1: Table showing identifications of differentially expressed proteins between stimulated and unstimulated cells from CLL clinical sample 003

Spot	PDQuest Spot Number	MW (KDa)	Estimated pI	Stimulated Cells	Protein Identified	Theoretical MW (KDa)	Theoretical pI	Score	Peptides Matched	Sequence Coverage
1	2222	27.3	~5.0	↓	Unnamed Protein Product	28.101	5.65	75	15	64%
2	5017	13	<6	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
3	7014	15.6	>6.5	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
4	7114	17.6	>6.5	↓	Fatty Acid Binding Protein 5	15.155	6.6	68	21	100%
5	3111	20.9	~5.5	↓	Interferon Alpha-1/13 Precursor	21.711	5.32	66	15	52%
6	2512	56.1	~5.0	↑	Tripartite Motif Protein (TRIM19 Zeta)	69.953	5.17	73	32	39%
7	3418	43.9	~5.5	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
8	4504	52.5	~5.5	↑	Astrotactin 2 (ASTN2 Protein)	49.557	5.53	73	28	56%
9	4517	54.5	~5.5	↓	Astrotactin 2 Isoform d	44.532	5.57	68	27	60%
10	4210	29.9	~5.5	↓	Glyoxalase Domain Containing 4	33.228	5.4	70	25	55%
11	5531	56.4	~6	↓	Aspartyl-tRNA Synthetase (DARS)	57.088	6.11	68	26	51%
12	6617	64.4	<6.5	↑	Erythrocyte Membrane Protein Band 4.1 (EPB41)	69.847	6.32	59	31	43%
13	6323	32.4	<6.5	↑	KNG1 Protein (Kininogen 1)	33.034	6.27	67	11	54%
14	5116	26.2	~6	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
15	5104	25.5	~6	↓	Phosphatidylinositol Transfer Protein Beta	22.23	5.89	70	21	64%
16	6218	26.6	<6.5	↓	Coenzyme Q7 Homolog Ubiquinone (Yeast)	22.839	6.45	61	23	62%
17	6216	26.6	<6.5	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
18	6116	24.9	<6.5	↓	TatD Dnase Containing 3 (TATDN3 Protein)	30.314	6.67	47	12	37%
19	8408	44.2	>6.5	↓	DnaJ (HSP40) Homolg Subfamily C Member 7	56.405	6.565	58	24	44%



Spot 1



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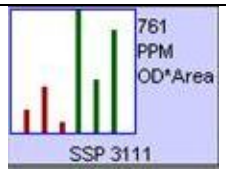

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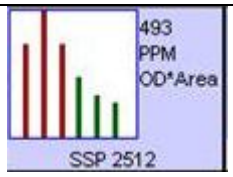

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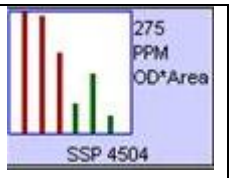

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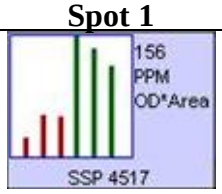
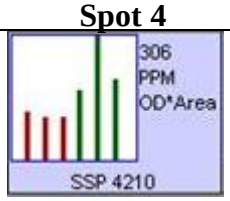
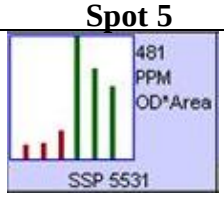


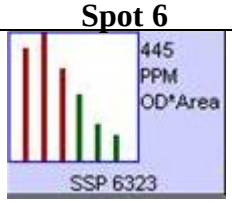
Spot 9



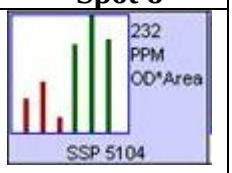
Spot 10



Spot 11



Spot 13



Spot 15

Figure 9.6: PDQuest histograms of the 10 proteins identified by MADLI-TOF-MS showing differential expression between stimulated and unstimulated cells in CLL clinical sample 003

The histograms show protein expression in the three test replicates (stimulated cells, red) and three control replicates (unstimulated cells, green). The protein spots had a >2-fold change in expression between stimulated and unstimulated cells ($p<0.05$) and were identified by MALDI-TOF-MS (Table 9.1). Protein spots 6, 8 and 13 were over expressed in stimulated cells and spots 1, 4, 5, 9, 10, 11 and 15 were over expressed in unstimulated cells.

9.3.3 CLL Sample 078 (see Table 8.1)

PDQuest highlighted 15 proteins spots which were differentially expressed between stimulated and unstimulated cells by over 2-fold with $p<0.05$ (Figure 9.7). All 15 protein spots were excisable and were analysed by MALDI-TOF-MS. Only two spots were identified to significance (score >66). Table 9.2 shows the 15 protein spots which were excised (see Figure 9.7). Spots which were not identified are shown in red. Proteins highlighted in blue were not identified to a significance (score >66). The increased (↑) or decreased (↓) expression of protein in stimulated cells is indicated. Also shown are the sequence coverage, estimated weights (KDa) and pI from the spot location in the 2D gel, and the theoretical weights and pI according the NCBInr

database. Figure 9.8 shows histograms from PDQuest illustrating the expression of proteins identified to significance in the stimulated and unstimulated replicates.

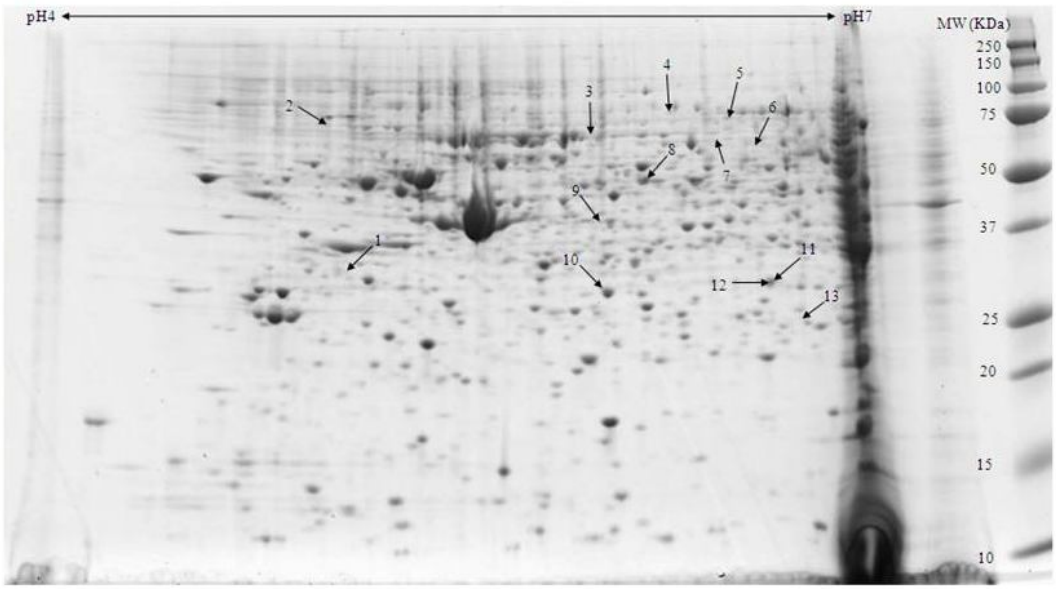


Figure 9.7: 2D gel showing differentially expressed proteins between stimulated and unstimulated cells from CLL clinical sample 078

The picture shows the 15 proteins spots (arrows) which were identified by PDQuest with differential expression between stimulated and unstimulated cells by over 2-fold ($p<0.05$).

Table 9.2: Table showing identifications of differentially expressed proteins between stimulated and unstimulated cells from CLL clinical sample 078

Spot Number	PDQuest Reference	MW (KDa)	Estimated pI	Stimulated Cells	Protein Identified	Theoretical MW (KDa)	Theoretical pI	Score	Peptides Matched	% Sequence Coverage
1	1323	30.3	5.5	↓	Adenylate Kinase Isoenzyme 5	22.07	5.38	70	15	72
2	1820	70.8	5.3	↑	Not Identified	N/A	N/A	N/A	N/A	N/A
3	5810	65.9	6	↑	RABGAP1L	67.96	5.36	36	11	20
4	6809	74.5	6.5	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
5	6707	71.9	6.5	↓	RAS Homolog Gene Family Member T1	74.17	6.31	44	14	23
6	7720	59.5	6.5	↓	HCG29146	53.44	6.15	34	16	37
7	6604	61.7	6.5	↓	Putative Ribonucleus III	62.84	6.75	60	11	25
8	6609	47.7	6.3	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
9	5517	38.1	6.2	↓	Unknown Protein	35.84	5.69	29	N/A	39
10	5313	27.7	6.2	↓	Programmed Cell Death 2	25.52	5.99	45	10	33
11	7315	28.7	6.7	↑	Kininogen 1	47.87	6.29	76	26	41
12	6206	28.4	6.7	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
13	8201	24.5	6.7	↓	Unknown Protein	27.77	6.12	41	13	50

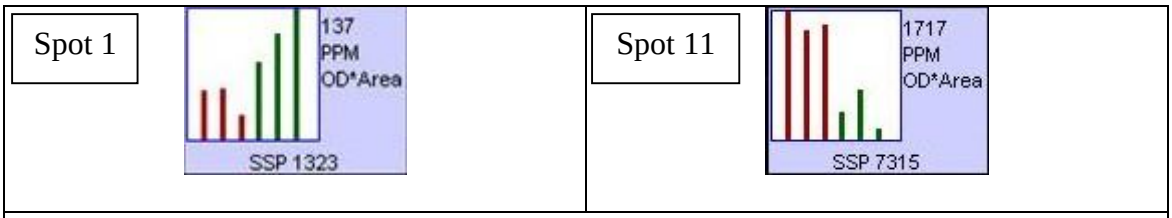


Figure 9.8: PDQuest histograms of the 2 proteins identified by MADLI-TOF-MS showing differential expression between stimulated and unstimulated cells in CLL clinical sample 078

The histograms show protein expression in the three test replicates (stimulated cells, red) and three control replicates (unstimulated cells, green). The protein spots had a >2-fold change in expression between stimulated and unstimulated cells ($p<0.05$) and were identified by MALDI-TOF-MS (Table 9.2). Protein spot 1 was over expressed in unstimulated cells and spot 11 was over expressed in stimulated cells.

9.3.4 CLL Sample 089 (see Table 7.1)

PDQuest highlighted 17 proteins spots which were differentially expressed between stimulated and unstimulated cells by over 2-fold with $p<0.05$ (Figure 9.9). Fourteen spots were excisable and were analysed by MALDI-TOF-MS. Eight spots were identified to significance (score >66). Table 9.3 shows the 17 protein spots which were differentially expressed (Figure 9.9). Spots highlighted in blue were not excised and spots which were not identified are shown in red. The increased (↑) or decreased (↓) expression of protein in stimulated cells is indicated. Also shown are the sequence coverage, estimated weights (KDa) and pI from the spot location in the 2D gel, and the theoretical weights and pI according the NCBInr database. Figure 9.10 shows histograms from PDQuest illustrating the expression of proteins identified to significance in the stimulated and unstimulated replicates.

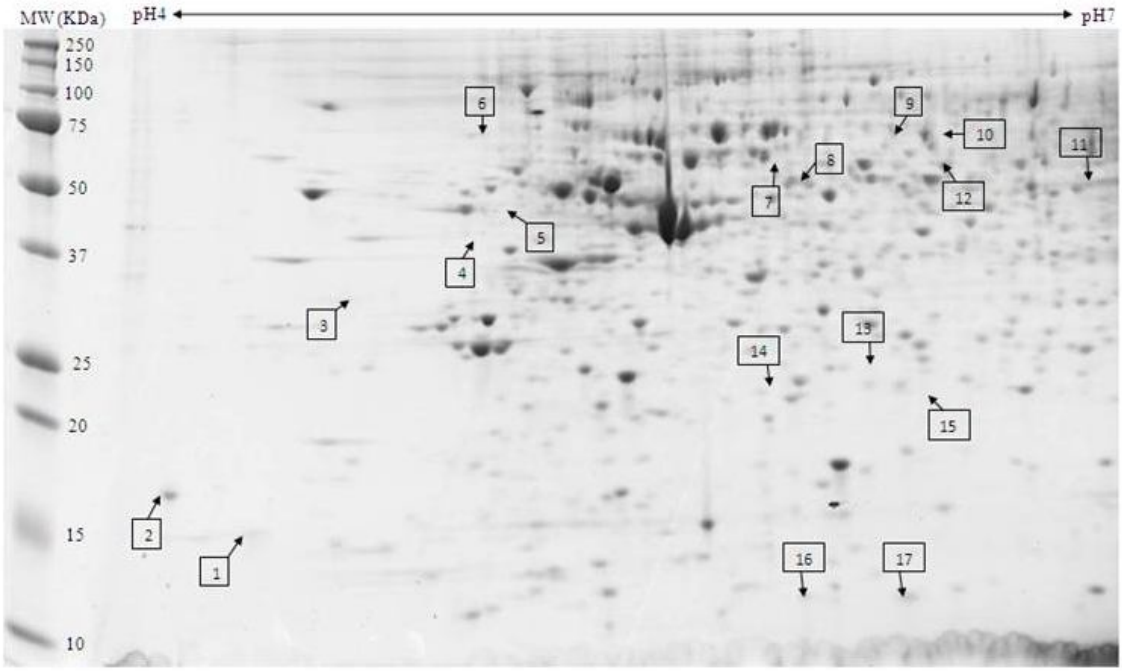


Figure 9.9: 2D gel showing differentially expressed proteins between stimulated and unstimulated cells from CLL clinical sample 089

The picture shows the 17 proteins spots (arrows) which were differentially expressed between stimulated and unstimulated cells by over 2-fold ($p<0.05$). Three of the protein spots (spots 3, 7 and 15) were not excised as they were either too faint, in a cluster of spots or in a streaky area of the gel.

Table 9.3: Table showing identifications of differentially expressed proteins between stimulated and unstimulated cells from CLL clinical sample 089

Spot	PDQuest Number	MW (KDa)	Estimated pI	Stimulated Cells	Protein Identified	Theoretical MW (KDa)	Theoretical pI	Score	Peptides Matched	Sequence Coverage
1	0020	14.8	<5.5	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
2	0118	16.5	<5.5	↓	Tropomyosin 4-anaplastic Lymphoma Kinase Fusion Protein	12.295	5.2	69	13	88%
3	0308	31	<5.5	↓	Unable to Excise	N/A	N/A	N/A	N/A	N/A
4	1515	38.2	<6	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
5	1615	43.8	<6	↓	FYVE-Ring Finger Protein Sakura (Rififylin)	39.687	5.51	69	27	52%
6	1712	67.8	<6	↑	Not Identified	N/A	N/A	N/A	N/A	N/A
7	5715	56.9	~6	↓	Unable to Excise	N/A	N/A	N/A	N/A	N/A
8	5713	51.2	~6	↑	Kazrin	46.738	6.31	74	18	44%
9	6716	68.6	~6	↑	Leukotriene A4 Hydrolase	69.109	5.87	143	32	57%
10	7725	67.7	<6.5	↑	RAS Homolog Gene Family Member T1	79.496	6.46	69	26	32%
11	8504	49.6	<6.5	↑	Kininogen 1	47.871	6.29	65	24	49%
12	6516	55.4	~6	↑	Protein Similar to CG 5439-PA	51.557	6.31	103	31	55%
13	6118	24.2	~6	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
14	4118	22.3	~6	↓	Thiopurine S-Methyltransferase	28.215	6.01	66	15	53%
15	6116	21.6	~6	↓	Unable to Excise	N/A	N/A	N/A	N/A	N/A
16	5008	11.7	~6	↓	Not Identified	N/A	N/A	N/A	N/A	N/A
17	6009	11.7	~6	↓	Not Identified	N/A	N/A	N/A	N/A	N/A

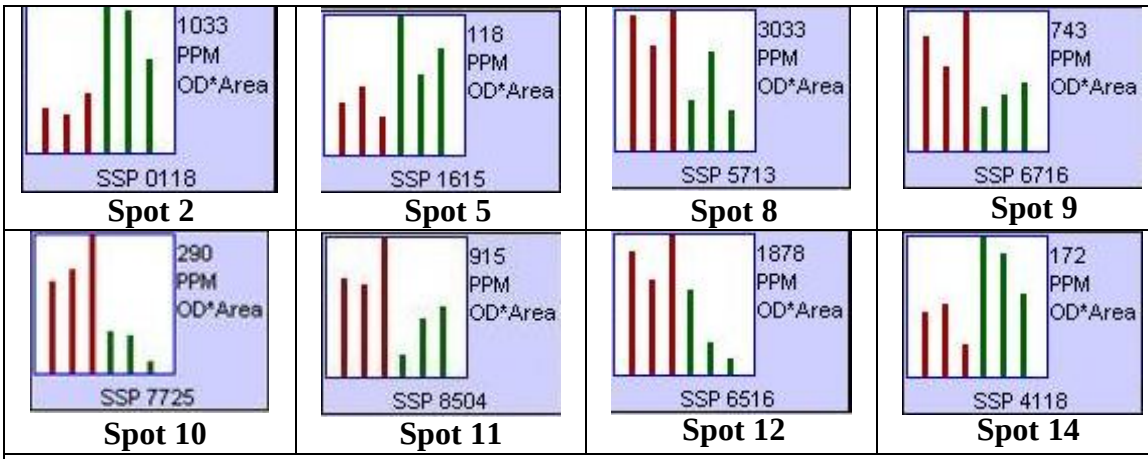


Figure 9.10: PDQuest histograms of the 8 proteins identified by MADLI-TOF-MS showing differential expression between stimulated and unstimulated cells in CLL clinical sample 089

The histograms show protein expression in the three test replicates (stimulated cells, red) and three control replicates (unstimulated cells, green). The protein spots had a >2-fold change in expression between stimulated and unstimulated cells ($p<0.05$) and were identified by MALDI-TOF-MS (Table 9.3). Protein spots 2, 5 and 14 were over expressed in unstimulated cells and spots 8, 9, 10, 11 and 12 were over expressed in stimulated cells.

