BBTM 04 Placement & Projects

Method Development for the Proteomic Analysis of Acute Rejection in Renal Allograft And Biomarkers Discovery

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ABSTRACT

Biomarker discovery for the noninvasive diagnosis of acute rejection will improve clinical management of renal transplant patients. Proteomics is a promising new discipline and is actively been studied for biomarker discovery in renal disease. To perform successful proteomic studies the sample preparation procedures need to be optimized. This study sought to develop methods for the proteomic analysis of acute rejection in renal allograft. The objectives of this study were achieved using the U937 cell line. The cell line was stimulated with INF-γ and TNF-α as model of acute inflammation and compared to an un-stimulated control. Proteins were extracted using the PARIS™ method and concentrated by lyophilisation. Protein samples separated by 1D SDS-PAGE after lyophilisation showed significant protein degradation. Protein degradation was reduced using protease inhibitors. However, these samples did not separate well during isoelectric focusing and the resulting 2D gel images. Isoelectric focusing was significantly improved by desalting the samples on P^™ desalting columns. This improved protein visualization on subsequent 2D gel separation visualized using the fluorescent stain SYPRO Ruby. Subsequent parallel 2D gels were then analyzed with Progenesis PG220 and SameSpots (Nonlinear Dynamic) software packages. 2D gel analysis with the computer software identified differential expressed of proteins between stimulated and non-stimulated U937 protein samples, detecting both up and down regulated proteins. Information derived from market research show biomarkers as an emerging market and has high commercial potential from clinical applications to drug discovery as well as in the field of renal transplantation. Screening of renal transplant patients for acute rejection will reduce the burden of the disease to help moving towards healthier patient lifestyle and more cost-effective healthcare delivery.
ACKNOWLEDGEMENTS

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<th>Description</th>
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<tr>
<td>1D</td>
<td>One dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>Two Dimensional</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric Focusing</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon- gamma</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted Laser Desorption/ionization</td>
</tr>
<tr>
<td></td>
<td>- Time of Flight</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly-acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor- alpha</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary Track Infection</td>
</tr>
</tbody>
</table>
PART 1

INTRODUCTION
1.1 Renal Diseases & Transplantation: General Background

Progression of renal diseases leads to end stage renal failure when the impairment to renal function is sufficiently severe. Among the primary causes of end stage renal disease (ESRD) included diabetic nephropathy, hypertensive nephrosclerosis, glomerulonephritis, benign or malignant. Symptoms associated with ESRD include peripheral neuropathy, affected mental status (encephalopathy), hyperkalemia and edema due to body toxic retention (e.g. elevated blood urea nitrogen and plasma serum creatinine) or inadequate potassium and sodium secretion (Rose & Renke, 1994).

ESRD is an important health problem causing worldwide morbidity and mortality. According to National Kidney Foundation (2002), the numbers of patients with ESRD has quadrupled from 100,000 to 400,000 over the last 20 years with the rate increases by 3 to 5 % per year. Patients with ESRD may subject to dialysis therapy which is attempted to remove fluid, potassium, and uremic toxins to relieve the symptoms of ESRD. But patients receiving dialysis may experience continue metabolic effects of renal failure and there are probable side-effects from the treatment itself. The side-effects include osteomalacia or encephalopathy due to aluminum in the water supply (De Broe & Van de Vyver, 1985), worsening bone problems, fungal infection, peritonitis, amyloidosis, e.g. joints pain etc. (Dische, 1994; Rose & Renke, 1994).

With the first successful tissue transplantation in man in 1954, renal transplantation is now the treatment of choice in most patients with ESRD. Renal transplantation has proved as the better therapy over dialysis and patients have greater potential to return to a healthy life (Dische, 1994). Despite issues in shortage of donor organ supply, legal, cultural and socioeconomic aspects, renal transplantation is the best form of renal replacement therapy and has continues to grow worldwide.

With improvements in management together with advances in transplant immunology and pharmacotherapy, there are now steady and continuous improvements in short term renal allograft survival rates. The rate of renal allograft failure in the first year post transplant is 18 % for cadaveric transplants and 6 % for living-donor transplants. Long term renal survival rate has also modestly improved with an annual 3 to 4 % persistent graft loss (Cecka, 2000).

1.2 Renal Allograft Rejection

Despite the progressive improvement in short-term and long-term renal allograft survival rate, transplant half-life is still poor and is significantly influenced by immune rejection (Hariharan et al., 2000). Immune responses causing rejection of the graft are mediated by lymphocytes, antibodies or both and result in inflammatory reactions that damage the allograft. Based on clinical and pathologic features (Table 1), there are hyperacute rejections which happen during the early post-transplant period, acute rejection, which may occur at any time, and chronic rejection, which is an indolent form of graft damage that occurs over months or years and leads to progressive decline of graft function.
### Table 1.1 Pathological features of kidney allograft rejection

*GBM: glomerular basement membrane

Acute allograft rejection has been recognized as major impediment to improved success in renal transplantation. It is characterized by a sudden deterioration in renal allograft function and is the principal cause of early graft lost and long term graft dysfunction, chronic allograft nephropathy (Hariharan et al., 2000; Meier-Kriesche et al., 2000). In addition, acute rejection was also recognized as one of the four most common causes of end-stage renal disease and an important factor in the organ shortage problem in the US (Agodoa et al., 1997).

#### 1.2.1 Immune Mechanisms of Acute Renal Allograft Rejection

Acute renal allograft rejection typically develops soon after transplantation, normally within days or weeks. It is a process of injury caused by infiltration of cells from the recipient’s immune systems which leads to multiple responses within the allograft and subsequent graft dysfunction. Acute rejection is mediated mainly by T-cell lymphocytes (Thomas et al., 1992). The T-cell lymphocytes are activated by donor transplant antigens (alloantigens) and shows direct specificity against donor antigens.
Alloantigens are proteins encoded in the recipient antigen presenting cells or major histocompatibility complex (MHC, the major determinant of rejection) and are recognized by recipient T-cells as allogeneic MHC molecules. A large proportion of the T-cell population is activated by an allograft in acute allograft rejection. The alloactivated T cells includes donor specific cytotoxic T-lymphocytes (CTLs) which will migrate through graft vascular wall. These graft-infiltrating lymphocytes will directly destroy or react against graft vascular cells and leads to the inflammation and damage of renal vasculature.

The graft-infiltrating lymphocytes also mediate a delayed type hypersensitivity mechanism of graft rejection, also known as persistent sub-clinical rejection. Sub-clinical rejection involves the recruitment of macrophage and release of various inflammatory factors such as B-lymphocytes, interleukins, chemokines and cytokines which causes nephron loss, chronic interstitial fibrosis or tubular atrophy and lead to chronic allograft nephropathy (Abbas & Lichtman, 2004).

1.2.2 Importance of Acute Rejection in Renal Transplantation

According to Hariharan et al., (2000), about 35% of allograft recipients have an episode of acute rejection in the first year after transplantation. Cadaveric renal allografts, which are performed most in renal transplantation, are associated with a 20% reduction in the one-year graft survival rate and four years shorter of projected half-life in patients who have had an episode of acute rejection compared to those who have not (Cecka, 2000).