9.4 Western Blot Confirmations

Western blots were used to confirm the change in expression of proteins identified by mass spectrometry (see section 9.3). Proteins were prioritised for western blot confirmation based on occurrence in the three biological replicates, availability of antibody, MASCOT score and % sequence coverage matched. Table 9.4 shows the identified proteins found to be differentially expressed between stimulated and unstimulated CLL clinical samples. Proteins highlighted in red had no western blot antibody commercially available. Proteins highlighted in blue were differentially expressed in more than one CLL clinical sample.

Table 9.4: Table to show differentially expressed proteins identified in all three CLL clinical samples

Sample	Spot	PDQuest Number	Stimulated Cells	Protein Identified	Theoretical MW (KDa)	Theoretical pI	Score	Sequence Coverage
089	9	6716	↑	Leukotriene A4 Hydrolase	69.1	5.9	143	57%
078	11	7315	↑	Kininogen 1	47.9	6.3	76	41%
078	1	1323	↓	Adenylate Kinase Isoenzyme 5	22.1	5.4	70	72%
089	2	0118	↓	Tropomyosin 4-anaplastic Lymphoma Kinase Fusion Protein	12.3	5.2	69	88%
003	4	7114	↓	Fatty Acid Binding Protein 5	15.2	6.6	68	100%
003	11	5531	↓	Aspartyl-tRNA Synthetase (DARS)	57.1	6.1	68	51%
003	13	6323	↑	KNG1 Protein (Kininogen 1)	33.0	6.3	67	54%
089	14	4118	↓	Thiopurine S-Methyltransferase	28.2	6.0	66	53%
089	11	8504	↑	Kininogen 1	47.9	6.3	65	49%
089	12	6516	↑	Protein Similar to CG 5439-PA	51.6	6.3	103	55%
003	1	2222	↓	Unnamed Protein Product	28.1	5.7	75	64%
089	8	5713	↑	Kazrin	46.7	6.3	74	44%
003	8	4504	↑	Astrotactin 2 (ASTN2 Protein)	49.6	5.5	73	56%
003	6	2512	↑	Tripartite Motif Protein (TRIM19 Zeta)	70.0	5.2	73	39%
003	15	5104	↓	Phosphatidylinositol Transfer Protein Beta	22.2	5.9	70	64%
003	10	4210	↓	Glyoxalase Domain Containing 4	33.2	5.4	70	55%
089	5	1615	↓	FYVE-Ring Finger Protein Sakura (Rififylin)	39.7	5.5	69	52%
089	10	7725	↑	RAS Homolog Gene Family Member T1	79.5	6.5	69	32%
003	9	4517	↓	Astrotactin 2 Isoform d	44.5	5.6	68	60%
003	5	3111	↓	Interferon Alpha-1/13 Precursor	21.7	5.3	66	52%

The proteins prioritised for western blot confirmation based on Table 9.4 were:

1. Kininogen 1
2. Leukotriene A4 hydrolase
3. Adenylate kinase isoenzyme 5
4. Tropomyosin 4-anaplastic lymphoma kinase fusion protein
5. Fatty acid binding protein 5
6. Aspartyl-tRNA synthetase
7. Thiopurine s-methyltransferase.

Two of the seven antibodies have been fully optimised; these are adenylate kinase isoenzyme 5 (see section 9.4.2) and aspartyl-tRNA synthetase (see section 9.4.3). Five of the antibodies (including kininogen - see section 9.4.1) have been purchased and assessed but still require further optimisation (data not shown).

9.4.1 Kininogen

Kininogen 1 was prioritised as it was found to be up-regulated by 2D-PAGE by >2-fold in all three clinical CLL biological replicates. Clinical sample 003 showed differential expression of the 33KDa form of kininogen, whilst samples 078 and 089 showed differential expressions of the 47.9KDa form. Samples were screened using a mouse monoclonal antibody to kininogen (Abcam) (see Table 4.1 and Table 4.2).

Unfortunately, after a series of optimisation steps the antibody was still not reliable enough to be able to measure an accurate fold change (Figure 9.11). Western blots were

performed using different blocking solution (BSA and milk), incubating with the primary antibody for different times (2-16hrs) and using different dilutions (1:150-1:1000) of the primary antibody. To be able to confirm kininogen expression in the samples a new antibody from a different supplier will need to be sourced.

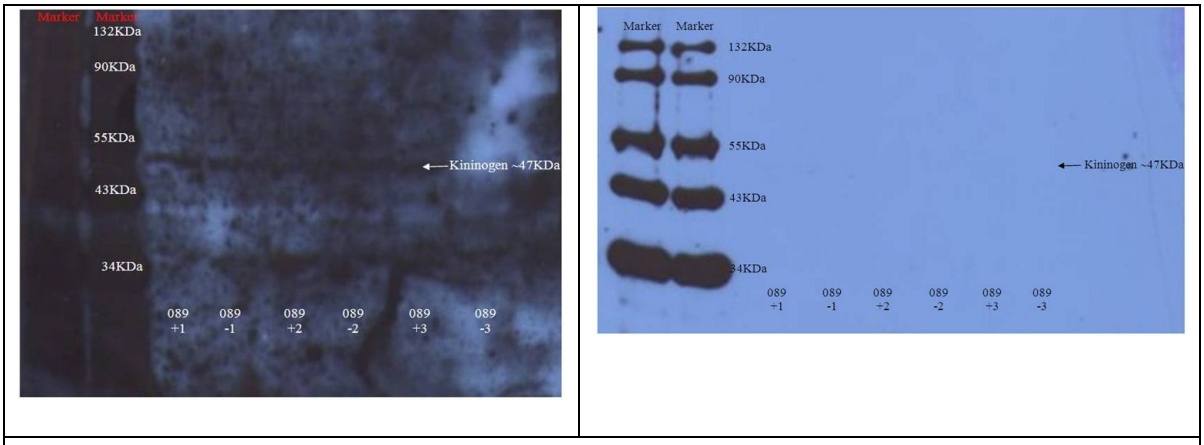


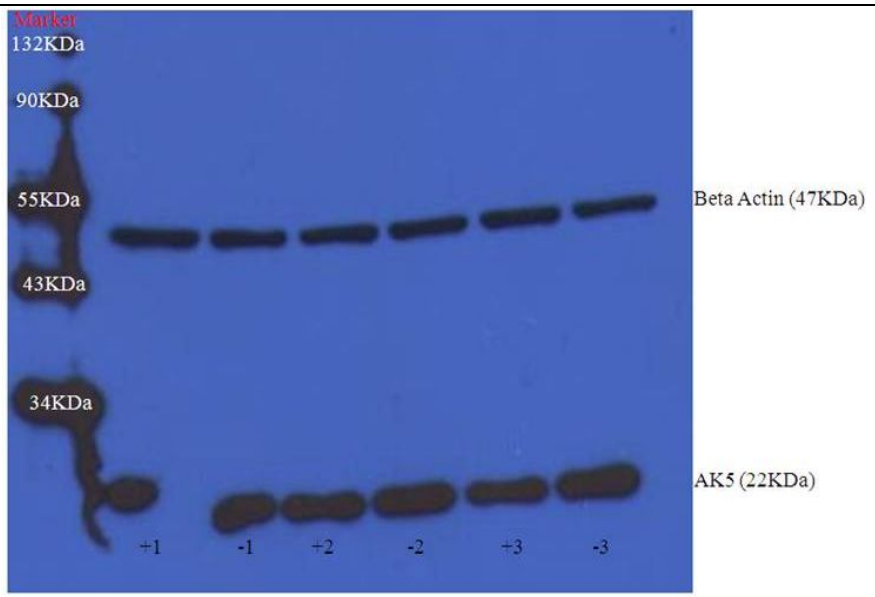
Figure 9.11: Western blots analysis of kininogen expression in clinical CLL sample 089

Western blots showing separate attempts at a kininogen screening assay. Western blots were tried using different blocking solution (BSA and milk) and incubating with the primary antibody for different times (2-16hrs) and different dilutions (1:150-1:1000). The blot on the left shows a membrane which had been incubated in BSA with an incubation of 16hrs with 1:1000 dilution of kininogen. The blot shows non-specific binding and the expected bands at ~47KDa can barely be seen and cannot be used to determine a fold change. The blot on the right shows a membrane which had been incubated in milk with an incubation of 2hrs with 1:150 dilution of kininogen. No protein bands are visible.

9.4.2 Adenylate Kinase Isoenzyme 5

Adenylate kinase isoenzyme 5 (AK5) was prioritised as it was found to be down-regulated by 2D-PAGE by >2-fold in clinical sample 078 with a score of 70 and a sequence coverage of 72%. A rabbit polyclonal antibody to AK5 (Abgent) (see Table 4.1 and Table 4.2) was used to screen clinical sample 078. The membrane was blocked in milk and incubated for 16hrs in the primary antibody in BSA.

Results show a decrease in AK5 in stimulated cells from clinical sample 078, which correlates with the results obtained from 2D-PAGE (see Table 9.2). Replicate 1 did not transfer well (possibly due to an air bubble between the gel and membrane) and could not be included in the assay; however the two technical replicates, on average, show a 1.6-fold decrease in expression of AK5 in stimulated cells (Figure 9.12).



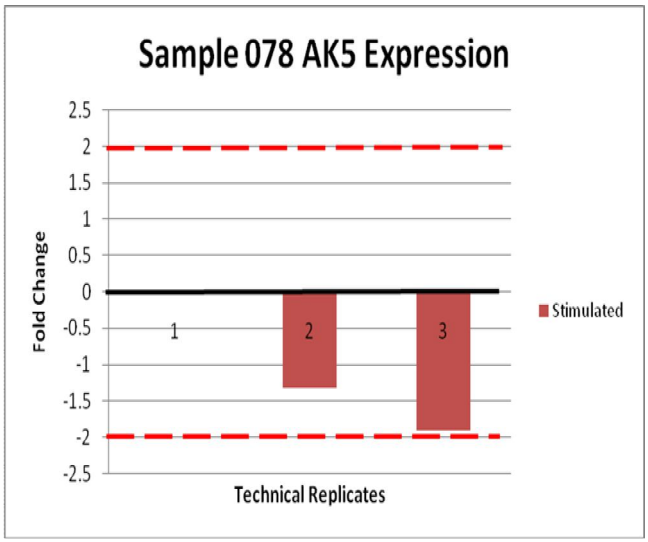


Figure 9.12: Western blot of adenylate kinase isoenzyme 5 (AK5) expression in clinical CLL sample 078

Sample 078 was screened for AK5 expression (22KDa) by Western blot. AK5 was found to be down-regulated by over 2-fold in stimulated cells by 2D-PAGE. Beta actin (47KDa) was used as a loading control. Replicate 1 on the Western blot did not transfer well so only two replicates could be included in the assay. The blot and graph show a decrease in AK5 expression in stimulated cells in replicate 2 and 3 by 1.3-fold and 1.9-fold respectively. The Western blot technique is not as sensitive as 2D-PAGE/PDQuest therefore it can be expected that fold changes may be a little lower.

AK5 was not identified as being differentially expressed by 2D-PAGE in clinical sample 089; however, a western blot was performed to assess AK5 expression in this

clinical sample. The three technical replicates show decreased expression (average 2.6-fold) of AK5 in stimulated cells (Figure 9.13). This correlates with the results from sample 078.

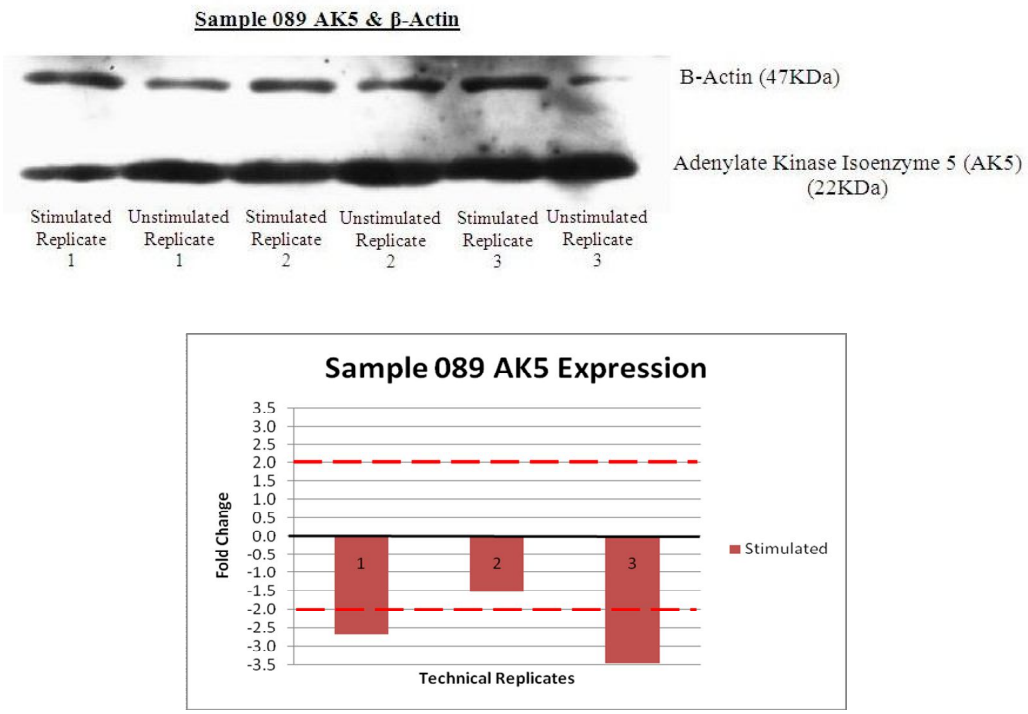


Figure 9.13: Western blot of adenylate kinase isoenzyme 5 (AK5) expression in clinical CLL sample 089

Sample 089 was screened for AK5 (22KDa) expression by western blot. Beta actin (47KDa) was used as a loaing control. The results show a down-regulation of AK5 in stimulated cells with an average fold change of 2.6.

9.4.3 Aspartyl-tRNA Synthetase

Aspartyl-tRNA synthetase (DARS) was found to be down-regulated by 2D-PAGE by >2-fold in clinical sample 003 with a score of 68 and sequence coverage of 51%. A mouse monoclonal antibody to DARS (Abova) (see Table 4.1 and Table 4.2) was used in a dilution of 1:200 in BSA for 16hrs. The membrane was blocked in milk. Results

show a decrease in DARS in stimulated CLL 003 cells, which correlates with the results obtained from 2D-PAGE (Table 9.1). The three technical replicates show an average 2.1-fold decrease in expression of DARS in stimulated cells (Figure 9.14).

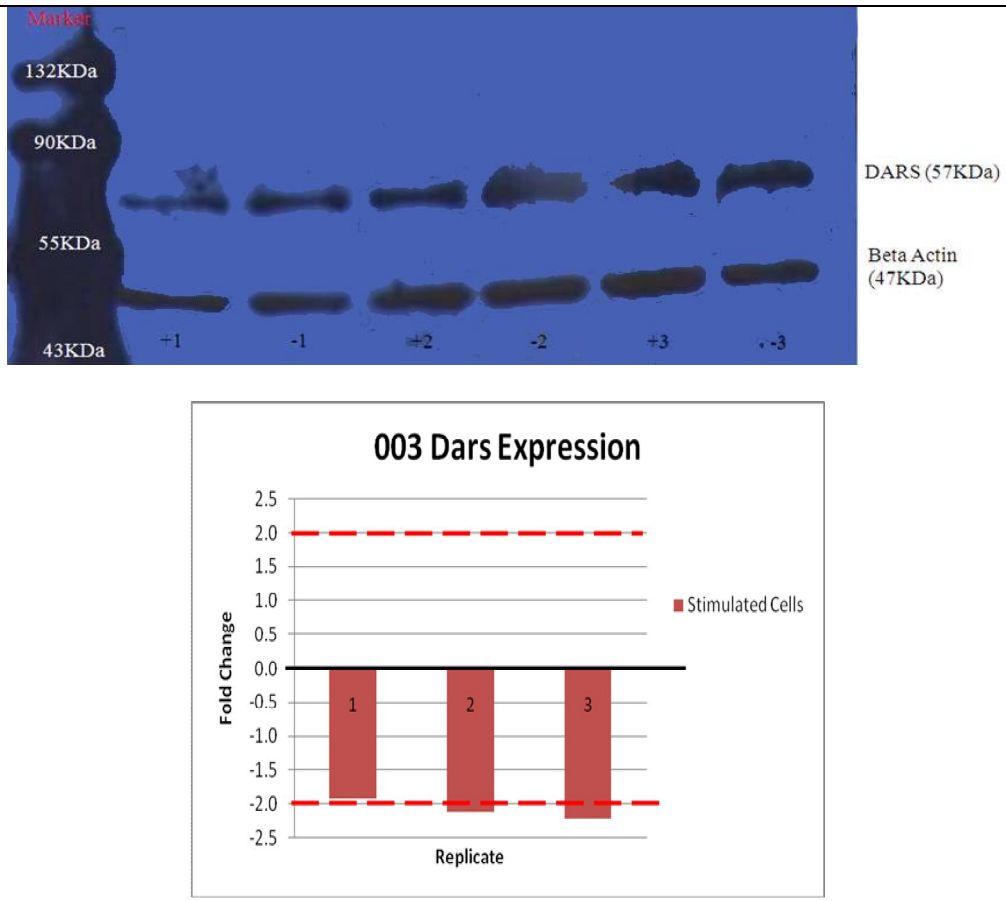


Figure 9.14: Western blot of aspartyl-tRNA synthetase (DARS) expression in clinical CLL sample 003

Sample 003 was screened for DARS (57KDa) expression by Western blot. Beta actin (47KDa) was used as a loading control. The results show a down-regulation of DARS in stimulated cells with an average fold change of 2.1. This correlates with the results from the 2D-PAGE experiment.

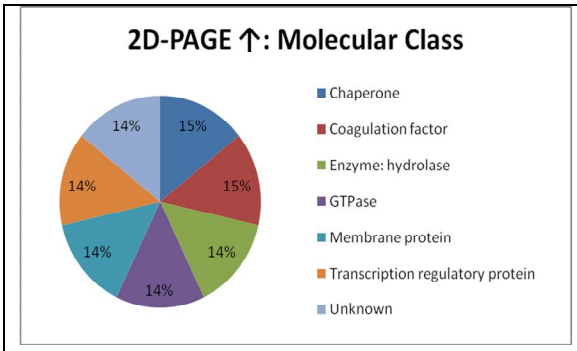
9.5 Summary

Table 9.5 shows all of the proteins found in the three clinical CLL 2D-PAGE experiments and the RAJI B-cell 2D-PAGE experiment (see sections 7.3 and 9.3). Twenty seven proteins have shown a change in expression between stimulated and unstimulated cells and have been identified by MALDI-TOF-MS. Of the 27 proteins, 7 were up-regulated and 20 were down-regulated.

The molecular class (Figure 9.15), molecular function (Figure 9.16), biological process (Figure 9.17) and location (Figure 9.18) of all the proteins found in the 2D-PAGE experiments were sourced from the Human Protein Reference Database (HPRD) (Mishra. 2006). The HPRD shows post-translational modifications, interaction networks and disease association for each protein in the human proteome. All the information in HPRD has been manually extracted from the literature by expert biologists who read, interpret and analyse the published data. The majority of the proteins found to be up-regulated were located in the cytoplasm and nucleus (Figure 9.18) and involved in protein metabolism (Figure 9.17). The majority of the proteins found to be down-regulated were enzymatic proteins (Figure 9.15) which were located in the cytoplasm (Figure 9.18) and involved in metabolism energy pathways (Figure 9.17).

Table 9.5: Table showing differentially expressed proteins between stimulated and unstimulated cells in all 2D-PAGE experiments

Sample	Protein	Alternate Name	Stimulated Cells
078	Adenylate Kinase Isoenzyme 5	AK1	↓
003	Aspartyl-tRNA Synthetase	DARS	↓
003	Astrotactin 2 Isoform d	ASTN2d	↓
RAJI	Chaperonin Containing TCP Subunit 8 (Theta)	CCT-theta	↓
RAJI	Chaperonin Containing TCP Subunit 8 (Theta)	CCT-theta	↓
RAJI	Chimerin (Chimaerin) 1	Rho GTPase activating protein 2	↓
003	Coenzyme Q7 Homolog Ubiquinone	COQ7	↓
003	Fatty Acid Binding Protein 5	EFABP	↓
089	FYVE-Ring Finger Protein Sakura	Rififylin	↓
RAJI	Glutaminyl-tRNA Synthetase, Isoform CRA-a	QARS	↓
003	Glyoxalase Domain Containing 4	N/A	↓
RAJI	Inner Membrane Protein, Mitochondrial Isoform 1	Oxidase cytochrome c assembly 1 like	↓
003	Interferon Alpha-1/13 Precursor	N/A	↓
RAJI	Keratin 14 - Epidermolysis Bullosa Simplex, Dailing-Meara, Koeber	KRT14	↓
RAJI	Nuclear Receptor Binding Protein 2	NRBP2	↓
003	Phosphatidylinositol Transfer Protein Beta	PITP beta	↓
RAJI	SARM 1 Protein	Sterile alpha and TIR motif containing 1	↓
RAJI	SARM 1 Protein	Sterile alpha and TIR motif containing 1	↓
089	Thiopurine S-Methyltransferase	TPMT	↓
089	Tropomyosin 4-anaplastic Lymphoma Kinase Fusion Protein	TPM4-ALK	↓
003	Astrotactin 2	ASTN2	↑
RAJI	Heat Shock 70KDa Protein	HSP1	↑
089	Kazrin	N/A	↑
78, 089, 003	Kininogen 1	KNG1	↑
089	Leukotriene A4 Hydrolase	N/A	↑
089	RAS Homolog Gene Family Member T1	RhoT	↑
003	Tripartite Motif Protein (TRIM19 Zeta)	Probable transcription factor, PML (Promyelocytic leukemia protein)	↑



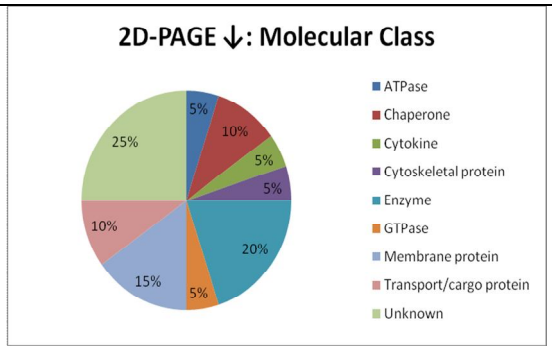
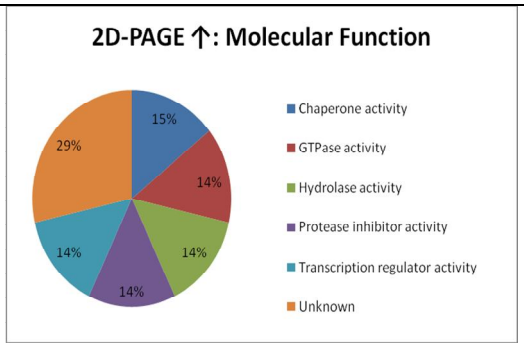


Figure 9.15: Pie charts showing molecular class of proteins found to be differentially expressed using 2D-PAGE.

The chart shows the molecular classes of the 7 up-regulated proteins (left) and the 20 down-regulated proteins (right). Proteins which were up-regulated had an equal distribution of protein molecular class; however the majority of the down-regulated proteins are enzymatic proteins.



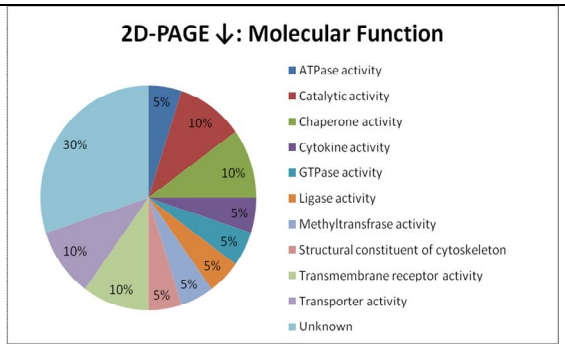
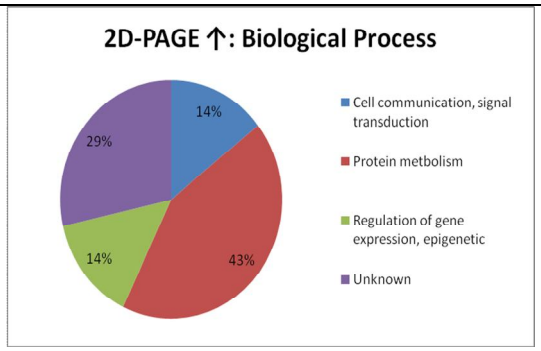


Figure 9.16: Pie charts showing molecular function of proteins found to be differentially expressed using 2D-PAGE.

The chart shows the molecular functions of the 7 up-regulated proteins (left) and the 20 down-regulated proteins (right). Proteins which were up-regulated had an equal distribution of protein molecular functions; however, the majority of the down-regulated proteins have unknown functions.



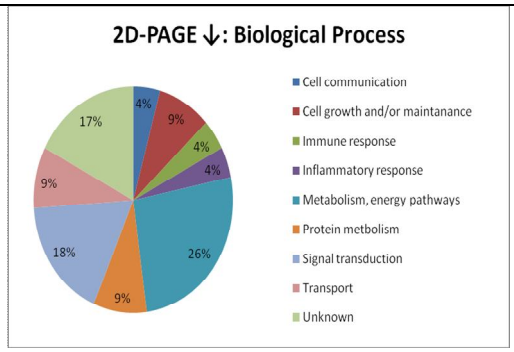
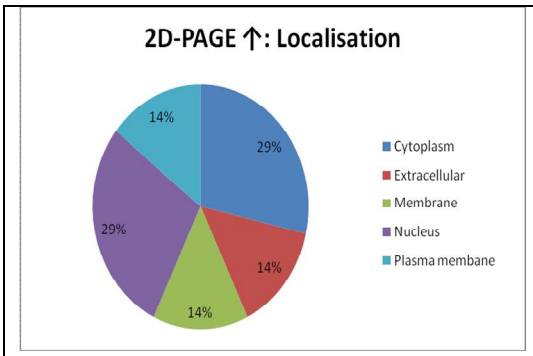


Figure 9.17: Pie charts showing biological process of proteins found to be differentially expressed using 2D-PAGE.

The chart shows the biological processes of the 7 up-regulated proteins (left) and the 20 down-regulated proteins (right). The majority of up-regulated proteins are involved in protein metabolism and the majority of down-regulated proteins are involved in metabolism energy pathways.



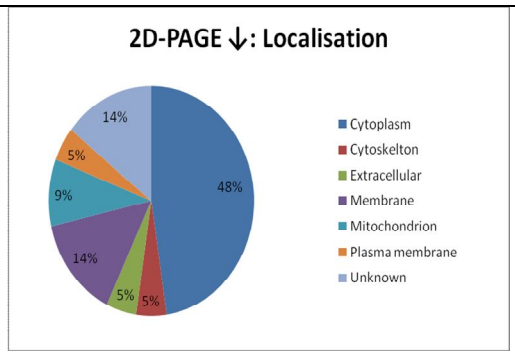


Figure 9.18: Pie charts showing localisation of proteins found to be differentially expressed using 2D-PAGE.

The chart shows the localisations of the 7 up-regulated proteins (left) and the 20 down-regulated proteins (right). The majority of up-regulated proteins are cytoplasmic proteins and nucleic proteins, and the majority of down-regulated proteins are cytoplasmic.

Two of the 27 proteins which had changed in expression between stimulated and unstimulated cells from clinical CLL samples were confirmed by western blot. These were AK5 (see section 9.4.2) and DARS (see section 9.4.3).

AK5 was found to be down-regulated in stimulated cells from clinical samples 078 and 089 (see Figure 9.8 and section 9.4.2). AK5 is the cytosolic form of adenylate kinase which plays an important role in homeostasis of cellular energy (Stanojevic *et al*. 2008). It is a phosphotransferase enzyme which catalyses the reaction:

$$2ADP \leftrightarrow ATP + AMP$$

Where:

ADP = adenosine diphosphate

ATP = adenosine triphosphate

AMP = adenosine monophosphate

AK5 was found to be down-regulated by 2D-PAGE by >2-fold in stimulated cells from clinical sample 078. However, expression of AK5 in clinical sample 089 was also shown to be down-regulated in stimulated cells by western blot (see section 9.4.2), despite the change in expression not being shown by 2D-PAGE. This shows that a change in expression of a protein can be missed by 2D-PAGE, and a protein that has shown an expression change in only one clinical CLL sample may still be relevant and further samples would need screening by western blot.

DARS was found to be down-regulated in stimulated cells from clinical sample 003 (see Figure 9.6 and section 9.4.3). DARS is part of the aminoacyl-tRNA synthetases which are essential for the translation of mRNA into proteins (Hausmann *et al*. 2008). Studies have shown that removal of fatty acids alters the structure and function of aminoacyl-tRNA synthetase complexes (Sivaram *et al*. 1990).

One protein, kininogen, was shown to be up-regulated by >2-fold in stimulated cells in all three clinical CLL samples by 2D-PAGE (see Table 9.4). Kininogen is the critical regulator of the plasma kallikrein-kinin system (Schmaier. 2002) which plays roles in inflammation, blood pressure control, coagulation and pain. It has also been suggested that kinins (a family of octato-decapeptides arisen from kininogen) influence cells of the immune system such as T-cells and B-cells, by modulating the activation, proliferation, migration and effector functions of these cells (Schulze-Topphoff *et al*. 2008). No references to CLL have been published.

Other proteins which showed a >2-fold change in expression by 2D-PAGE and were prioritised (based on Table 9.4) but not yet confirmed by western blot were leukotriene A4 hydrolase, tropomyosin 4-anaplastic lymphoma kinase fusion protein, fatty acid binding protein 5 and thiopurine s-methyltransferase.

Leukotriene A4 hydrolase was found to be up-regulated in stimulated cells from clinical sample 089 (Table 9.4). Leukotriene A4 hydrolase catalyses the final step in the biosynthesis of leukotriene B4 (Haeggstrom. 2000). Leukotriene B4 had been shown to interact with the B-cell differentiation process, and amplify activation (Yamaoka *et al*. 1989), suggesting that cells which show high levels of leukotriene A4 hydrolase could be hyper-active to stimulation.

Tropomyosin 4-Anaplastic Lymphoma Kinase Fusion Protein was found to be down-regulated in stimulated cells from clinical sample 089 (Table 9.4). Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase identified in a chromosomal translocation associated with some anaplastic large cell lymphomas. In cancers, all

translocations involving ALK produce fusion proteins with constitutive tyrosine kinase activity (Chiarle *et al*. 2008). Fusion proteins of ALK are oncogenic and strongly linked to out-of control cell proliferation (Morris *et al*. 1994) suggesting that the cells that show an increase in tropomyosin-ALK could show pro-survival characteristics. However, tropomyosin-ALK was found to be down-regulated in stimulated cells.