The projected half-life of cadaveric renal transplants for recipients with and without an episode of clinical rejection was 7.0 versus 8.8 years in the year 1988 and 8.0 versus 17.9 years in the year 1995 (Hariharan et al., 2000) (Figure 1.1). As can be seen, although there was improvement in the projected half life of cadaveric renal transplants for recipients with an episode of clinical rejection over the years, the improvement is not significant. Also, compared to those who have rejection the overall projected half-life of cadaveric grafts transplanted in 1988 was found to be almost double among those without rejection.

Reports also suggest that rejection is detected in 30% of biopsies from patients thought to have stable renal function or to have been successfully treated for rejection (Li et al., 2001). All the figures mentioned show the severity and impact of acute renal allograft on the improved success in renal transplantation. According to Harper et al. (2000), about 20% of the patients in the US who are on the waiting list are those with a failed graft due to acute rejection and about 15% of the procedures performed are repeated transplantations. Chronic allograft nephropathy and death with functioning allograft has accounts for a total of 80% allograft failure (Howard et al., 2002).
1.2.3 Prevention & Treatment of Graft Rejection

The basis of preventing and treating the rejection of organ transplants is immunosuppressants. New immunosuppression drugs include corticosteroids, tacrolimus, azathioprine, rapamycin, deoxysoergualin, cyclosporine (CsA). Current immunosuppressive therapy is designed mainly to prevent and reduce acute T cell-mediated rejection (Table 1.2). CsA for example, functions by blocking the T-cell phosphatase that is requires to activate the transcription factor NFAT and inhibit the transcription of immune regulatory genes e.g. cytokines. Introduction of CsA has improved the success of kidney transplantation by reducing the incidence and severity of allograft rejection (Yoshimura et al., 2004).

Nonetheless, the advantages and limitations of these pharmacotherapy drugs are well known. All these immunosuppressants carry the problem of non-specific immunosuppression where the drugs may inhibit responses to more than the graft. There are also clinical problems in over- and under-immunosuppression. Consequently, patients treated with these drugs may become susceptible to infections, particularly by intracellular microbes, and have an increased incidence of cancers, especially tumors caused by oncogenic viruses.

Sometimes immunosuppressive agents may also cause nephrotoxicity itself and lead to acute and chronic allograft dysfunction. Accurate and relatively noninvasive diagnostics using easily accessible bodily fluids such as serum, plasma, urine or saliva for the serial monitoring of graft function and early detection of rejection before it becomes clinically apparent would be timely. Immunosuppression doses can be adjusted and optimized accordingly to specific individual needs and reduce complications.
Table 1.2 Treatments for graft rejection. Immunosuppression drugs commonly use to treat the rejection of organ grafts and the mechanisms of action.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
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<tbody>
<tr>
<td>Cyclosporine and FK 506*</td>
<td>Blocks T cell cytokine production by inhibiting activation of the NFAT transcription factor</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>Blocks lymphocyte proliferation by inhibiting guanine nucleotide synthesis in lymphocytes</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Blocks lymphocyte proliferation by inhibiting IL-2 signaling</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>Reduce inflammation by inhibiting macrophage cytokine secretion</td>
</tr>
<tr>
<td>Anti-CD3 monoclonal antibody</td>
<td>Depletes T cells by binding to CD3 and promoting phagocytosis or complement-mediated lysis (used to treat acute rejection)</td>
</tr>
<tr>
<td>Anti-IL-2 receptor antibody</td>
<td>Inhibits T-cell proliferation by blocking IL-2 binding. May also opsonize and help eliminate activated IL-2R-expressing T-cells</td>
</tr>
<tr>
<td>CTLA4-Ig</td>
<td>Inhibits T-cell activation by blocking B7 costimulator binding to T-cell CD28; used to induce tolerance (experimental)</td>
</tr>
<tr>
<td>Anti-CD40 ligand</td>
<td>Inhibits macrophage and endothelial activation by blocking T-cell CD40 ligand binding to macrophage CD40 (experimental)</td>
</tr>
</tbody>
</table>

* FK 506 is a drug that works like cyclosporine, but FK 506 is not used as widely. Adapted from: Abbas, A.K. and Lichtman, A.H., (2004) Basic Immunology, page 190.
1.3 Diagnosis of Acute Allograft Rejection

Clinical and pathological diagnosis for the classification of acute rejection (e.g., Banff 97 classification, Racusen et al., 1999) remains unreliable in predicting transplant outcomes and graft responses to therapy. Current diagnostic standard depends on a set of histologic parameters on percutaneous renal allograft biopsy, clinical presentation and measurement of biochemical organ function. However, these methods are not flawless, lack of sensitivity and often fail to detect acute rejection until late stages of progression. Percutaneous renal biopsy is invasive, costly and associated with significant patients’ risk such as pain, bleeding and even graft loss. Long-term renal allograft survival is still dependent on the early detection of acute rejection along with adequate treatment.

1.3.1 Molecular & Noninvasive Diagnostic Methods

Molecular and noninvasive diagnostic strategies are now being actively tested in multicenter clinical trials sponsored by the National Institutes of Health and the Immune Tolerance Network (Strom, 2005). Earlier studies in the screening of acute graft rejection from clinical symptoms to the genome-wide analysis of biopsy specimens and peripheral blood by using different approaches including DNA microarray, flow cytometry, & immunoblotting (ELISA) have been described. The studies shown altered expression of a number of gene products in the specimens during the clinical course of the disease such as the expression of T-cell activated genes CD3 (Li et al., 2001a), CD25 and FOXP3 (Muthukumar et al., 2005) in biopsy samples, and amplified expression of CTL in circulating blood cells by Vasconcellos et al. (1998).

Studies using urine-sediment cells included the mRNA measurement of CD 103 levels, a cell surface marker for CTLs (Ding et al., 2003), mRNA-encoding cytotoxic protein granzyme B and perforin (Li et al., 2001b), and urinary monokine induced by IFN-γ (MIG) as the predictor of acute rejection (Ding et al., 2003). Lately, low concentrations of donor-derived DNA (microchimerism) in serum were detected by Gadi et al. (2006) demonstrating the potential generic biomarkers of cell-free DNA in serum as the setting of organ rejection monitoring.

1.4 Proteomics: A Perspective in Molecular & Noninvasive Diagnostics

‘Proteome’ can be simply defined as “a set of proteins encoded by the genome” while ‘proteomics’ is “any-large scale protein-based systematic analysis of the entire proteome or a defined sub-proteome from a cell, tissue, or entire organism” (Speicher, 2004). Proteomic analysis is a promising new tool for functional genomics and is now widely applied to several fields of biological and biomedical research since its birth in the mid-1990s. This new discipline of research was enabled by the completion of human genome project together with the development of methodologies such as mass spectrometry, protein chips and 2D gel electrophoresis (Speicher, 2004).

Recently, the concept of clinical proteomics has permitted the translation of the proteomic technologies to bedside utility (clinical practice or ‘near-patient’ diagnostic).
Clinical application of proteomics can be exploited as a fundamental knowledge to better understand normal physiology and the complexity of disease mechanisms at protein level, and in searches for novel biomarkers of diseases and new therapeutic targets. Proteomics has already proved valuable in the field of neurology and cancer diagnosis e.g. ovarian cancer (Petricoin et al., 2002).