Fatty Acid Binding Protein 5 was found to be down-regulated in stimulated cells from clinical sample 003 (Table 9.4). The exact physiological role of fatty acid binding proteins is still unknown (Krieg *et al*. 1993), however, studies have indicated that fatty acid binding proteins may stabilise leukotriene A4 hydrolase in leukaemia cells and are important in trans-cellular biosynthesis where leukotriene A4 hydrolase is transferred from one cell to another (Zimmer *et al*. 2004). This suggests that cells with a decrease in expression of fatty acid binding proteins may be unable to stabilise leukotriene A4 hydrolase. Leukotriene A4 hydrolase was found to be up-regulated in stimulated cells in this study.

Thiopurine s-methyltransferase was found to be down-regulated in stimulated cells from clinical sample 089 (Table 9.4). Deficiency in thiopurine s-methyltransferase (TPMT) is linked to toxicity and lack of response in cancer treatment as it is the main enzyme responsible for inactivating the toxic products of the immunosuppressant azathioprine (A. Ansari. 2002) suggesting that these cells could show therapy resistance.

Seven proteins which showed a >2-fold change in expression by 2D-PAGE and which may be of potential interest were TRIM19 zeta, astrotactin 2, glyoxalase domain containing 4, phosphatidylinositol transfer protein beta, rififylin, kazrin and RAS

homolog gene family member T1. However, a commercially available antibody was not available for these proteins and western blot confirmation was not possible (see Table 9.4).

TRIM19 zeta was found to be up-regulated in stimulated cells from clinical sample 003 (Table 9.4). TRIM19 zeta is also known as promyelocytic leukaemia protein. It is a tumour suppressor induced by interferons, involved in multiple apoptosis pathways. Studies have shown that cells that are deficient in promyelocytic leukaemia protein are resistant to apoptosis (Wu *et al*. 2002), while cells over expressing promyelocytic leukaemia protein activate caspase 8 (Kuwayama *et al*. 2008). Caspase 8 is involved in the initiation of apoptosis; however, it can also initiate signals that protect the cell from autophagic or necrotic cell death (Maelfait *et al*. 2008) suggesting that these cells could show pro-survival characteristics.

Astrotactin 2 was found to be up-regulated and astrotactin 2 isoform d was found to be down-regulated in stimulated cells from clinical sample 003 (Table 9.4). Astrotactin is a neuron cell surface antigen, named because of its role in mediating neuron-astroglial contacts. It binds granule neurons to astroglia (cells found in the brain and spinal cord) and maintains the cerebellar astroglial form (Edmondson *et al*. 1988). No references to oncology or haematology have been published.

Glyoxalase domain containing 4 was found to be down-regulated in stimulated cells from clinical sample 003 (Table 9.4). The glyoxalase system catalyses the conversion of methylglyoxal to D-lactic acid via the intermediate S-D-lactoylglutathione. Addition of S-D-lactoylglutathione to HL60 leukaemia cells in culture induced growth arrest and

toxicity (Thornalley *et al*. 1988), suggesting that a lack of S-D-lactoylglutathione could promote cell survival.

Phosphatidylinositol transfer protein beta was found to be down-regulated in stimulated cells from clinical sample 003 (Table 9.4). Phosphatidylinositol transfer protein beta regulates pathways involved in proliferation, apoptosis and cell survival (Snoek. 2004). Studies have shown that an over expression of phosphatidylinositol transfer protein beta results in the inhibition of anti-apoptotic pathways, stimulating apoptosis (Schenning et al. 2007), suggesting that down-regulation may result in anti-apoptotic characteristics.

Rififylin was found to be down-regulated in stimulated cells from clinical sample 089 (Table 9.4). Rififylin is a novel protein characterised in 2004 which is reported to be involved in endocytosis (a fundamental cellular process, whereby extracellular material as well as plasma membrane proteins and lipids are internalised and transported to various intracellular compartments) (Coumailleau et al. 2004). Studies show that when rififylin is over-expressed in HeLa cells, it localized to and induced the condensation of endosomal structures (Coumailleau et al. 2004).

Kazrin was found to be up-regulated in stimulated cells from clinical sample 089 (Table 9.4). Karin is a novel protein reported in 2004 and is described as a periplakin interacting protein (Groot *et al*. 2004). Periplakin is a 195KDa membrane associated protein found in keratinocytes (Ruhrberg *et al*. 1997) which suggests that kazrin may have other functions which are not yet reported.

RAS homolog gene family member T1 was found to be up-regulated in stimulated cells from clinical sample 089 (Table 9.4). RAS homolog gene family member T1 (RhoT) is

a homolog of Tc10 (Abe *et al*. 2003) which regulates cell signaling of the actin cytoskeleton and processes associated with cell growth (Murphy *et al*. 1999) suggesting that an over-expression could result in irregular cellular signaling.

Chapter 10 - The Proteomic Analysis of CLL Clinical Samples Using Antibody Microarrays

Aims:

- To identify changes in protein expression between stimulated and unstimulated cells in a clinical CLL sample selected in section 8.4 using The Panorama® Antibody Microarray – XPRESS Profiler725

The Proteomic Analysis of CLL Clinical Samples Using Antibody Microarrays

The Panorama® Antibody Microarray – XPRESS Profiler725 (Sigma-Aldrich) was used to determine differentially expressed proteins between stimulated and unstimulated CLL cells (see section 3.3). Clinical CLL sample 003 was used in this experiment (see 8.4.1). The XPRESS Profiler725 was chosen because of the large number of antibodies represented and also the selection of antibodies representing proteins from a variety of different biological pathways (see section 4.12). Results obtained from the antibody array experiment should be complementary to the results obtained from 2D-PAGE/MS.

Ideally three technical replicates would be run, including one where the fluorescent dyes labelled on the test and control samples are swapped around. However, since 2D-PAGE/MS data was available for the same clinical sample and the antibody microarray slides were expensive, only one microarray was used. This would serve as a preliminary screening method and any proteins shown to be of interest could be verified by a more economical method such as western blotting. All the antibodies spotted on the microarray were available from Sigma-Aldrich for western blotting confirmation.

10.1 Experimental

CLL sample 003 was used for this experiment because;

- It showed differential expression of numerous proteins in the 2D-PAGE experiment (9.3.2)
- There was a sufficient number of cells left in the sample for further analysis
- A large quantity of protein could be extracted due to the high WBC of the patient (antibody microarrays require a minimum of 1mg of protein from each test sample)

Approximately $2.4x10^8$ cells were stimulated or exposed to an isotype control in a concentration of $5x10^6$cells/ml for 5.5hrs (see section 4.4) and the protocol as described in section 4.12 was used to extract and quantify protein, and analyse the Panorama XPRESS Profiler725 antibody microarray.

One ml of protein at a concentration of 1µg/µl was needed for the array. Stimulated and unstimulated extracts were quantified as 4.9µg/µl and 3.2µg/µl respectively. Stimulated cells were labelled with a Cy5 dye (red) and unstimulated cells were labelled with a Cy3 dye (green). A dye to protein ratio of >2 was needed for the array (see section 4.12.4). Stimulated and unstimulated extracts were labelled with a ratio of 3.5 and 4.86 respectively. Fifty micrograms of each labelled protein extract was loaded onto the array.

10.2 Results

Of the 725 antibodies on the array, 686 antibody spots were classed as;

- Less than 3% saturated pixels
- Not flagged as bad or absent
- Uniform intensity
- Uniform background
- Detectable above background

Of the 686 spots, 70 showed a ≥2-fold change in expression in the stimulated (test) sample when compared to the unstimulated (control) sample (see Figure 10.1). Twenty eight proteins were up-regulated (see Table 10.1) and 42 were down-regulated (Table 10.2) in the stimulated cells. Protein spots which appeared red were up-regulated in stimulated cells and protein spots which appeared green were down-regulated in stimulated cells (Figure 10.2).

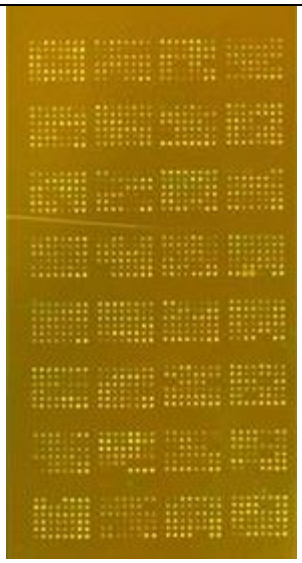


Figure 10.1: Panorama® Antibody Microarray – XPRESS Profiler725 slide image with labelled protein extracts from stimulated and unstimulated cells from clinical CLL 003 sample

On the Panorama® Antibody Microarray – XPRESS Profiler725 (Sigma-Aldrich), 686 antibodies were classed in the experiment. Of the 686, 70 proteins were differentially expressed between stimulated and unstimulated 003 CLL cells.

Table 10.1: Table showing proteins identified using the Panorama® Antibody Microarray – XPRESS Profiler725 which were up-regulated in stimulated cells from clinical sample 003

Protein	Antibody	Alternate Name	Lowess M Log Ratio	Fold Change (2^n)
PUMA bbc3	#P4818	BCL2 binding component 2	3.067	8.4
TRAIL	#T9191	TNF-related apoptosis inducing ligand	2.922	7.6
PUMA bbc3	#P4743	BCL2 binding component 2	2.664	6.3
Siah2	#S7945	Seven in absentia homlog 2	2.483	5.6
MyD88	#M9934	Myeloid differentiation primary response protein	2.23	4.7
P53R2	#P4993	Ribonucleotide reductase M2 B	1.69	3.2
CtBP1	#C8741	C-terminal binding protein 1	1.637	3.1
Chk1	#C9358	Cell cycle checkpoint kinase	1.613	3.1
ILP2	#I4782	Baculoviral IAP repeat containing 8	1.608	3.0
TSG101	#T5826	Tumour susceptibility gene 101	1.585	3.0
ARP3	#A5979	Actin related protein 3	1.579	3.0
iASPP	#A4605	Inhibitor of apoptosis stimulating protein of p53	1.55	2.9
Bim	#B7929	BCL2-like 11 (apopotosis facilitator)	1.512	2.9
Nuf2	#N5287	Cell division cycle associated 1	1.464	2.8
p115 TAP	#P3118	Vesicle docking protein p115	1.42	2.7
bCOP	#G6160	Coatomer protein complex, subunit beta	1.42	2.7
Caspase 13	#C8854	N/A	1.305	2.5
Ubiquitin Cterminal Hydrolase L1	#U5258	Ubiquitin carboxyl terminal hydrolase isozyme L1	1.175	2.3
Cortactin	#C6987	Oncogen EMS1	1.163	2.2
FXR2	#F1554	Fragile X mental retardation syndrome related protein 2	1.15	2.2
FADD	#F8053	FAS associating protein with death domain	1.146	2.2

Sir2	#85313	Sirtuin 2	1.141	2.2
TRAIL	#T3067	TNF-related apoptosis inducing ligand	1.089	2.1
Spectrin a & b	#S3396	Spectrin	1.077	2.1
p53DINP1SIP	#P4868	p53-Dependent damage-inducible nuclear protein 1	1.074	2.1
dCatenin NPRAP	#C4864	Catenin (cadherin associated protein) delta 2 (neural plakophilin related arm repeat protein)	1.048	2.1
Glutamate receptor NMDAR 2a	#G9038	Glutamate receptor ionotropic, N-methyl D-aspartate subunit 2A	1.016	2.0
Centrin	#C7736	Centrin 1	1.003	2.0

Table 10.2: Table showing proteins identified using the Panorama® Antibody Microarray – XPRESS Profiler725 which were down-regulated in stimulated cells from clinical sample 003

Protein	Antibody	Alternate Name	Lowess M Log Ratio	Fold Change (2^n)
BACE 1	#B0806	Beta site App cleaving enzyme 1	-2.754	-6.7
PIASx	#P9498	Protein inhibitor of activated STAT X	-2.393	-5.3
Rad17	#R8029	RAD1 homolog	-2.253	-4.8
hBRM hSNF2a	#H9787	SWI/SNF related matrix associated actin dependent regulator of chromatin a2	-2.053	-4.1
Smad4	#S3934	Deletion target in pancreatic carcinoma 4	-1.97	-3.9
SLIPR MAGI3	#S4191	Membrane associated guanylate kinase related 3 (scaffolding protein)	-1.832	-3.6
Tropomyosin	#T9283	TPM1	-1.78	-3.4
RhoE	#R6153	Ras homolog gene family, member E	-1.755	-3.4
BACH1	#B1310	Transcription regulator protein BACH1	-1.686	-3.2
RALAR	#R8529	Ras related protein Ral A	-1.639	-3.1
Rab7	#R8779	Ras associated protein Rab7	-1.634	-3.1
Zyxin	#Z0377	P83	-1.81	-3.5
Importin a3	#I9783	Karyopherin alpha3	-1.56	-2.9
SKM1	#S9568	Sodium channel, voltage gated, type IV, alpha subunit	-1.55	-2.9
Protein Kinase C PKC	#P5704	Protein Kinase C	-1.522	-2.9
Caldesmon	#C6542	Caldesmon 1	-1.386	-2.6
E6AP	#E8655	Ubiquitin protein ligase E3A	-1.374	-2.6
Paxillin	#P1093	FLJ16691	-1.367	-2.6
hABH1	#A8103	Alpha/beta hydrolase domain containing protein 1	-1.354	-2.6
Growth Factor Independence1	#G6670	Zinc finger protein Gfi 1	-1.336	-2.5
Slipr MAGI3	#S1190	Membrane associated guanylate kinase related 3 (scaffolding protein)	-1.331	-2.5

AOP1	#A7674	Peroxiredoxin 3 (antioxidant protein 1)	-1.324	-2.5
FBI1 POKEMON	#F9429	Zinc finger and BTB domain containing 7	-1.322	-2.5
NBS1	#N3037	Nibrin	-1.276	-2.4
MRP2	#M3692	ATP binding cassette, sub family C, member 2 (Multidrug resistance associated protein 2)	-1.267	-2.4
p34cdc2	#C3085	CDC2	-1.263	-2.4
LIS1	#L7391	Nude like protein	-1.238	-2.4
Vimentin	#V6389	N/A	-1.192	-2.3
ING1	#I3659	Inhibitor of growth 1	-1.189	-2.3
DNASE I	#D0188	Deoxyribonuclease I	-1.182	-2.3
aInternexin	#I0282	Alpha internexin	-1.145	-2.2
Mad2	#M8694	MAD2 mitotic arrest deficient like 1	-1.143	-2.2
Cytokeratin CK5	#C7785	N/A	-1.13	-2.2
gParvin	#P5746	Parvin gamma	-1.113	-2.2
BUB1	#B0561	Budding uninhibited by benzimidazoles 1 homolog	-1.053	-2.1
MAP1	#M4278	Microtubule asociated protein 1	-1.051	-2.1
GRP94	#G4420	Tumor rejection antigen 1	-1.051	-2.1
Nitric Oxide Synthase bNOS	#N2280	Nitric oxide synthase 1	-1.031	-2.0
Heat Shock Factor 1	#H4163	N/A	-1.016	-2.0
Nitrotyrosine	#N0409	N/A	-1.015	-2.0
Cyclin A	#C4710	N/A	-1.003	-2.0