Proteomics in renal transplantation is in its early phase. Nonetheless, renal and urinary proteomics has demonstrated strength and great potential in nephrology research as well as in acute renal allograft rejection. Identification of distinctive disease proteomic profiles that correlate with clinical features will enable the development of biomarkers relevance for molecular and noninvasive diagnosis methods (Thongboonkerd, 2005).

1.4.1 Theories & Methodologies

Information from the advent of human genome sequencing is only partial and will only be completed as a whole when all of the possible gene products, i.e. proteins, are known. Genomes of simple prokaryotes to higher eukaryotes although complex are finite and largely static over the lifetime of an organism. Transcription from DNA to RNA may remain stable but regulation at the level of translation, for example post-translational modifications, protein-protein interaction, alternative gene splicing etc can generate several proteins from a single “gene”. Measurement of mRNA expression provides no information about posttranslational protein modification. Thus, proteomics can be used to provide information at the protein level and in a global manner that is not easy to determine from genomic sequences alone.

Compared to genomics where a uniform experimental technique can be used for nucleic acids, proteomics is far more challenging and complex. There is not a single biochemical method for the analysis of all proteins due to the diversity of protein expressions. Analytical methods of proteome analysis generally begin with protein extraction, followed by protein separation using 2D polyacrylamide gel electrophoresis. Specific proteins that showed altered expression at the protein level between two parallel sample that have been separated by gels can then be digested to produce peptide fragments that can be “sequenced” by mass spectrometry (MS) allowing protein identification.

In MS, peptides will go through an ionization source followed by molecular mass (or mass-to-charge ratios, m/z) measurement of the resulting ionized peptides (Figure 1.2). This is usually performed by MALDI-TOF-MS for whole proteins. Finally, by utilizing bioinformatics tool, peptide ions observed in the spectrum will be compared with a database of predicted proteins masses from known protein sequences (Speicher, 2004; Hoorn et al., 2005). Protein composition and altered protein expression between normal and diseases biological samples will then compared and known.
Figure 1.2 Flow diagrams of protein identification by mass spectrometry analysis from a simple mixture to the correlative study with real time PCR in clinical applications

Adapted from: Hoorn et al., (2005)

1.4.2 TaqMan-PCR in the Analysis of Potential Biomarkers

Although proteomics can be used for biomarker discovery, a proteomic assay alone is not practical for everyday screening of transplant patient. cDNA can be generated from RNA extract from the biological sample using the enzyme reverse transcriptase (RT) for real-time PCR analysis of potential biomarkers identified from a proteomic analysis. This is very important as gene expression through measurements of RNA levels may not necessarily predict the protein translation levels. Commercially available TaqMan real-time PCR assays are available for all human gene and could be screened for i.e. a correlative studies with proteomics. It is a relatively advanced technology and widely accepted for routine detection and quantitative analysis of DNA and RNA nucleic acid.

The key features of TaqMan-PCR include the fluorescent dye-labeled TaqMan probe which is complementary to the target nucleic acid sequence and allowed the real-time accumulation and measurement of fluorescent data points from every PCR cycle. This will enable the detection of specific DNA or RNA and through this gene expression the biomarkers from proteomics can then be identified. A PCR based methods for the detection of acute rejection biomarkers will be more useful clinically for routine monitoring of transplant patients.
1.4.3 Proteomics in Acute Renal Allograft Rejection

Several studies with urinary proteomics had been performed to determine whether the urine of renal transplants patients undergoing acute allograft rejection has a characteristic profile. An earlier study (Lapin et al., 1989) had found protein spots that might be an indicator for acute rejection with 2D PAGE. In a current study by Clarke et al. (2003), urinary proteome profiles analyzed by SELDI-TOF-MS have identified peptides with molecular weight (M_r) of 6.5, 6.6, 6.7, 7.1 and 13.4 kDa as potential candidates of diagnostic markers.

Another study with the same SELDI-TOF proteome technique and very rigid patient selection criteria, which included allograft function, histology and clinical course was able to distinguish different renal diseases from acute rejection, Schaub et al. (2005) found the presence or absence of three prominent peak clusters correlated with the clinicopathologic course in most patients. The three prominent peaks clusters were found in 17 of 18 patients (94%) with acute rejection episodes, none from 28 normal controls, and with only four out of 22 patients (18%) without clinical or histologic evidence of rejection (stable transplant group). But none of the potential biomarkers was identified in the study.

Additional to the immunological mechanism in the development of acute rejection, other complications may lead to infiltration of lymphocytes into the graft. For instance inflammation of graft may be mediated by virus infected cells, e.g. Cytomegalovirus (CMV) in a urinary tract infection (UTI), and at times graft infiltrating lymphocytes itself will mediate the processes of recurrent autoimmune disease. The immune mechanisms and characteristics of renal diseases had become confounding factors in the blood or urinary proteomic analysis of acute cellular rejection. Previous studies have recognized the importance and difficulties in the differential diagnosis of graft inflammation between the acute rejection and other renal diseases, which is essential as all complications needs a different therapy.

CMV is the most important in UTI viral infection. CMV-infection together with acute allograft rejection has been identified as the most common complications after renal transplantation. Transplantation of kidneys infected with the virus may lead to lung infections in the immunosuppressed recipient (Millard et al., 1973). Post transplant CMV viremia is also one of the most common causes of systemic inflammation which lead to the filtration of inflammatory proteins (e.g. cytokines, chemokines) by the transplant kidney. As acute graft rejection is also defined as infiltration of the graft by immune inflammatory factors, CMV viremia had become a potential confounder of diagnostic from specificity of acute renal allograft rejection (Karpinski et al., 2003).

Acute tubular necrosis (ATN) and glomerulopathies (glomerular diseases) are also important in the differential diagnosis of allograft dysfunction from acute renal allograft rejection. For example, urine proteins associated with rejection may also be found in tubular-based pathology e.g. tubular atrophy, calcineurin-inhibitor-toxicity, pyelonephritis. Nevertheless, proteomics in the field of nephrology has enabled the
findings of some proteins correlated with specific renal diseases and which would enable
distinguish between alloimmune from other causes of graft inflammation. Summary of
some findings of common renal diseases are given in Table 1.3.