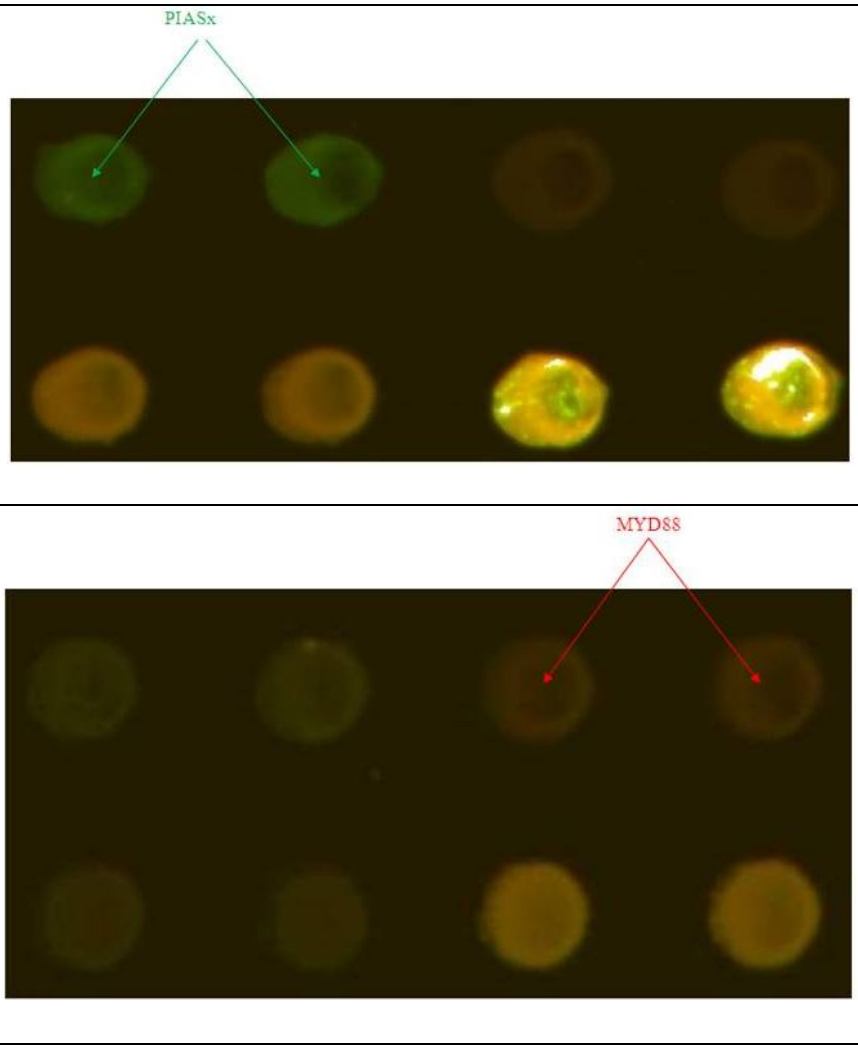


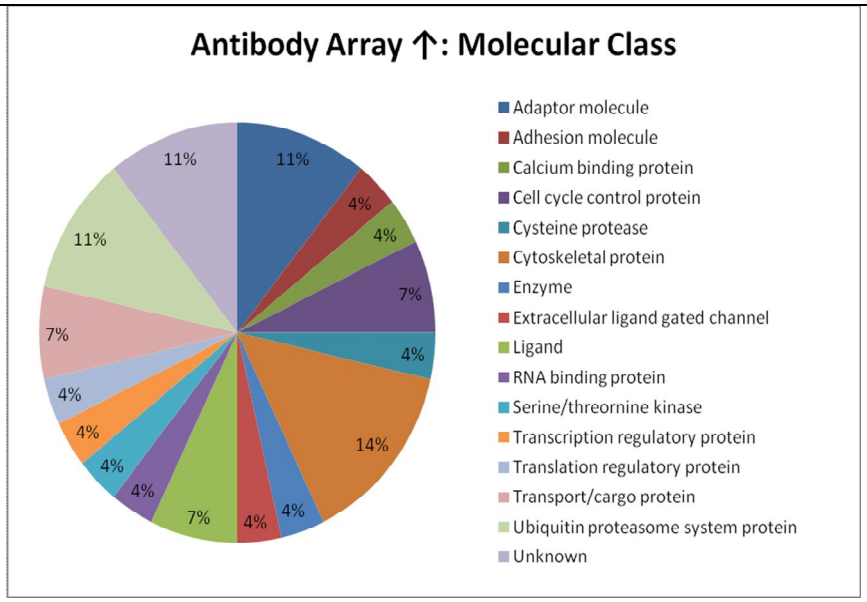
Figure 10.2: Picture showing down-regulation of PIASx and up-regulation of MYD88 on the XPRESS Profiler725 microarray

Stimulated cells were labelled with a Cy5 dye (red) and unstimulated cells were labelled with a Cy3 dye (green). Antibodies were spotted onto the array in duplicate. The picture shows down-regulation of PIASx (Sigma-Aldrich #P9498) (top) in stimulated cells, so the spot appears green and up-regulation of MYD88 (Sigma-Aldrich #M9934) (bottom), so the spot appears red.

10.3 Summary

Seventy proteins were shown to have ≥2-fold change in expression in stimulated 003 CLL cells when compared to unstimulated cells on an XPRESS Profiler725 microarray. The molecular class, molecular function, biological process and location of the proteins was sourced from the Human Protein Reference Database (Mishra. 2006).

The majority of the proteins found to be up-regulated were cytoskeletal proteins (Figure 10.3) related to receptor signaling complex scaffold activity and ubiquitin specific protease activity (Figure 10.4), involved in cell communication and signal transduction (Figure 10.5) and found in the cytoplasm (Figure 10.6). The majority of the proteins found to be down-regulated were cytoskeletal proteins (Figure 10.3) involved in cell communication and signal transduction (Figure 10.5) and found in the nucleus (Figure 10.6). The majority of the proteins found to be down-regulated in the 2D-PAGE/MS experiment (see section **Error! Reference source not found.**) were also located in the nucleus (Figure 9.18).



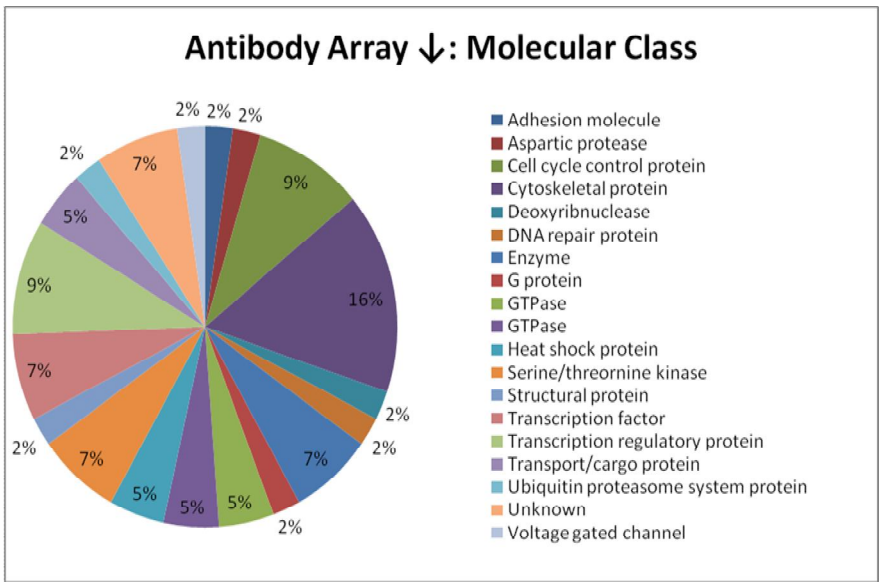
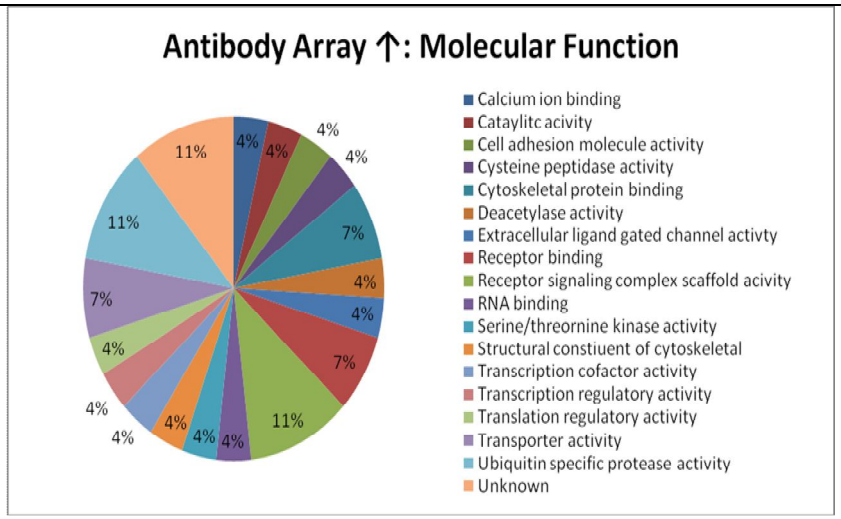


Figure 10.3: Pie charts showing molecular class of proteins found to be differentially expressed on the XPRESS Profiler725 microarray

The chart shows the molecular classes of the 28 up-regulated proteins (top) and the 42 down-regulated proteins (bottom). The majority of differentially expressed proteins are cytoskeleton protein.



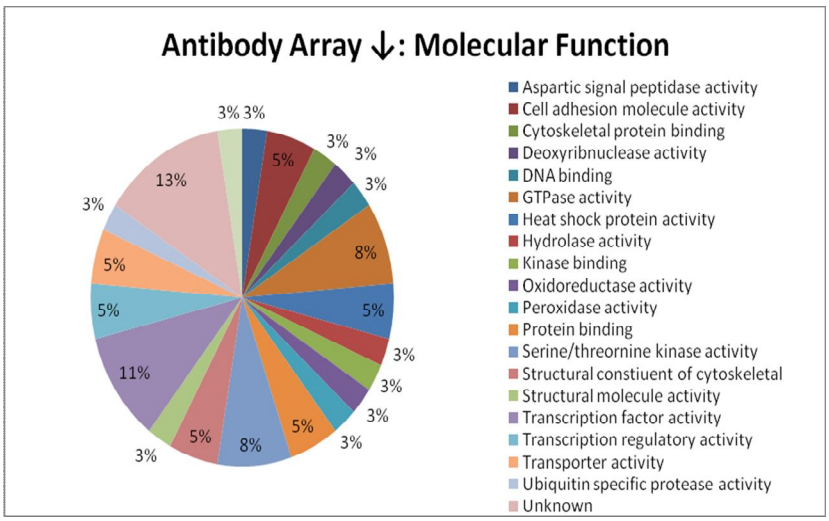
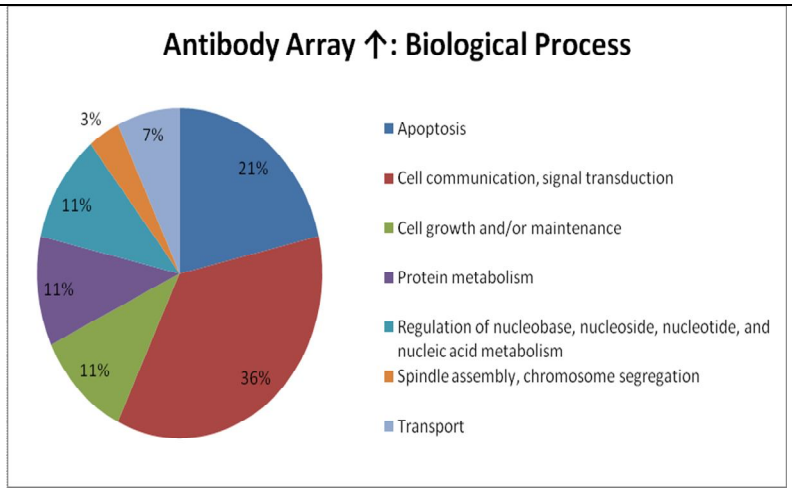


Figure 10.4: Pie charts showing molecular function of proteins found to be differentially expressed on the XPRESS Profiler725 microarray

The chart shows the molecular functions of the 28 up-regulated proteins (top) and the 42 down-regulated proteins (bottom). The majority of up-regulated proteins are a mixture of proteins related to receptor signaling complex scaffold activity, ubiquitin specific protease activity and proteins with unknown functions (top). The majority of down-regulated proteins have unknown functions (bottom).



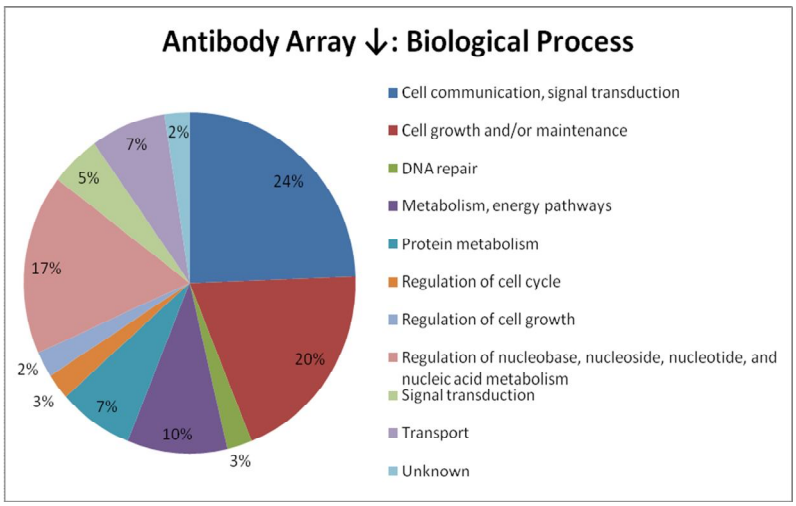
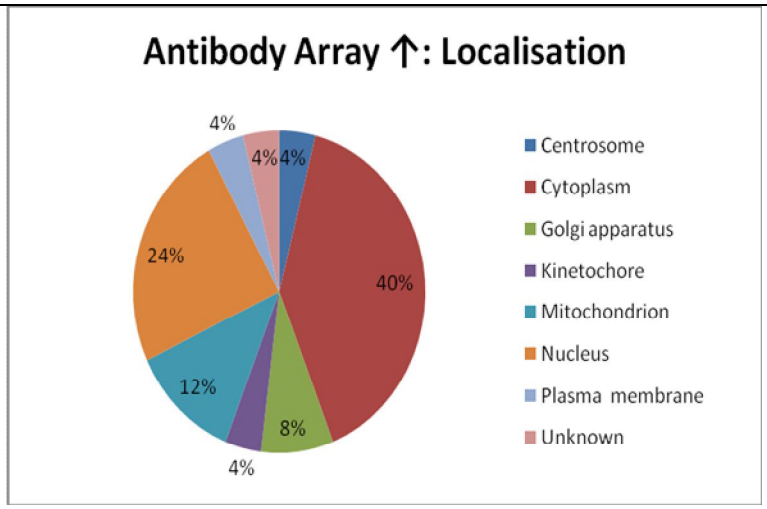


Figure 10.5: Pie charts showing biological process of proteins found to be differentially expressed on the XPRESS Profiler725 microarray

The chart shows the molecular functions of the 28 up-regulated proteins (top) and the 42 down-regulated proteins (bottom). The majority of differentially expressed proteins are involved in cell communication, signal transduction. However, 21% of up-regulated proteins are related to apoptosis (top) and 20% of down-regulated proteins are involved in cell growth and/or maintenance (bottom).



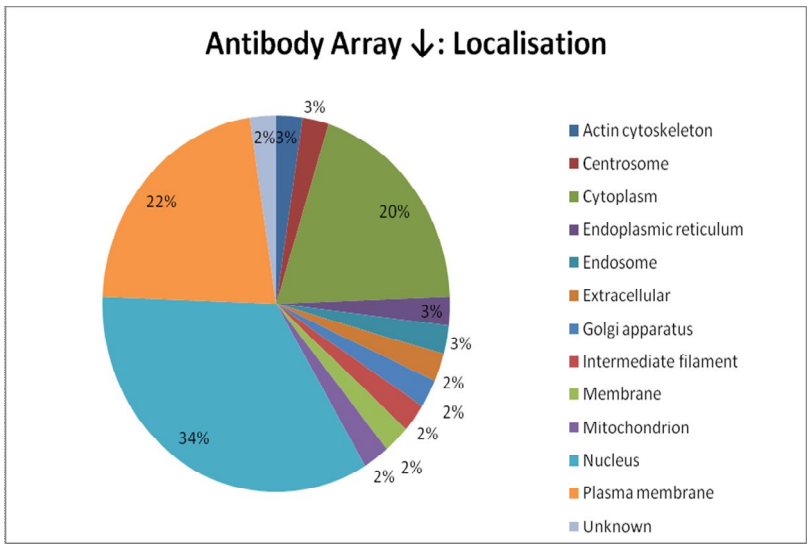


Figure 10.6: Pie charts showing localisation of proteins found to be differentially expressed on the XPRESS Profiler725 microarray

The chart shows the molecular functions of the 28 up-regulated proteins (top) and the 42 down-regulated proteins (bottom). The majority of up-regulated proteins are cytoplasmic proteins (top) and the majority of down-regulated proteins are nucleic proteins (bottom). Twenty-four percent of up-regulated proteins were also nucleic and 20% of down-regulated proteins were cytoplasmic proteins.