Table 1.3 Summary: some findings from recent proteomic studies in renal diseases

<table>
<thead>
<tr>
<th>Renal Diseases</th>
<th>Findings of involved proteins</th>
</tr>
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<tbody>
<tr>
<td><strong>Glomerular diseases</strong></td>
<td></td>
</tr>
<tr>
<td>• IgA nephropathy</td>
<td>IgA1</td>
</tr>
<tr>
<td>• FSGS</td>
<td>MBL-associated serine protease*, Albumin*, α-fibrinogen, Fibulin</td>
</tr>
<tr>
<td>• Diabetic nephropathy</td>
<td>C1q-binding protein</td>
</tr>
<tr>
<td></td>
<td>Contraception-associated protein Elastase, Myosin, Troponymosin</td>
</tr>
<tr>
<td><strong>Tubulointerstitial diseases</strong></td>
<td></td>
</tr>
<tr>
<td>• CsA nephrotoxicity</td>
<td>Calbindin-D28k*</td>
</tr>
<tr>
<td>• Lead nephrotoxicity</td>
<td>Aflatoxin B1 aldehyde reductase, Transketolase</td>
</tr>
<tr>
<td>• Radiocontrast-induced</td>
<td>Calcineurin</td>
</tr>
<tr>
<td>• Gentamicin nephrotoxicity</td>
<td>HSP70, α₂-microglobulin*, Transferrin*</td>
</tr>
<tr>
<td>• Dent’s disease</td>
<td>β₂-microglobulin*</td>
</tr>
<tr>
<td></td>
<td>Actin, α₂-tubulin*, Acetyl-CoA carboxylase, ATP synthase</td>
</tr>
<tr>
<td></td>
<td>α subunit, Fatty acid transport protein</td>
</tr>
<tr>
<td><strong>Vascular disease</strong></td>
<td></td>
</tr>
<tr>
<td>• Renovascular hypertension</td>
<td>3010027A04Rik, TypeA/B hnRNP p40</td>
</tr>
<tr>
<td></td>
<td>Phosphatase Ser/Thr protein kinase 10</td>
</tr>
<tr>
<td><strong>Renal Cancer</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytokeratin 8, Vimentin, α-tubulin*</td>
</tr>
<tr>
<td></td>
<td>Aldolase A, Aconitase, Aldehyde dehydrogenase 1</td>
</tr>
<tr>
<td></td>
<td>LaminB1, Elongation factor 2</td>
</tr>
<tr>
<td><strong>Renal Transplantation</strong></td>
<td>Unidentified</td>
</tr>
<tr>
<td><strong>ESRD</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cystatin C*, α1-antitrypsin, Retinol-binding protein*</td>
</tr>
<tr>
<td></td>
<td>α-fibrinogen, salivary, praline-rich protein</td>
</tr>
</tbody>
</table>

* Proteins whose altered expression was observed in more than one disease.
CsA: Cyclosporine A; FSGS: Focal segmental glomerulosclerosis; hnRNP: Heterogeneous nuclear ribonucleoprotein; Ig: Immunoglobulin; MBL: Mannan-binding lectin; HSP: Heat shock protein.
Adapted from: Thongboonkerd, V. (2005)

ATN can be regarded as a model of injury to the tubules due to ischemia-reperfusion. Findings in the renal tubular diseases with proteomic studies include the Dent’s diseases (Table 1.3) which is associated with renal Fanconi syndrome. Studies with proteomic analysis found out that several vitamins, apolipoproteins, cytokines, prosthetic group-carrier proteins etc are at the higher amount in the Dent’s urine compared with normal control. The proteins are suggested to cause reabsorption and thus impaired function of proximal tubular in the renal with Fanconi syndrome (Cutillas et al., 2004).
Whereas the injuries cause by the glomerulopathies is centered largely on the glomerular capillary. The most common glomerular disease worldwide is immunoglobulin nephropathy (IgAN) which is cause by the deposition of immune complexes, IgA1 and other unknown foreign antigens in the glomerular mesangium. With proteomics, it was now confirmed that predominant glomerular deposition of IgA1 is due to the underglycosylation of O-glycans in serum IgA1 (Hiki et al., 2001).

In the study by Schaub et al., (2005), patients with ATN, glomerulopathies and lower urinary tract infection (UTI) with Cytomegalovirus (CMV) were also included in the study. The resulting study showed there was no comparable expression pattern when compared to acute renal allograft rejection in the three groups of renal diseases patients. Both ATN and glomerular diseases represent pathophysiology models of allograft injury distinct from that due to the alloimmune response. Another study using CE-MS also identified a distinct urinary polypeptide pattern in patients with acute rejection and a different polypeptide pattern in patients with UTI compared both to patients without evidence of rejection and infection (Wittke et al., 2005).

Systematic analysis of protein-expression patterns provides a window on the biology and pathogenesis of renal allograft rejection. Although the exact protein has not been identified for acute renal allograft rejection, the finding of proteins associated with certain renal diseases has proved the potential of proteomic technologies in the field.
1.5 The Project

1.5.1 Biomarker Discovery & the Business Idea

A biomarker is a biologic characteristic that can be measured and evaluated as an indicator of normal and pathogenic biologic processes, or pharmacologic response to treatment (De Gruttola et al., 2001). It can be any parameter of a patient that can be measured ranging from gene expression products (mRNAs), genetic defects (mutations), proteins, proteomic patterns, lipids, imaging methods, or electrical signals (De Gruttola et al., 2001; Hewitt et al., 2004).

Table 1.4 Work flow of Biomarker Development Pathway

| 1. Discovery phase | - Initial preclinical exploratory  
|                   |   - discovery biomarker on tissue, serum or urinary samples (noninvasive specimen)  
|                   |   - Confirmation of preclinical discovery  
|                   |   - validate biomarker on same type of samples  
|                   |   - promising direction identified and prioritized  
| 2. Clinical assay development and initial validation | - Optimize assay  
|            |   - increase throughput, reproducibility  
|            |   - Set up clinical assay and test on existing samples  
|            |   - Clinical assay detect established disease  
| 3. Clinical utility Determination | - Retrospective longitudinal  
|                          |   - test biomarker in completed clinical trial  
|                          |   - detects disease early before it becomes clinically obvious  
|                          |   - ‘screen positive’ rule is determined; evaluate  
|                          |   - Sensitivity/specificity  
|                          |   - Prospective screening  
|                          |   - use biomarker to screen population  
|                          |   - extent and characteristics of disease detected by test  
|                          |   - false referral rate identified  
|                          |   - Disease control  
|                          |   - impact of screening on reducing the burden of disease  
| 4. Product Development | - Assay concept phase  
|                         |   - market analysis, intellectual property analysis  
|                         | - Assay development  
|                         |   - product and regulatory plans, stability testing  
|                         | - Alpha and Beta testing  
|                         |   - design verification protocols  
|                         |   - GMP materials and regulatory oversight  
|                         |   - test reproducibility/cross reactivity, defined protocol  
|                         | - Product launch  
|                         |   - assay fit for intended use, QC/QA in place  
| 5. Commercialization | - Commercial development of the assay  

Adapted from: Pepe et al. (2001)
Despite advances in the field of medicine we still lack significant diagnosis tools for major diseases. This is coupled with increasing clinical development costs and a recent decline in drug discovery success rate. This means the medical field must re-evaluate their clinical diagnosis assays and drug development process to save lives and in order to reduce attrition rates for pharmaceutical or biotechnology companies. Biomarkers in the next decade will change clinical diagnostics and the way in which pharmacy companies can determine the economic viability of their drug discovery process.

Discovery of biomarkers in the past has been a byproduct of unrelated research. With recent high throughput technologies of proteomics and other discovery technologies for example microarrays, histology, flow cytometry, chromatography and other instruments, the overall biomarker discovery effort has improved. Table 1.4 summarizes the work flow in the biomarker discovery pathway. Successful biomarker development requires an understanding of the entire biomarker development process, involving the primary knowledge of disease definition, the phases in the discovery process, clinical assay development and validation, determination of clinical utility, and finally product development and commercialization.