Several proteins were shown to be differentially expressed on two occasions on the microarray. This is because some protein targets had two different antibodies referenced on the slide. For example, PUMA bbc3 was shown to be up-regulated in stimulated cells by 8.4-fold (#P4618) and 6.3-fold (#P4743). Antibody #P4618 is an anti-PUMA bbc3, C-terminal antibody produced in rabbit and #P4743 is an anti-PUMA bbc3, N-terminal antibody produced in rabbit. TRAIL was shown to be up-regulated in stimulated cells by 7.6-fold (#T9191) and 2.1-fold (#T3067). Antibody #T9191 is an anti-TRAIL antibody produced in rabbit and #T3067 is a monoclonal anti-TRAIL antibody produced in mouse. SLIPR MAGI3 was found to be down-regulated in stimulated cells by 3.6-fold (#S4191) and 2.5-fold (#S1190). Antibody #S4191 is a monoclonal anti-SLIPR MAGI3 antibody produced in mouse and #S1190 is an anti-SLIPR MAGI3 antibody produced in rabbit.

These three proteins are therefore of specific interest and confirmation by western blot should be prioritised as they have shown differential expression by over 2-fold in more than one type of antibody.

PUMA bbc3 is a BCL-2 family protein and is activated by P53 in cells undergoing P53 induced apoptosis (Yu *et al*. 2003). PUMA bbc3 is said to be silenced in many human malignancies resulting in anti-apoptotic effects (Garrison *et al*. 2008). However, it was shown to be up-regulated in stimulated cells from CLL clinical sample 003.

TRAIL is known to induce apoptosis in cancer cells independent of P53 (Wang. 2008). However studies have shown that TRAIL also has survival and proliferation properties. TRAIL failed to induce apoptosis in small cell lung cancer cells in a study by

Belyanskaya et al, and instead resulted in a 40% increase in proliferation. In the same study it was shown that TRAIL-induced cell proliferation was mediated by extracellular signal-regulated kinase 1 and 2 (Belyanskaya *et al*. 2008). TRAIL was shown to be up-regulated in stimulated cells from CLL clinical sample 003 suggesting that these cells could show pro-survival characteristics.

SLIPR MAGI3 is part of the MAGUK (Membrane-Associated Guanylate Kinase) family proteins. It has been shown to interact with tumour suppressor phosphatase, phosphatase and tensin homolog (phosphatase PTEN) (Wu *et al*. 2000). SLIPR MAGI3 was shown to be down-regulated in stimulated cells from CLL clinical sample 003. Loss of phosphatase PTEN results in enhanced AKT/PKB kinase activation and resistance to apoptosis (DiCristofano *et al*. 2000), suggesting that these cells could show anti-apoptotic characteristics.

Chapter 11 - Conclusions

Conclusion

11.1 Aims of Project

The aim of this project was to identify potential biomarkers which could be used in a clinical test to predict patient prognosis. IgV_H gene mutational status has proven to be a good prognostic marker but the test is expensive and time consuming and can not be used routinely in clinical practice. If the B-cell has not been sensitised to a specific antigen (i.e. unmutated IgV_H genes on BCR) it is hyper-responsive to stimulation through the BCR by antigen. Stimulation of the BCR may prevent apoptosis of malignant cells; therefore a hyper-responsive BCR is linked to poor prognosis. Proteomics was used to investigate the signaling pathways activated by the BCR to gain a greater understanding of the anti-apoptotic nature of the malignant B-cells and to find potential prognostic biomarkers related to a hyper-active BCR.

11.2 Concluding Remarks

This project generated a list of candidate proteins (see Table 11.1) which may be related to prognosis in CLL. Further western blot confirmation remains to be completed but the results give a greater understanding of the mechanisms of the BCR and therefore, why an over-active BCR is related to poor prognosis and survival of malignant B-cells.

The BCR signaling pathways, rather than the individual protein targets, are important in the determining the prognosis of a patient with CLL. The cascades of signals activated by the BCR are related to anti-apoptotic characteristics and malignant cell survival. The cellular pathways of the BCR are not yet fully understood, which makes it difficult to

determine pathway links between the protein targets found in this project. However, we have shown connections between some of the proteins targets found. For example 2D-PAGE/MS showed a decrease in a fatty acid binding protein and an increase in leukotriene A4 hydrolase - fatty acid binding proteins stabilise leukotriene A4 hydrolase, a decrease in a fatty acid binding protein would result in a change of expression of leukotriene A4 hydrolase (see section **Error! Reference source not found.**). It is very possible that many of the targets found act in concert in specific pathways which are not yet understood. The list of proteins targets generated from this project may bring us a step closer to solving the puzzles of the BCR signaling cascades and making those links. Targets found have been ranked in order of their importance to prioritise for further study (Table 11.1) Level of importance was based on confirmation status, the number of clinical samples the target was found in and level of expression change.

Table 11.1: Protein targets ranked in order of importance for further study showing fold change in stimulated cells

Target	CLL Clinical Sample	2D-PAGE/MS	Western Blot	Antibody Microarray
Kininogen	003 078 089	↑ >2-fold ↑ >2-fold ↑ >2-fold		
AK5	078 089	↓ >2-fold	↓ 1.6-fold ↓ 2.6-fold	
DARS	003	↓ >2-fold	↓ 2.1-fold	
PUMA bbc3	003 003			↑ 8.4-fold ↑ 6.3-fold
TRAIL	003 003			↑ 7.6-fold ↑ 2.1-fold
SLIPR MAGI3	003 003			↓ 3.6-fold ↓ 2.5-fold
BACE1	003			↓ 6.7-fold
SIAH	003			↑ 5.6-fold

PIASx	003			↓ 5.3-fold
Leukotriene A4 Hydrolase	089	↑ >2-fold		
Tropomyosin 4-anaplastic Lymphoma Kinase Fusion Protein	089	↓ >2-fold		
Fatty Acid Binding Protein 5	003	↓ >2-fold		
Thiopurine S-Methyltransferase	089	↓ >2-fold		
Rad17	003			↓ 4.8-fold
MyD88	003			↑ 4.7-fold
hBRM hSNF2a	003			↓ 4.1-fold
Smad4	003			↓ 3.9-fold
Zyxin	003			↓ 3.5-fold
RhoE	003			↓ 3.4-fold
Tropomyosin	003			↓ 3.4-fold
P53R2	003			↑ 3.2-fold
BACH1	003			↓ 3.2-fold
Chk1	003			↑ 3.1-fold
CtBP1	003			↑ 3.1-fold
Rab7	003			↓ 3.1-fold
RALAR	003			↓ 3.1-fold
ARP3	003			↑ 3-fold
ILP2	003			↑ 3-fold
TSG101	003			↑ 3-fold
Bim	003			↑ 2.9-fold
iASPP	003			↑ 2.9-fold
Importin a3	003			↓ 2.9-fold
Protein Kinase C PKC	003			↓ 2.9-fold
SKM1	003			↓ 2.9-fold
Nuf2	003			↑ 2.8-fold
bCOP	003			↑ 2.7-fold
p115 TAP	003			↑ 2.7-fold
Caldesmon	003			↓ 2.6-fold
E6AP	003			↓ 2.6-fold
hABH1	003			↓ 2.6-fold
Paxillin	003			↓ 2.6-fold
Caspase 13	003			↑ 2.5-fold
AOP1	003			↓ 2.5-fold
FBI1 POKEMON	003			↓ 2.5-fold
Growth Factor Independence1	003			↓ 2.5-fold
LIS1	003			↓ 2.4-fold
MRP2	003			↓ 2.4-fold
NBS1	003			↓ 2.4-fold
p34cdc2	003			↓ 2.4-fold
Ubiquitin Cterminal Hydrolase L1	003			↑ 2.3-fold
DNASE I	003			↓ 2.3-fold
ING1	003			↓ 2.3-fold

Vimentin	003			↓ 2.3-fold
Cortactin	003			↑ 2.2-fold
FADD	003			↑ 2.2-fold
FXR2	003			↑ 2.2-fold
Sir2	003			↑ 2.2-fold
aInternexin	003			↓ 2.2-fold
Cytokeratin CK5	003			↓ 2.2-fold
gParvin	003			↓ 2.2-fold
Mad2	003			↓ 2.2-fold
dCatenin NPRAP	003			↑ 2.1-fold
p53DINP1SIP	003			↑ 2.1-fold
Spectrin a & b	003			↑ 2.1-fold
BUB1	003			↓ 2.1-fold
MAP1	003			↓ 2.1-fold
Glutamate receptor NMDAR 2a	003			↑ 2-fold
Centrin	003			↑2-fold
GRP94	003			↓ 2.1-fold
Cyclin A	003			↓ 2-fold
Heat Shock Factor 1	003			↓ 2-fold
Nitric Oxide Synthase bNOS	003			↓ 2-fold
Nitrotyrosine	003			↓ 2-fold
Kazrin	089	↑ >2-fold		
Astrotactin 2 (ASTN2 Protein)	003	↑ >2-fold		
Tripartite Motif Protein (TRIM19 Zeta)	003	↑ >2-fold		
Phosphatidylinositol Transfer Protein Beta	003	↓ >2-fold		
Glyoxalase Domain Containing 4	003	↓ >2-fold		
FYVE-Ring Finger Protein Sakura (Rififylin)	089	↓ >2-fold		
RAS Homolog Gene Family Member T1	089	↑ >2-fold		
Astrotactin 2 Isoform d	003	↓ >2-fold		
Interferon Alpha-1/13 Precursor	003	↓ >2-fold		

Many of the protein targets found have no previous connection to CLL or BCR signaling (e.g. astrotactin, kazrin and kininogen). In both 2D-PAGE/MS and antibody microarray experiments, more proteins were found to be down-regulated in the stimulated cells suggesting that BCR activation not only activates signaling pathways but also inhibits cellular pathways resulting in decrease in expression of proteins. Some of the proteins found to be increased in expression are anti-apoptotic or cell survival

proteins (e.g. TRIM19 zeta and iASPP) and some proteins found to be decreased in expression are related to cell death (e.g. phosphatidylinositol transfer protein beta and glyoxalase domain containing 4) suggesting that activation of the BCR is resulting in malignant cell survival.

11.3 Antibody Microarray vs. 2D-PAGE/MS

2D-PAGE/MS is a global proteomic technique and remains the gold standard for the analysis of protein expression. However there are several drawbacks associated with the technique. It is very time consuming and requires highly experienced analysts to perform experiments reproducibly. Many differentially expressed proteins are lost in the numerous stages of 2D-PAGE/MS;

- Proteins may not enter the gel due to hydrophobic nature or pH
- Protein spots may not get excised if they are in a cluster, a streak or are they too faint
- Proteins may not be identified by MS if there are too many contaminating peptides, too little sample or if the protein isn't in the database

The antibody microarray is a promising new technology which is designed to overcome these problems. However, the antibody microarray is not a global proteomic technique and results are limited to what antibodies are referenced on the slide. The two techniques therefore cannot not be compared, but may work complimentary to one another, both providing information on protein expression.

11.4 Future Work

Table 11.1 shows protein targets highlighted in this project which require further validation. Due to the time implications with 2D-PAGE, only three clinical CLL samples were analysed with this technique. However, protein targets generated can be used to develop a fast screening assay for the other clinical samples. For example, an enzyme-linked immunosorbent assay (ELISA) can be used to detect an antibody in numerous samples in a 96-well microtitre plate format, resulting in many samples being screened for one protein target at the same time. This technique would be particularly useful when screening for a target protein such as kininogen, which was found to be up-regulated in all three clinical CLL sample by 2D-PAGE/MS. The prognostic value of kininogen could be determined by screening the bank of CLL samples for kininogen, using an ELISA technique, and relating the expression of kininogen in each sample to the classifications of the sample, based on IgV_H mutational status and BCR responsiveness. Western blots can also be used to determine protein target levels in clinical samples. Sigma-Aldrich have released a “Prestige” set off antibodies for commercial use. These antibodies have been designed and validated by the Human Proteome Resource and are supplied with standardised universal protocols. The Human Protein Atlas (proteinatlas.org) is publicly available and shows approximately 1600 human proteins which have been screen for protein profiling. Kininogen is one of the antibodies offered by Sigma Prestige (#HPA001616) and could be used in the future for validation and identifying clinical significance in the CLL clinical samples. 2D-PAGE targets can also be confirmed by MALDI-TOF-MS/MS analysis. Some of the differentially expressed proteins found in this project have been analysed by MALDI-TOF-MS/MS (data not shown). Pilot studies were used to determine methods and techniques to identify and validate future protein extracts (collaboration with the

Institute of Cancer Therapeutics, the University of Bradford). The antibody microarray results can be validated by western blot, and the clinical relevance of the proteins found can be assessed by screening numerous clinical samples for the target proteins.

To date there is over 215 CLL samples stored in Hull (with 155 already characterised) and access to further CLL samples via collaborations with Cardiff, Birmingham, Liverpool and Newcastle. A large collection of samples (~750) from around the UK could be screened for the potential prognostic biomarkers found in this investigation. Blood samples continue to be collected in Hull from CLL patients and new samples will require classifying into "good and poor prognosis" based on IgV_H mutational status and BCR responsiveness.

Additional prognostic biomarkers could be discovered by further 2D-PAGE/MS and antibody microarray experiments on CLL clinical samples. Results from these experiments would give more pieces to add to the BCR signaling pathway puzzle.

The maximum stimulation time was used in this project. This was defined as the maximum time that the stimulant could remain on the cells without causing cellular stress. For RAJI B-cells this was 8hrs (see section 7.2) and for CLL clinical samples this was 5.5hrs (see section 8.2.3). In future experiments this time point could be reduced to identify protein changes at shorter stimulation times. A stimulation time of 5mins shows changes in expression of phosphoproteins (see Chapter 6) but not of unphosphorylated proteins (see section 7.1.2). The time point at which total protein expression changes are seen could be determined. Changes in expression at the proteome level could then be determine for specific time points, working up to the maximum stimulation time. This

would create a greater understanding of the mechanism of the BCR from pathways and proteins activated quickly to ones activated further downstream.

11.5 Conclusion

In conclusion, some of the proteins found to have changed in expression between stimulated and unstimulated cells in this project are related to cell signaling pathways and have connections with other proteins targets found. Some targets found were novel proteins which have not previously been associated with CLL or BCR signaling. The project has provided some evidence that stimulation of the BCR in CLL results in cell survival proteins being up-regulated and cell death proteins being down-regulated. These potential prognostic biomarkers now need validating and assessing across a wider range of clinical samples. The identification of more proteins relating to BCR signaling will not only increase our understanding of the mechanisms of the BCR, but will also provide potential prognostic biomarkers for CLL allowing for earlier treatment for patients with a poor prognosis and peace of mind for patients with a good prognosis.

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APPENDICES

Appendix A - Suppliers and Contact Details

Supplier	Address
Abcam	332 Cambridge Science Park, Milton Road, Cambridge, CB4 0FW, UK
Abgent	10239 Flanders Court, San Diego, CA 92121, USA
Abnova	9th Fl, No. 108, Jhouzih St, Neihu District, Taipei City, 114 Taiwan
Amersham Biosciences	Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK
Axon Instruments	71 Four Valley Drive, Concord, ON, L4K 4V8, Canada
Bio-Rad Laboratories	1000 Alfred Nobel Drive, Hercules, CA 94547, USA
Bruker Daltronics Ltd	Banner Lane, Coventry, CV4 9GH, UK
Fisher Scientific	Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, UK
Invitrogen	3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK
Jackson ImmunoResearch laboratories Inc.	7 Acorn Business Centre, Oaks Drive, Newmarket, Suffolk, CB8 7SY, UK
Kodak	Headstone Drive, Harrow, Middlesex, HA1 4TY, UK
Nonlinear Dynamics	Cuthbert House, All Saints, Newcastle upon Tyne, NE1 2ET, UK
Pierce Biotechnology	PO Box 117, Rockford, IL 61105, USA
Promega Corporation	Delta House, Southampton Science Park, Southaptom, SO10 7NS, UK
Santa Cruz Biotechnology Inc.	2145 Delaware Avenue, Santa Cruz, CA 95060, USA
Sarstedt	68 Boston Road, Beaumont, Leys, Leicester, LE4 1AW, UK
Scientific Laboratory Supplies (SLS)	Wilford Industrial Estate, Ruddington Lane, Wilford, Nottingham, NG1 7EP, UK
Sigma-Aldrich	Fancy Road, Poole, Dorset, BH12 4QH, UK
Thermo Scientific	81 Wyman Street, Waltham, MA 02454, USA
UniEquip	Fraunhoferstrasse 11, Martinried, Munich, D-82152, Germany

Appendix B – CLL Sample Database

Sample	Sex	Date of Diagnosis	Date of Collection 1 (C1)	Age at C1	Binet Stage at C1	IgVH Mutational Status (% Standard Germline Concordance)
001	Male	01/12/1999	17/06/2005	74	B	93.5
002	Male	01/10/2000	30/09/2005	70	A	93.9
003	Male	01/01/2001	30/09/2005	80	C	100
004	Male	29/01/2004	07/10/2005	64	B	93.7
005	Male	07/05/2004	08/07/2005	81	A	98.3
006	Female	03/05/2001	14/10/2005	61	A	90.5
007	Male	13/02/2002	14/10/2005	78	A	93
008	Female	26/04/1999	14/10/2005	74	A	96
009	Male	21/08/2002	21/10/2005	71	A	94
010	Female	31/10/2001	28/10/2005	83	A	99
011	Male	30/01/2002	02/09/2005	59	A	98.3
012	Female	04/11/2005	04/11/2005	73	A	95
013	Male	03/02/1998	04/11/2005	79	A	96
014	Female	20/05/2005	11/11/2005	58	A	93
015	Female	05/01/1995	11/11/2005	84	A	96.6
016	Female	07/01/2004	18/11/2005	77	A	99
017	Male	23/02/2001	18/11/2005	69	A	92
018	Female	01/01/1985	18/11/2005	82	A	92.6
019	Male	16/04/2003	25/11/2005	61	A	92
020	Female	05/05/1987	25/11/2005	71	A	94.4
021	Male	15/03/2004	25/11/2005	50	A	94.2
022	Male	11/11/2005	11/11/2005	55	A	90
023	Female	09/05/1991	02/12/2005	68	A	89
024	Male	20/06/2001	02/12/2005	69	A	99.6
025	Male	11/08/2000	02/12/2005	65	A	97.8
026	Female	23/07/2004	16/12/2005	59	A	91.6
027	Female	23/04/2004	16/12/2005	81	A	99.5
028	Female	02/09/1994	16/12/2005	66	A	90.9
029	Male	26/06/2002	16/12/2005	79	C	87
030	Male	18/06/2004	06/01/2006	66	A	99.4
031	Male	12/12/2002	06/01/2006	67	B	92.7
032	Male	10/12/2003	06/01/2006	68	A	90.8
034	Male	11/06/2004	13/01/2006	80	A	97.2
035	Male	24/05/1994	20/01/2006	80	A	92.3
036	Female	19/06/1997	20/01/2006	77	A	95.3
038	Female	23/09/2005	27/01/2006	56	A	99.4
039	Female	01/01/1996	27/01/2006	70	A	89
041	Female	01/02/2005	03/02/2006	73	A	89
042	Female	19/01/2001	10/02/2006	76	A	90.4
043	Female	08/12/2000	10/02/2006	66	A	96.5
044	Male	09/01/2002	17/02/2006	63	B	96.4
045	Female	28/05/2003	17/02/2006	76	A	93
046	Female	29/08/1982	17/02/2006	77	B	92.1
048	Male	14/02/2006	10/03/2006	100	C	99.6
049	Female	26/05/1970	10/03/2006	85	A	94.7
050	Male	10/04/2001	17/03/2006	69	A	97.2
052	Male	16/01/2002	31/03/2006	73	A	97.6
054	Male	21/08/2002	07/04/2006	39	C	99.6
055	Female	02/12/1999	26/05/2006	76	C	96.7

056	Male	25/07/1997	28/04/2006	75	A	100
057	Male	17/02/2006	12/05/2006	63	B	96.2
058	Male	22/10/2004	12/05/2006	62	A	99.6
059	Female	30/05/1996	19/05/2006	72	A	85
060	Female	09/06/1994	19/05/2006	74	A	93.3
062	Female	14/05/1986	07/07/2006	75	B	96
063	Female	14/06/2004	07/07/2006	75	B	100
064	Male	10/09/1986	14/07/2006	68	B	94.4
066	Male	13/02/2004	25/08/2006	87	B	94.7
069	Male	25/01/2006	01/09/2006	77	C	100
070	Male	20/08/2004	02/03/2007	69	A	90
071	Male	13/08/2004	08/09/2006	83	A	90.3
072	Male	01/01/1987	08/09/2006	77	A	89.3
073	Female	30/06/2006	15/09/2006	73	A	97.5
074	Male	11/02/2005	22/09/2006	62	B	93.6
076	Female	03/02/2006	29/09/2006	71	A	96.6
077	Male	31/03/2006	06/10/2006	63	B	94.1
078	Male	21/07/2006	13/10/2006	77	A	100
080	Male	11/02/1999	10/11/2006	76	A	89.6
081	Male	02/07/1992	17/11/2006	57	A	94.6
082	Female	15/01/2003	17/11/2006	83	A	94.9
086	Male	20/10/2006	01/12/2006	61	A	88.5
088	Male	17/07/2002	12/01/2007	78	B	92.1
089	Female	22/10/2004	15/12/2006	78	A	97.5
090	Female	03/06/1998	11/11/2006	70	A	93.4
091	Male	04/12/2002	28/09/2007	75	C	100
092	Female	01/12/2006	12/01/2007	79	B	100
093	Male	08/01/2007	19/01/2007	37	B	97.8
096	Male	22/07/2005	16/02/2007	76	A	100
099	Male	20/06/2001	09/03/2007	74	C	100
100	Female	26/01/2007	02/03/2007	79	A	100
101	Female	01/01/2003	09/03/2007	78	B	100
103	Male	14/10/2005	16/03/2007	48	A	94.6
104	Female	18/08/2006	16/03/2007	87	A	94.1
107	Male	30/06/1998	30/03/2007	66	A	94
111	Female	29/10/1998	20/04/2007	91	A	92.4
112	Male	05/02/2003	27/04/2007	63	A	100
113	Male	10/10/2001	27/04/2007	69	A	96.3
114	Female	02/12/2005	27/04/2006	59	A	99.6
115	Female	06/03/2002	27/04/2007	86		93.9
116	Male	08/12/2006	11/05/2007	53	A	91.1
117	Male	28/09/2000	18/05/2007	58	B	91
118	Male	31/12/2003	18/05/2007	59	A	100
120	Male	24/04/2007	25/04/2007	58	A	98
121	Male	23/01/2002	01/06/2007	70	A	96.3
122	Male	30/03/2007	01/06/2007	76	A	93.4
123	Female	11/04/2001	01/06/2007	64	A	99.2
124	Female	06/09/2000	08/06/2007	69	A	97.1
131	Female	27/06/2001	13/07/2007	55		91.1
132	Female	13/04/2007	27/07/2007	81	A	91.2
133	Male	06/07/2007	20/07/2007	68	A	99.2
134	Male	04/12/2000	20/07/2007	70	A	94
135	Male	04/11/1994	20/07/2007	72	A	89.9
139	Male	25/07/2007	10/08/2007	62	C	99.6
140	Female	16/04/1999	10/08/2007	72	A	95

141	Male	16/04/2003	10/08/2007	73	C	94.5
142	Male	25/07/2007	10/08/2007	64	A	100
144	Male	20/07/2007	28/07/2007	59	A	100
145	Male	11/07/2007	24/08/2007	75	A	95.5
146	Female	04/12/2002	31/08/2007	81	A	97.6
147	Male	07/09/2007	07/09/2007	87	C	100
148	Female	08/06/2007	07/09/2007	44	A	100
149	Female	24/08/2007	14/09/2007	75	A	91
150	Female	17/08/2007	14/09/2007	73	C	100
151	Male	18/05/2007	21/09/2007	71	A	92.4
155	Male	21/09/2007	19/10/2007	70	A	99.6

Appendix C – Tissue Culture Solutions

Tissue culture (TC) media/Washing Media

- 1 bottle RPMI 1640 media (Invitrogen), 50ml Foetal calf serum, 5ml Pen/Strep (Invitrogen), 5ml Glutamine (Invitrogen), 5ml Fungizone (Invitrogen).
- 20% freezing media as washing media + 20% filtered, sterile DMSO (Sigma-Aldrich).
- 10% freezing media as washing media but + 10% filtered, sterile DMSO (Sigma-Aldrich).

Phosphate Buffered Saline

1 tablet PBS per 500ml DH_2O

Appendix D – 2D-PAGE Solutions

2D Lysis Buffer
2.52g Urea, 0.912g Thiourea, 0.24g CHAPS, 46.2mg Dithiothreitol (DTT), 60µl Biolyte (pH3-10), 12µl 1% Bromophenol Blue (BPB), 3.3ml Distilled Water, 60µl Protease Inhibitor (Sigma-Aldrich #P8340), 60µl Phosphatase Inhibitor 1 (Sigma-Aldrich #P2850), 60µl Phosphatase Inhibitor 2 (Sigma-Aldrich #P5726)

Equilibration Buffer
6.7ml 1.5M Tris-HCl (pH 8.8), 72.07g urea (60.06MW), 69ml 87% glycerol, 4.0g SDS, trace BPB. Made up to 200ml DH_2O.

Equilibration Buffer 1
100mg DTT to 10ml equilibration buffer
Equilibration Buffer 2
250mg idoacetamide to 10ml equilibration buffer

1% Agarose Solution
1g Agarose, 100ml Running Buffer, trace of BPB

Running Buffer
1 part TGS (x10) (Bio-Rad) to 9 parts DH_2O
(10x TGS = 25mM Tris, 192mM Glycine, 0.1% (w/v) SDS (pH8.3))

0.1M AMBIC Solution
0.16g Ammonium Bicarbonate, 20ml DH_2O

10mM DTT / 0.1M Ammonium Bicarbonate solution
0.0077g DTT in 5ml 0.1M ammonium bicarbonate

55mM Iodoacetamide / 0.1M Ammonium Bicarbonate
0.051g IAA in 5ml 0.1M ammonium bicarbonate – protect from light

50mM acetic acid
Molarity = % solvent X 10 / MW
Acetic acid = 16.6M = 16,600mM
50mM / 16,600mM X 1000 = 3µl
3µl acetic acid in 997µl DH_2O

1mg/ml Trypsin Solution
100µg trypsin (Promega #V5280) + 100µl 50mM Acetic Acid

Digestion Buffer 1
400µl 0.1M Ammonium Bicarbonate
100µl ACN
25µl Trypsin Solution
475µl DH_2O

Digestion Buffer 2

400µl 0.1M Ammonium Bicarbonate
100µl ACN
500µl DH_2O

0.1M AMBIC
10ml DH_2O + 0.8g Ammonium Bicarbonate

0.05M & 0.025M AMBIC
5ml 0.1M AMBIC + 5ml DH_2O = 0.05M
5ml 0.05M AMBIC + 5ml DH_2O = 0.025M

DHB Matrix
50% ACN:DH_2O + 1% formic acid. Saturate with DHB (Sigma-Aldrich #85707)

Appendix E – Protein Staining Solutions

Krypton Fixing Solution
40% Ethanol + 10% Acetic Acid + 50% DH_2O

Krypton Destaining Solution
5% Acetic Acid + 95% DH_2O

Pro-Q Diamond Fixing Solution
50% Methanol + 10% Acetic Acid + 40% DH_2O

Pro-Q Diamond Destaining Solution
50ml 1M Sodium Acetate (pH 4.0) + 200ml ACN +250ml DH_2O

Flamingo and Sypro Ruby Fixing Solution
40% Ethanol + 10% Acetic Acid + 50% DH_2O

Appendix F – Western Blot Solutions

Laemmli Buffer
4.0ml DH_2O, 1ml 0.5M Tris-HCl pH 6.8, 0.8ml Glycerol, 1.6ml 10% SDS, trace of BPB.
To every 190µl add 10µl 5% beta-mercapto ethanol (β-Me), 10µl phosphatase inhibitor 1 (Sigma-Aldrich #P2850) and 2 (Sigma-Aldrich #P5726) and 10µl protease inhibitor mix (Sigma-Aldrich #P8340).

Running Buffer
1 part TGS (x10) (Bio-Rad) to 9 parts DH_2O
(10x TGS = 25mM Tris, 192mM Glycine, 0.1% (w/v) SDS (pH8.3))

Transfer Buffer
200ml Methanol + 800ml DH_2O + 3g Tris + 14.4g Glycine

Tris Buffered Saline (TBS) (x20)
121g Tris, 170g Sodium Chloride, made up to 1L in DH_20, pH 7.6 with conc HCl

TBS/TWEEN
250ml TBS (x20) + 4750ml DH_2O + 2ml TWEEN-20

5% Blocking Solution
2g Marvel non-fat dried milk powder
40ml TBS/ 0.05% TWEEN-20

Appendix G – Antibody Microarray (XPRESS profiler725) solutions

Protease inhibitor cocktail
Add 0.3ml DH_2O to the lyophilised vial (Store at -20C for long term)

Benzonase working solution
2ul of stock benzonase (-20C) added to 18uL of Extraction/Labeling Buffer (from the array kit)

Lysis buffer
10ml labelling/extraction buffer
50uL of protease inhibitor cocktail solution.
100uL of phosphatase inhibitor cocktail
1.2uL of benzonase working solution.

Wash Buffer:
Phosphate Buffered Saline, pH 7.4, with Tween 20 (from kit) + 1L of water. Filter through a 0.45μM filter.

Appendix H – MALDI-TOF-MS

Peptide Contaminants Lists

Group 1

M/Z	Contaminant	Modification	Unmodified Peak M/Z
993.5	Keratin 10	N/A	N/A
1036.5421	Type 1 Hair Keratin 1	N/A	N/A
1066.4986	Keratin 9	N/A	N/A
1179.6004	Keratin 1	N/A	N/A
1277.6332	Keratin 1	N/A	N/A
1475.7489	Keratin 1	N/A	N/A
1638.8598	Keratin 1	N/A	N/A
1837.9654	Keratin 9	N/A	N/A
1851.9269	Keratin 9	N/A	N/A
1993.9766	Keratin 1	N/A	N/A
2211.104	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
2225.3	Background Peak	N/A	N/A
2283.1801	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
2367.2626	Keratin 10	N/A	N/A
2383.9518	Keratin 1	N/A	N/A

Group 2

M/Z	Contaminant	Modification	Unmodified Peak M/Z
1107.8	Background Peak	N/A	N/A
1109.49	Keratin 10	N/A	N/A
2055.962	Type 1 Hair Keratin 1	CAM	1998.941
2342.984	Keratin 10	CAM	2285.9628

Group 3

M/Z	Contaminant	Modification	Unmodified Peak M/Z
1090.531	Keratin 10	N/A	N/A
1141.5194	Keratin 1	N/A	N/A
1165.5848	Keratin 10	N/A	N/A
1234.679	Keratin 10	N/A	N/A
1262.5971	Keratin 10	N/A	N/A
1265.6372	Keratin 1	N/A	N/A
1307.6776	Keratin 9	N/A	N/A
1320.1	Background Peak	N/A	N/A
1357.7182	Keratin 10	N/A	N/A
1434.7699	Keratin 10	N/A	N/A
1707.7503	Keratin 9	CAM	1650.7289
1716.8511	Keratin 1	N/A	N/A
1794.9	Background Peak	N/A	N/A
1941.9876	Keratin 1	N/A	N/A
2021.9886	Keratin 1	N/A	N/A
2082	Background Peak	N/A	N/A