Although complex, the biomarkers may make a great contribution to clinical diagnosis and in the discovery of promising medical product. Biomarkers at the same time are also an evolving business idea in the commercialization of prospective solutions in various fields (Part 4). Biomarker discovery for the molecular and noninvasive diagnosis of acute renal allograft rejection will be of great advantage for patients, and also of commercial value in supporting patient care in renal transplant centers.

**1.5.2 Role of Biomarkers**

Technological advances have led to the emergence and rapid proliferation of biomarkers in clinical use and commercial from drug discovery, clinical development and molecular diagnostics. In clinical diagnosis, there are many advantages to having rapid and reliable test systems particularly for disorders that do not reveal obvious physical symptoms, and which are readily available to doctors at the point of care or in the ‘near-patient’ setting. Biomarkers will enable serial measurement, faster turnaround time, and relatively noninvasive and easy-to-perform tests.

Other than providing valuable clinical decision tools for diagnosis and monitoring of treatment, the role of biomarkers may also spans from drug discovery and development to target discovery and validation, study of drug and disease mechanisms, toxicity profiling and also to the use in clinical trials enrollment as secondary and surrogated endpoints (Hewitt et al., 2004) (Table 1.5).

Surrogate end-point marker is a rare biomarker that is used in substitution for other clinical end points, such as survival, cancer recurrence, stroke, or fracture (De Gruttola et al., 2001). Major concerns for researchers in the attempt to boost productivity and efficiency in the field of drug discovery and development are the escalating costs and lengthy clinical trials. The clinical trials phase currently accounts for over 60 per cent of
drug discovery and development cost, and it may take up to USD 1,700 million to get a drug to market [4].

Biomarkers and surrogate end point markers are emerging as the solution to drug discovery problems with their ability to reduce drug development costs and time, in which biomarkers will be able to supply efficacy, toxicity, and mechanistic information for the preclinical and clinical phases (Hewitt et al., 2004). Other roles of biomarkers also included its application with therapeutics to produce commercial tests which will aid patient selection or personalized medicine e.g. drug dosing (Table 1.5).

**Table 1.5 Roles of Biomarkers**

| Research/Preclinical phase | • Endpoint marker in animal studies  
|                          | - proof of concept testing  
|                          | - screening tool for leads  
|                          | - rank compounds in portfolio  
|                          | • Pharmacodynamic evaluation  
|                          | • Toxicity profile  
| Clinical phase           | • Early detection  
|                          | • Differential diagnostic  
|                          | • Identify subpopulations for clinical study  
|                          | - type/location of injury  
|                          | - mechanism of disease, mechanism of action  
|                          | • Predict severity and prognosis, regression, etc.  
| Surrogate endpoint       | • Drug effect, dose ranging studies  
|                          | • Focused hypothesis may shorten and decrease size of trial  
|                          | • Speed agents through testing and approval process  
| Commercial phase         | • e.g. test to aid drug discovery & dosing  

Adapted from: Hewitt et al. (2004)
1.6 Regulations & Legislative Requirement

Since it is relatively new, the regulatory environment for diagnostic assay utilizing high-throughput biomarker analysis is still emerging. Regulatory agencies include the major regulatory player US Food and Drug Administration, FDA (www.fda.gov) are currently on the stages of compromising the biomarkers safety and efficacy to the public. Key issues categorized by FDA includes regulations of biomarkers applications in clinical settings, regulation of product life cycle, regulation on biomarkers-based testing technologies, and regulation of data.

Other existing regulations applicable in the drugs discovery, development and clinical diagnostics of biomarkers in the UK includes:

- Medicines and Healthcare Product Regulatory Authority
- Medicines for Human Use (Clinical Trials) Regulations 2004 (MHRA)

Principal role of Medicines and Healthcare Product Regulatory Authority is to ensure that all medicines, medical devices and equipment on the UK market meet appropriate standards of safety, quality and performance. MHRA require that the medicines used in clinical trials are manufactured to Good Manufacturing Practice (GMP) standards by a licensed manufacturer and that each trial has an identified sponsor (or sponsoring group) with overall responsibility (KeyNotes, 2005).

Regulatory guidance from other major overseas markets are:

- Europe – European Medicines Agency (EMEA)
- Japan – Ministry of Health and Welfare (MHLW)

European Medicines Evaluation Agency (EMEA) was established by European Commission in 1995 for the administration of the centralized procedure for highly innovative or biotechnology-based drugs as well as a ‘mutual recognition’ procedure for most pharmaceutical regulation in EU states. Currently EMEA and FDA are working together in the initiatives to accelerate the approvals of diagnostics and new drugs discovery integrating biomarkers.

1.6.1 Patent

Biomarkers can be a valuable intellectual property but the patenting of biomarkers and their uses is complex as the body of case law is relatively new. Each marker and its uses claimed in a patent application must be checked against prior literature and patents to confirm novelty. Other than that, the assay enabled by well designed laboratory studies can also be the essence of the valuable intellectual property. Patent application for a novel biomarkers discovery can be applied from http://www.patent.gov.uk/ in the UK and http://www.uspto.gov/ in the US.
1.7 Aims and Objectives

1.7.1 Objectives of this Project

Aims and objective of the project includes:

- Development of methods for the proteomic analysis for acute renal allograft rejection, from sample preparation to method optimizations using the U937 lymphocyte cell line as a model.

- Identification of differences in protein expression with Progenesis PG220 SameSpots (Nonlinear Dynamics) software packages.

- Assessment of proteomics as a means for characterizing biomarker potential.

1.7.2 Long Term Objectives

Long term objective of this project is to develop a non-invasive diagnostic and monitoring method for the early detection of acute renal transplant rejection which constitutes the major impediment to the success of renal transplantation:

- Identification of biomarkers with proteomics technologies using urinary samples and analysis of the potential biomarkers by real-time TaqMan PCR.

- To usefully identify a combination of commercially available TaqMan PCR gene expression assays that can be used to non-invasively to identify patients with acute renal allograft rejection using as an alternative diagnostics or prior to renal biopsy.

- Non-invasive biomarker as an indicator of rejection and monitoring of graft immune response which will allowed optimization of immunosuppressive therapy to reduce the side-effects of immunosuppressants.

- Initiate clinical trials to enable translation of the assays into a clinical setting.
PART II

MATERIALS AND METHODS
2.1 Experiment Methods

2.1.1 Cell Culture

U937 lymphocytes cell line (ECACC 85011440) were grown in RMPI 1640 (with 2mM Glutamine and 10% Foetal Bovine Serum, FBS) culture media with or without the cytokines INF-\(\gamma\) and TNF-\(\alpha\) (Peprotech). Those not treated with cytokines acted as normal control. INF-\(\gamma\) and TNF-\(\alpha\) are pro-inflammatory and would stimulate an event such as acute rejection in organ transplantation. Both were incubated overnight (24 hours) at 37.5ºC.