2095.0376	Keratin 9	CAM	2038.0161
2171.0246	Keratin 9	N/A	N/A
2292.2081	Keratin 1	N/A	N/A

Group 4

M/Z	Contaminant	Modification	Unmodified Peak M/Z
842.5094	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
847.4519	Keratin 10	N/A	N/A
847.4593	Type 1 Hair Keratin 1	N/A	N/A
874.4992	Keratin 1	N/A	N/A
881	Background Peak	N/A	N/A
895.4592	Keratin 1	N/A	N/A
897.4135	Keratin 9	N/A	N/A
906.5043	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
910.4152	Keratin 1	N/A	N/A
913.4084	Keratin 9	MSO	897.4135
932.5047	Keratin 1	N/A	N/A
935.4251	Keratin 10	N/A	N/A
945.4999	Keratin 1	N/A	N/A
948.4493	Keratin 10	N/A	N/A
951.42	Keratin 10	MSO	935.4251
962.4901	Keratin 9	N/A	N/A
966.4567	Keratin 10	N/A	N/A
967.4519	Keratin 9	N/A	N/A
973.5313	Keratin 1	N/A	N/A
976.4231	Keratin 10	N/A	N/A
982.4233	Type 1 Hair Keratin 1	N/A	N/A
985.5789	Keratin 1	N/A	N/A
988.5309	Type 1 Hair Keratin 1	N/A	N/A
998.4836	Keratin 1	N/A	N/A
999.4451	Keratin 1	N/A	N/A
999.4966	Keratin 1	N/A	N/A
999.5833	Type 1 Hair Keratin 1	N/A	N/A
1003.5531	Keratin 10	N/A	N/A
1006.4296	Keratin 1	N/A	N/A
1006.4873	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
1009.5313	Type 1 Hair Keratin 1	N/A	N/A
1014.4785	Keratin 1	MSO	998.4836
1015.4401	Keratin 1	MSO	999.4451
1033.516	Keratin 1	N/A	N/A
1045.5636	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
1047.5754	Type 1 Hair Keratin 1	N/A	N/A
1048.5785	Keratin 10	N/A	N/A
1060.5633	Keratin 9	N/A	N/A
1063.5088	Porcine Trypsin (Sigma-Aldrich and Promega)	CAM	1006.4873
1064.6098	Keratin 10	N/A	N/A
1065.4993	Keratin 9	N/A	N/A
1065.5211	Keratin 1	N/A	N/A
1066.5163	Keratin 1	N/A	N/A
1071.5251	Keratin 10	N/A	N/A

1073.5949	Keratin 1	N/A	N/A
1077.5245	Porcine Trypsin (Sigma-Aldrich and Promega)	PAM	1006.4873
1078.6255	Type 1 Hair Keratin 1	N/A	N/A
1079.5116	Keratin 10	N/A	N/A
1081.4942	Keratin 9	MSO	1065.4993
1081.5133	Keratin 9	N/A	N/A
1082.4935	Keratin 9	MSO	1066.4986
1087.52	Keratin 10	MSO	1071.5251
1087.5279	Keratin 1	N/A	N/A
1092.5028	Keratin 1	N/A	N/A
1095.5469	Keratin 9	N/A	N/A
1096.4662	Type 1 Hair Keratin 1	CAM	982.4233
1097.5082	Keratin 9	N/A	N/A
1104.596	Type 1 Hair Keratin 1	CAM	1047.5754
1106.5259	Keratin 10	MSO	1090.531
1118.6125	Type 1 Hair Keratin 1	PAM	1047.5754
1121.5837	Keratin 9	N/A	N/A
1124.4976	Type 1 Hair Keratin 1	PAM	982.4233
1125.5422	Keratin 1	N/A	N/A
1127.5401	Keratin 1	N/A	N/A
1131.5463	Keratin 10	N/A	N/A
1133.5	Background Peak	N/A	N/A
1134.5935	Type 1 Hair Keratin 1	N/A	N/A
1138.5925	Keratin 9	N/A	N/A
1140.5354	Type 1 Hair Keratin 1	N/A	N/A
1143.535	Keratin 1	MSO	1127.5401
1147.5412	Keratin 10	MSO	1131.5463
1152.5531	Keratin 1	N/A	N/A
1153.5306	Type 1 Hair Keratin 1	N/A	N/A
1153.7	Background Peak	N/A	N/A
1154.5874	Keratin 9	MSO	1138.5925
1157.5143	Keratin 1	MSO	1141.5194
1157.5909	Keratin 9	N/A	N/A
1158.6041	Keratin 10	N/A	N/A
1159.6542	Keratin 1	N/A	N/A
1169.5255	Type 1 Hair Keratin 1	MSO	1153.5306
1175.6015	Keratin 1	N/A	N/A
1185.5229	Keratin 10	N/A	N/A
1186.6062	Type 1 Hair Keratin 1	N/A	N/A
1190.6085	Keratin 9	N/A	N/A
1191.615	Type 1 Hair Keratin 1	CAM	1134.5953
1194	Background Peak	N/A	N/A
1197.5568	Type 1 Hair Keratin 1	CAM	1140.5354
1201.6171	Keratin 10	N/A	N/A
1203.6765	Type 1 Hair Keratin 1	N/A	N/A
1204.6419	Keratin 9	N/A	N/A
1205.6307	Type 1 Hair Keratin 1	PAM	1134.5953
1206.6034	Keratin 9	MSO	1190.6085
1206.6147	Keratin 9	N/A	N/A
1211.5725	Type 1 Hair Keratin 1	PAM	1140.5354
1217.6161	Keratin 1	N/A	N/A
1218.6212	Type 1 Hair Keratin 1	N/A	N/A
1222.6096	Keratin 9	MSO	1206.6147
1223.4	Background Peak	N/A	N/A
1235.5287	Keratin 9	N/A	N/A

1237.5847	Keratin 10	N/A	N/A
1238.6739	Type 1 Hair Keratin 1	N/A	N/A
1243.6641	Type 1 Hair Keratin 1	N/A	N/A
1260.698	Type 1 Hair Keratin 1	CAM	1203.6765
1261.5668	Keratin 10	N/A	N/A
1274.7137	Type 1 Hair Keratin 1	PAM	1203.6765
1277.5869	Keratin 9	N/A	N/A
1277.7099	Keratin 1	N/A	N/A
1280.6481	Keratin 1	N/A	N/A
1300.5296	Keratin 1	N/A	N/A
1302.7012	Keratin 1	N/A	N/A
1302.7151	Keratin 1	N/A	N/A
1309.6382	Type 1 Hair Keratin 1	N/A	N/A
1315.6852	Keratin 9	N/A	N/A
1323.6725	Keratin 9	MSO	1307.6776
1324.6168	Keratin 10	N/A	N/A
1326.6396	Type 1 Hair Keratin 1	N/A	N/A
1332.5195	Keratin 1	MSO	1300.5296
1340.6692	Keratin 1	N/A	N/A
1350.6284	Keratin 10	N/A	N/A
1350.6827	Keratin 9	N/A	N/A
1357.6957	Keratin 1	N/A	N/A
1367.6736	Type 1 Hair Keratin 1	N/A	N/A
1381.6481	Keratin 10	N/A	N/A
1383.6903	Keratin 1	N/A	N/A
1390.6808	Keratin 10	N/A	N/A
1391.7277	Keratin 9	N/A	N/A
1392.6502	Keratin 10	N/A	N/A
1393.7321	Keratin 1	N/A	N/A
1399.7652	Type 1 Hair Keratin 1	N/A	N/A
1421.7383	Keratin 1	N/A	N/A
1424.695	Type 1 Hair Keratin 1	CAM	1367.673
1438.7107	Type 1 Hair Keratin 1	PAM	1367.673
1450.7244	Keratin 10	N/A	N/A
1460	Background Peak	N/A	N/A
1464.6821	Type 1 Hair Keratin 1	N/A	N/A
1465.7394	Type 1 Hair Keratin 1	N/A	N/A
1469.7304	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
1469.8434	Keratin 1	N/A	N/A
1471.7499	Type 1 Hair Keratin 1	N/A	N/A
1475.7852	Keratin 1	N/A	N/A
1482.7143	Keratin 1	N/A	N/A
1494.5	Background Peak	N/A	N/A
1495.6983	Keratin 10	N/A	N/A
1504.7754	Type 1 Hair Keratin 1	N/A	N/A
1520.7452	Keratin 10	N/A	N/A
1523.7886	Keratin 1	N/A	N/A
1526.7519	Porcine Trypsin (Sigma-Aldrich and Promega)	CAM	1469.7304
1530.6902	Type 1 Hair Keratin 1	N/A	N/A
1537.4	Background Peak	N/A	N/A
1539.7835	Keratin 1	MSO	1523.7886
1540.7676	Porcine Trypsin (Sigma-Aldrich and Promega)	PAM	1469.7304
1542.6675	Keratin 1	N/A	N/A

1543.6996	Keratin 10	N/A	N/A
1546.6851	Type 1 Hair Keratin 1	MSO	1530.6902
1546.8509	Type 1 Hair Keratin 1	N/A	N/A
1556.8391	Type 1 Hair Keratin 1	N/A	N/A
1574.6574	Keratin 1	MSO	1542.667
1586.7656	Keratin 9	N/A	N/A
1599.8336	Keratin 1	N/A	N/A
1603.8723	Type 1 Hair Keratin 1	CAM	1546.8509
1605.7431	Keratin 9	N/A	N/A
1617.888	Type 1 Hair Keratin 1	PAM	1546.8509
1619.7156	Keratin 9	N/A	N/A
1622.8271	Keratin 10	N/A	N/A
1625.8493	Type 1 Hair Keratin 1	N/A	N/A
1636.8176	Keratin 10	N/A	N/A
1639.7	Background Peak	N/A	N/A
1640.7735	Keratin 10	N/A	N/A
1644.7331	Type 1 Hair Keratin 1	CAM	1530.6902
1650.7289	Keratin 9	N/A	N/A
1653.8152	Keratin 1	N/A	N/A
1657.7928	Keratin 1	N/A	N/A
1669.8101	Keratin 1	MSO	1653.8152
1672.7644	Type 1 Hair Keratin 1	PAM	1530.6902
1687.9701	Keratin 1	N/A	N/A
1707.7721	Keratin 10	N/A	N/A
1708.7132	Keratin 1	N/A	N/A
1712.8925	Type 1 Hair Keratin 1	N/A	N/A
1721.766	Keratin 9	PAM	1650.7289
1730.9548	Keratin 1	N/A	N/A
1731.9136	Type 1 Hair Keratin 1	N/A	N/A
1736.8424	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
1741	Background Peak	N/A	N/A
1751.8266	Keratin 1	N/A	N/A
1765.7346	Keratin 1	CAM	1708.7132
1768.7992	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
1779.7503	Keratin 1	PAM	1708.7132
1787.965	Type 1 Hair Keratin 1	N/A	N/A
1791.7276	Keratin 9	N/A	N/A
1793.8639	Porcine Trypsin (Sigma-Aldrich and Promega)	CAM	1736.8424
1797.0116	Keratin 10	N/A	N/A
1807.8796	Porcine Trypsin (Sigma-Aldrich and Promega)	PAM	1736.8424
1813.8939	Keratin 1	N/A	N/A
1830.9093	Keratin 1	N/A	N/A
1839.1	Background Peak	N/A	N/A
1844.9461	Keratin 1	N/A	N/A
1848.9926	Type 1 Hair Keratin 1	N/A	N/A
1853.9	Background Peak	N/A	N/A
1855.9218	Keratin 9	N/A	N/A
1867.9218	Keratin 9	MSO	1851.9269
1869.0552	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
1871.9167	Keratin 9	MSO	1855.9218
1880.9534	Keratin 10	N/A	N/A

1881.8865	Type 1 Hair Keratin 1	N/A	N/A
1881.9705	Keratin 1	N/A	N/A
1882.8422	Porcine Trypsin (Sigma-Aldrich and Promega)	CAM	1768.7992
1896.9484	Keratin 10	MSO	1880.9534
1910.8735	Porcine Trypsin (Sigma-Aldrich and Promega)	PAM	1768.7992
1938.9589	Keratin 9	N/A	N/A
1953.1127	Keratin 10	N/A	N/A
1954.9538	Keratin 9	MSO	1938.9589
1957.9688	Type 1 Hair Keratin 1	N/A	N/A
1966.0603	Keratin 9	N/A	N/A
1973.9637	Type 1 Hair Keratin 1	MSO	1957.9688
1985.8943	Keratin 1	N/A	N/A
1994.7	Background Peak	N/A	N/A
1996.9709	Keratin 10	N/A	N/A
1998.941	Type 1 Hair Keratin 1	N/A	N/A
2005.0937	Type 1 Hair Keratin 1	N/A	N/A
2013.907	Keratin 10	N/A	N/A
2017.8841	Keratin 1	MSO	1985.8943
2022.025	Type 1 Hair Keratin 1	N/A	N/A
2037.9876	Type 1 Hair Keratin 1	N/A	N/A
2038.0161	Keratin 9	N/A	N/A
2045.9637	Type 1 Hair Keratin 1	N/A	N/A
2047.0627	Type 1 Hair Keratin 1	N/A	N/A
2069.9781	Type 1 Hair Keratin 1	PAM	1998.941
2070.9284	Keratin 10	CAM	2013.907
2079.9146	Keratin 1	N/A	N/A
2084.9441	Keratin 10	PAM	2013.907
2091.8822	Keratin 9	N/A	N/A
2102.9851	Type 1 Hair Keratin 1	CAM	2045.9637
2104.0842	Type 1 Hair Keratin 1	CAM	2047.0627
2105.0621	Type 1 Hair Keratin 1	N/A	N/A
2109.0532	Keratin 9	PAM	2038.0161
2117.0008	Type 1 Hair Keratin 1	PAM	2045.9637
2118.0998	Type 1 Hair Keratin 1	PAM	2047.0627
2121.018	Keratin 1	N/A	N/A
2146.0159	Keratin 9	N/A	N/A
2150.0777	Keratin 1	N/A	N/A
2155.0812	Type 1 Hair Keratin 1	N/A	N/A
2158.0307	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
2174.0256	Porcine Trypsin (Sigma-Aldrich and Promega)	MSO	2158.0307
2176.0992	Keratin 10	N/A	N/A
2203.0144	Keratin 9	MSO	2171.0246
2205.0716	Keratin 9	N/A	N/A
2210.0472	Keratin 10	N/A	N/A
2212.0979	Keratin 10	N/A	N/A
2212.1026	Type 1 Hair Keratin 1	CAM	2155.0812
2215.0522	Porcine Trypsin (Sigma-Aldrich and Promega)	CAM	2158.0307
2221.0666	Keratin 9	MSO	2205.0716
2226.1183	Type 1 Hair Keratin 1	PAM	2155.0812
2229.0678	Porcine Trypsin (Sigma-Aldrich and Promega)	PAM	2158.0307

2234.1775	Type 1 Hair Keratin 1	N/A	N/A
2236.9536	Keratin 1	N/A	N/A
2247.1788	Type 1 Hair Keratin 1	N/A	N/A
2271.1291	Keratin 9	N/A	N/A
2283.0921	Keratin 10	N/A	N/A
2285.9628	Keratin 10	N/A	N/A
2286.1248	Keratin 1	N/A	N/A
2293.9751	Keratin 1	CAM	2236.9536
2298.1506	Type 1 Hair Keratin 1	N/A	N/A
2299.1196	Keratin 9	N/A	N/A
2299.175	Porcine Trypsin (Sigma-Aldrich and Promega)	MSO	2283.1801
2304.2002	Type 1 Hair Keratin 1	CAM	2247.1788
2307.1826	Type 1 Hair Keratin 1	N/A	N/A
2307.9907	Keratin 1	PAM	2236.9536
2318.2159	Type 1 Hair Keratin 1	PAM	2247.1788
2325.1577	Keratin 9	N/A	N/A
2329.1742	Keratin 1	N/A	N/A
2329.1816	Keratin 9	N/A	N/A
2331.1074	Type 1 Hair Keratin 1	N/A	N/A
2331.1094	Keratin 9	MSO	2299.1196
2338.2361	Type 1 Hair Keratin 1	N/A	N/A
2340.1136	Keratin 10	CAM	2283.0921
2341.1526	Keratin 9	MSO	2325.1577
2345.1765	Keratin 9	MSO	2329.1816
2349.2183	Keratin 1	N/A	N/A
2354.1292	Keratin 10	PAM	2283.0921
2355.1721	Type 1 Hair Keratin 1	CAM	2298.1506
2356.9999	Keratin 10	PAM	2285.9628
2357.2306	Keratin 9	N/A	N/A
2369.1877	Type 1 Hair Keratin 1	PAM	2298.1506
2377.1881	Keratin 9	N/A	N/A
2382.1792	Keratin 9	CAM	2325.1577
2388.1288	Type 1 Hair Keratin 1	CAM	2331.1074
2396.1948	Keratin 9	PAM	2325.1577
2400.2115	Keratin 1	N/A	N/A
2402.1445	Type 1 Hair Keratin 1	PAM	2331.1074
2409.164	Keratin 10	N/A	N/A
2416.2064	Keratin 1	MSO	2400.2115
2457.2	Porcine Trypsin (Sigma-Aldrich and Promega)	N/A	N/A
2463.2838	Type 1 Hair Keratin 1	N/A	N/A
2508.1453	Keratin 1	N/A	N/A
2510.1317	Keratin 9	N/A	N/A

Mascot Search Results – Beta Galactosidase (LacZ Protein)