2.1.2 Protein Extraction

Protein was extracted with Cell Disruption Buffer (PARIS™ Kit, Ambion) which allowed the direct disruption of U937 cells to obtain total protein. For each preparation \(10^6\) cells are spin down at 5,000rpm for 5 minutes and culture media were removed. Pelleted cells were washed by resuspending in ~1ml phosphate buffered saline (PBS), pelleted again and the supernatant were removed. To each pellet, 300ml of Cell Disruption Buffer were added as suggested by the manufacturer’s instruction and placed on ice for 15 minutes to enable lysis of the cells. Protein extraction was then ready and samples were kept at -70 ºC until required.

2.1.3 Protease Inhibitors

To inactivate protease activities in the protein sample, 6X protease inhibitors cocktail (Roche) were added together with Cell Disruption Buffer (PARIS™ Kit, Ambion) during protein extraction. Samples with PARIS™ reagent and protease inhibitors were placed on ice for 15 minutes to enable lysis of the cells and inactivation of protease activities. Samples were then spun down at 5000rpm for 5 minutes to clear the cell membrane and supernatant. Pellets were removed and supernatants (total protein samples) were kept at -70 ºC until required.

2.1.4 Desalting

Micro Bio-Spin™ Chromatography columns (Bio-Rad) were used for the purification of protein sample from salts or other protein contaminants. As refer to the manufacturer’s instruction, maximum 75μl of protein sample were loaded directly into each of the chromatography column after removal of the column’s gel packing buffer.

After loading of samples, columns were centrifuge for 4 minutes at 1,000 x g. Purified samples was then in either SSC (150mM sodium chloride, 17.5 mM sodium citrate, pH 7.0 with 0.02% sodium azide) buffer or Tris buffer (10mM Tris-HCl, pH 7.4 with 0.02% sodium azide) and where the molecules smaller than the column’s exclusion limit will be retained by the column. Purified samples were kept at -70 ºC until required.
2.1.5 Sample Concentration

Proteins samples need to be concentrated were lyophilized with CentriVap Centrifugal Concentrator (Labconco) at 60 ºC. Total volume of protein samples with the required protein concentration (through the determination of protein concentration with Bradford Protein Microassay, Bio Rad) were split into two and lyophilized in separate in eppendorf to speed up the protein concentration process via lyophilizing with the CentriVap. Protein sample was lyophilized to the suitable volume and was ready for further use.

2.1.6 1D Polyacrylamide Gel Electrophoresis

Protein samples were to be run on 1D polyacrylamide gel electrophoresis is prepared in duplicated. 15μl of each sample with 6X loading buffer were then incubated on heating block set to 95ºC for 5 minutes before loading into 12% hand cast gels (Table 2.1) and ran at 200V, 400mA. Gels were stained with Coomassie Brilliant Blue (Bio Rad).

<table>
<thead>
<tr>
<th></th>
<th>12% Running Gel (ml)</th>
<th>Stacking Gel (ml)</th>
</tr>
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<tbody>
<tr>
<td>Water (30%)</td>
<td>4.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Acrylamide (30%)</td>
<td>6.0</td>
<td>0.83</td>
</tr>
<tr>
<td>1.5M Tris HCl pH 8</td>
<td>3.8</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris HCl pH 6.8</td>
<td>-</td>
<td>0.63</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
<td>0.05</td>
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<tr>
<td>10% APS</td>
<td>0.05</td>
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<tr>
<td>TEMED</td>
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</tbody>
</table>

2.1.3 2D Polyacrylamide Gel Electrophoresis

First dimension total protein separation was performed using 7cm IPG strips, pH 4-9 (Bio Rad). Protein concentration of each protein sample was determined with Bradford Protein Microassay (Bio Rad) to ensure equal loading of samples into the 2D gel electrophoresis. The total volume with 125μg protein concentration of each sample (stimulated and non-stimulated) were lyophilized with CentriVap (Labconco) at 60 ºC to ~5μl. Lyophilized protein were then mix with 125μl of Sample Buffer (Bio Rad) for the rehydration of the 7cm IPG strips as suggested by the manufacturer’s instruction manual. Isoelectric focusing (IEF) was then performed with Protean IEF cell (Bio Rad).

Second dimension separation was performed using SDS-PAGE with 4-16% precast acrylamide gradient gels (Bio Rad) and 1XTGS (Bio Rad) running buffer. The use of precast consumables will provide a saving in time and labour, more consistent separation and speed up the subsequent analysis. IPG strips after IEF were treated with equilibrations buffer I and II as given in the Bio Rad instruction manual before SDS-PAGE electrophoresis at 200V, constant for approximately 40 minutes. The gels were stained with the red fluorescent dye SYPRO-Ruby (Bio Rad) which has a high dynamic range and sensitivity. Gel images were acquired using the Fluorchem gel documentation system (Alpha Innotech) and stored at 4 ºC until further use.
2.1.4 2D gels Analysis: Progenesis PG220 SameSpots (Non-Linear Dynamic)

A composite image for both 2D gels, stimulated and non-stimulated were generated and analyzed using the software 2D-Progenesis (Non-linear Dynamic) to compare for the differences between normal and pathogenic (stimulated) state. Statistical analysis of each protein was performed to identify which proteins are specific to or significantly increase or decrease in pathogenic state. Protein of interest can then brought for further studies with MALDI-TOF mass spectrometry.

2.2 Market Research

Market research was carried out to assess for the need for biomarkers applications in the diagnosis of acute renal allograft rejection in the transplant patients. The unique selling point of the biomarkers product was determined, as well as its market size, competitor analysis and SWOT (strengths, weakness, opportunities and threats) analysis. The aim of the market research is to assess the profitability of the biomarker market to its customers and provide advantages for potential customers.

2.2.1 Secondary Market Research

Secondary market research was performed via collection of information from published sources included the market and industry reports on biomarkers which includes electronic database from various providers (e.g. Mintels and KeyNotes), scientific magazines, trading magazines, government and company information and statistic etc. Secondary market research enables the knowledge of market size, trend and its development.

2.2.2 Primary Market Research

Primary market research enables the knowledge on the target market of biomarkers. Primary market can be approached via survey, observation, field test, questionnaires and interviews. However the later two methods were not carried out in this project for biomarkers market.

Target market for the biomarkers in the clinical applications of acute renal allograft rejection diagnosis in transplant patient were focus in the renal transplant statistic in the UK and overview of the health standard of UK populations as well as in the global overview for the renal transplant rate. Transplant centers were observed for its efficacy and distribution.
PART 3

RESULTS
3.1 Methods Optimization

U937 lymphocytes cell line (ECACC) grown in RMPI culture media and stimulated with cytokines INF-γ and TNF-α was treated as pathogenic state as in acute rejection while as those not treated with cytokines acted as normal control. Cells were then brought to protein extraction followed by proteomics methods development including the optimization and validation for sample preparation in 1D polyacrylamide gel electrophoresis (PAGE) and the subsequent visualization of results in 2D PAGE. Resulting 2D gels were then analyzed with the Progenesis PG220 SameSpots (Nonlinear Dynamics) software packages.

U937 protein samples were extract with Cell Disruption Buffer (PARIS™ Kit, Ambion) in protein sample preparation. PARIS™ method is based on the lysis of plasma cell membrane by nonionic detergents which allowed the direct disruption of cell in obtaining total protein sample. Total protein samples were then concentrated via lyophilizing with CentriVap (Labconco) at 60 ºC. Protein concentration via lyophilizing was performed as there is need of proper amount of sample volume together with protein loaded into IPG strips. Proper sample volume is important for optimum sample in-gel rehydration in the first dimension protein separation of 2D PAGE. Protein loaded is depends on the length of IPG strip and the stain that will be used in 2D gels visualization. Total volume of protein samples with the required protein concentration (through the determination of protein concentration with Bradford Protein Microassay) were thus concentrated into a smaller volume via lyophilizing in this experiment to enable the mixing of protein sample with proper amount of sample buffer for IPG strip rehydration.

![Figure 3.1](image)

**Figure 3.1** Comparison of U937 cell line total protein samples without lyophilized (lane 1, 2) and after lyophilized (lane 3, 4)

Lyophilized U937 protein sample were first optimized with the visualization of protein samples on 1D PAGE. Comparison of protein samples after lyophilized and without lyophilized was made (**Figure 3.1**). Proteins sample after lyophilized shows the visualization of fewer protein bands (lane 3 and 4) than in sample without lyophilized (lane 1 and 2). There are also observed vertical streaking and sample precipitations at the lower end of gel in lyophilized samples (lane 3 and 4). Vertical streaking may be due to
Thus, it can be deduced that there are increased enzyme protease at high temperature during lyophilisation of protein sample. Increased activities of enzyme proteases digest proteins generating small protein bands causing vertical streaking and also the observed fewer protein bands in samples without protease inhibitors (lane 1, 2). Vertical streaking may also due to protein samples overload and precipitation may be caused by denatured protein with high temperature during lyophilizing as mentioned. As protein sample after treating with enzyme protease gave better resolution of protein bands in lyophilized samples, U937 protein samples (stimulated and non-stimulated) with enzyme protease activities were then brought to the separation in 2D PAGE. However, the resulting 2D gels visualization with SYPRO Ruby (Figure 3.3) shown there are occurrence of both vertical and horizontal streaking in both stimulated (a) and non-stimulated (b) gels.

Thus, likely increased activities of enzyme protease activities were pursue by adding 6X protease inhibitors cocktail (Roche) together with Cell Disruption Buffer (PARIS™ Kit, Ambion) during protein extraction to inactivate protease activities in the protein sample. Comparison between samples after lyophilized but with (lane 3 and 4) and without enzyme protease inhibitors (lane 1 and 2) were made (Figure 3.2). There are also occurrence of vertical streaking and sample precipitations at the lower end of gel in both lyophilized samples with and without protease inhibitors. However, protein bands resolution is better in sample with protease inhibitors (lane 3 and 4) with less protein precipitation. There are observed fewer protein bands in samples without protease inhibitors (lane 1 and 2) than in sample with protease inhibitors.

**Figure 3.2** Comparison of U937 cell line total protein samples after lyophilized without protease inhibitors (lane 1, 2) and with protease inhibitors (lane 3, 4)

Thus, it can be deduced that there are increased enzyme protease at high temperature during lyophilisation of protein sample. Increased activities of enzyme proteases digest proteins generating small protein bands causing vertical streaking and also the observed fewer protein bands in samples without protease inhibitors (lane 1, 2). Vertical streaking may also due to protein samples overload and precipitation may be caused by denatured protein with high temperature during lyophilizing as mentioned. As protein sample after treating with enzyme protease gave better resolution of protein bands in lyophilized samples, U937 protein samples (stimulated and non-stimulated) with enzyme protease activities were then brought to the separation in 2D PAGE. However, the resulting 2D gels visualization with SYPRO Ruby (Figure 3.3) shown there are occurrence of both vertical and horizontal streaking in both stimulated (a) and non-stimulated (b) gels.
Figure 3.3 2D polyacrylamide gel electrophoresis of U937 cell line protein lysate stimulated (b) and non-stimulated (a) with cytokines INF-γ and TNF-α. First dimension: 7cm IPG strips pH 4-9. Sample load: 100μg of protein. Second dimension: vertical SDS-PAGE (4-16%). Red fluorescent dye SYPRO-Ruby stains.
Both 2D gels as in Figure 3.3 shown the occurrence of vertical streaking at the sample application point and intermittent horizontal streaking. 2D gel of stimulated U937 sample in Figure 3.3 also has the problem of large vertical blank stripe.

![Figure 3.4](image)

**Figure 3.4** vertical blank stripe in 2D polyacrylamide gel electrophoresis of stimulated U937 cell line protein lysate, without desalting.

According to the Bio Rad Proteomics Expression ([www.proteomicsexpression.com](http://www.proteomicsexpression.com)), horizontal streaks in 2D gels are associated with problem in the isoelectric point focusing (IEF) due to improper sample preparations. Problems with improper sample preparation include liquid in excess of the amount absorbed by the strip, incomplete focusing, overloading, high viscosity or sample solubility problems. While as vertical streaks are more related to second-dimension electrophoresis due to loss of solubility of a protein at its pl, interfering compounds, improper reducing agent, or incorrect placement of the IPG strips. However, IPG rehydration and IEF focusing were optimized according to the Bio Rad instructions manual during sample preparation for the 2D gels in Figure 3.3 and there are no physical problems in the incorrect placement of IPG strip during second dimension of 2D PAGE protein separations.

As the 2D gels in Figure 3.3 were not taken for any protein sample contaminants removal or inactivation, both horizontal streaking and the incidence of large vertical blank stripe (in stimulated U937 protein sample) are indicative as a result of interfering compounds. Major interfering compounds in 2D gels include salts, proteases, lipids, polysaccharides and phenols. These interfering compounds can be reduced by the removal of each class of interfering compound with dialysis or gel filtration techniques (Speicher, 2004). The incidence of streaking was assumed not due to the occurrence of proteases as protease inhibitors were added during sample preparation.

Consequently, U937 protein samples were brought for protein sample purification from major contaminant, salts, with chromatography columns (Bio-Rad) during sample preparation. Visualization of protein sample after desalting was first made in 1D PAGE. Comparison between U937 protein sample after desalting (lane 3 and 4) and without desalting (lane 1 and 2) was given in Figure 3.5. There are no observed significant differences of protein band visualization between the two treating suggested that salt made not much interference in 1D PAGE.
Table 3.5 Comparison of U937 cell line total protein between sample after desalting (lane 1, 2) and without desalting (lane 3, 4), both with protease inhibitors

Though, visualization of U937 protein samples with IPG strips with and without desalting with chromatography column (Bio Rad) shows there were better resolution of protein bands in sample after desalting. The resulting IPG strips staining with Coomassie Brilliant Blue (Bio Rad) are given in Figure 3.6. There was observed protein precipitation at both lower ends of the IPG strips with samples without desalting treatment (a) while as there was no occurrence of protein precipitation but fine divided protein band along the strip with the sample after desalting (b). High concentrations of salts in protein samples interfere with electrophoretic separation and thus the consequence isoelectric point protein precipitation at both ends of IPG strip (Speicher, 2004).

Figure 3.6 Isoelectric focusing (IEF) in first dimensional separation of U937 cell line protein lysate 2D gel electrophoresis (a) without desalting (b) after desalting with 7cm IPG strips pH 4-9. Sample load: ~100μg of protein. Coomassie Brilliant Blue stains.
Figure 3.7 2D polyacrylamide gel electrophoresis of U937 cell line protein lysate stimulated (b) and non-stimulated (a) with cytokines INF-γ and TNF-α. First dimension: 7cm IPG strips pH 4-9. Sample load: 100μg of protein. Second dimension: vertical SDS-PAGE (4-16%). Red fluorescent dye SYPRO-Ruby stains.
Figure 3.8 Comparison between 2D polyacrylamide gel electrophoresis of U937 cell line protein lysate in stimulated sample without desalting (a) and after desalting (b). First dimension: 7cm IPG strips pH 4-9. Second dimension: vertical SDS-PAGE (4-16%). Red fluorescent dye SYPRO-Ruby stains.
Optimization of protein samples with desalting and protease inhibitors gave an appreciable resolution of protein band separation with IPG strips in the first dimension of protein separation of 2D PAGE (Figure 3.6). Protein samples with desalting and protease inhibitors were then brought further for second dimension of 2D PAGE protein separation (Figure 3.7). Comparison with stimulated U937 protein samples between samples without desalting treatment and after desalting were given in Figure 3.8. The resolution of 2D gel image was better in sample after desalting (b) than in the 2D gel image with sample without desalting (a). Overall, 2D gel images after desalting gave better visualization and better divided protein spots.

Nevertheless, there are still occurrences of regional horizontal streaking in both stimulated and non-stimulated U937 samples (Figure 3.7) with the likely cause of protein overloading. The total amount of protein loaded into an IPG strip usually depends on the length of the strip and the stain that will be used to visualize the 2D gels. Hence, protein sample need to be properly quantify to ensure the proper loading of protein concentration accordingly to the two factors.

Figure 3.9 Occurrence of blank stripes in the vertical dimension in 2D polyacrylamide gel electrophoresis of U937 cell line protein lysate of non-stimulated sample after desalting. First dimension: 7cm IPG strips pH 4-9. Second dimension: vertical SDS-PAGE (4-16%). Red fluorescent dye SYPRO-Ruby stains.

On the other hand, there was also occurrence of blank stripes in the vertical dimension which can be clearly observed in stimulated U937 cell line protein sample with desalting (Figure 3.9). The problem indicated that a region of the IPG may not have been sufficiently rehydrated or may have torn during handling, resulting in the absence of focused protein in that region. There might also probable trapped air bubble in the agarose that joins the strip to the top of the second-dimension gel and thus the consequent vertical blank stripes (Bio Rad Proteomics Expression).
3.2 Computer Aided Analysis: Progenesis PG 220 SameSpots (Nonlinear Dynamics)

Acquired digital images for both 2D gels, stimulated and non-stimulated in Figure 3.7, were analyzed using the commercially available 2D images analysis software packages, Progenesis PG220 with the additional SameSpots packages (both from Nonlinear Dynamic). Differences between normal and pathogenic (stimulated) state of U937 cell line were compared. The workflow is summarized in Figure 3.10.

Figure 3.10 Flow diagrams in summary of the steps involve 2D gels analysis with Progenesis PG 220 SameSpots (Nonlinear Dynamics)

Align 2D images (SameSpots)
Import into Progenesis PG220
Spots detection
Edit spots
Spots matching
Finding proteins of interest & making comparisons
Data construction/Investigation

Stimulated and non-stimulated (set as reference gel) 2D gel images were first aligned with Progenesis SameSpots TT900 S2STM. The aligned images are corrected for positional variations using manual and automatic applied vectors between spots on two images followed by an image alignment (Figure 3.11). This was follow by 2D pattern detection and quantification with Progenesis PG220. Manual spot editing were performed which include contrast enhancement, artifact removal, background subtracting, etc (Figure 3.12).

Protein spots in the two different gels (stimulated and non-stimulated) were then matched for qualitative and/or quantitative differences between the 2D patterns. Gels were compared for the same proteins present on the two gel images and also for differentially expressed spots (Figure 3.13 – Figure 3.17). Tools include filtering data to reveal expression changes, observation of protein expression in 3 dimensions (Figure 3.14 b) and statistic analysis (Figure 3.16). Final steps involved finding proteins of interest and making comparison between the two gels and the subsequent establishment of 2D gel database with the information acquired (Figure 3.15 - Figure 3.17) and thus allowed further “spot picking”.

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* Windows: A) main alignment area and shows the current focus rectangle shown in Window C; B) Alpha blend to animate between the current and reference image; C) shows the focus for other windows; D) checkerboard view of the current image interleaved with the reference images.

Figure 3.11 2D images alignment with TT900 S2S (Progenesis SameSpots, Nonlinear) software packages. (a) Manual (red vectors) and automatically (blue vectors) generated vectors between spots on two images in the initial alignment followed by (b) aligned images using the alignment vectors to produce an aligned image where images have merged and are ready for further spots detection and analysis. Green = current/stimulated spots; magenta = reference/normal control spots.
Figure 3.12 Spots detection and editing after automatic analysis (alignment) with Progenesis PG220 (Nonlinear Dynamic) software packages. Before (a) and after (b) automatic and manual spots filtering and editing. Spots editing can be made via splits, auto splits, merge, draw or delete spots.
Figure 3.13 Spots matching with Progenesis PG220 (Nonlinear Dynamic) software packages. Images in warped view, slaves/stimulated spots (blue), references spots (red), showing differences between two images where protein spots are express in one and not in the other, while as hollow outlines showing protein spots which are express in both of the gels; match vectors (pink).

Figure 3.14 (a) image in warped view with only match vectors (pink) which allows to see the appropriate matches have been made and thus allowed the following edit matching e.g. via deleting matches, performing area of interest matching and adding user seed matches; (b) 3D view of the same area in (a).
Figure 3.15 Data investigation with Progenesis PG220 (Nonlinear Dynamic) software packages to look for differences and identify spots that are significantly different between images. Small window highlighting a green spot which is unique to current/stimulated image and not expressed in the reference/normal control gel. ‘Montage’ window shows the match series for the selected spot and verifies matching is correct.

Figure 3.16 Data investigation and construction with Progenesis PG220 (Nonlinear Dynamic) software packages. Data viewing in “measurement window” displaying matched spots and differential expressed spots (highlighted in green and yellow) after “data filtering” which could then be used for “spot picking”.
Figure 3.17 Data investigation and construction with Progenesis PG220 (Nonlinear Dynamic) to look for differences between images. Small window highlighting a spot which is potentially expressed at higher levels in the normal control/reference gel (magenta) compared to the stimulated gel (green). Histogram window (a) shows the trend in volume (red) and normalized volume (blue) across the match series, where the Reference bar 1 shows the mean value for both images and variations in terms of standard deviation; bar 2 shows value for stimulated gel; bar 3 shows value for normal control gel. ‘Montage’ window (b) shows the match series for the selected spot and verifies matching is correct.
PART 4

MARKET RESEARCH
4.1 Secondary Market Research

4.1.1 Definition & Market Size

Despite the early stages of market development, leading biotechnology and pharmaceutical companies as well as academic groups are now at the leading position of biomarker discovery, validation and utilization for various applications from molecular diagnostics to drug discovery and development.

According to internal Market and Technology Analysis of ITI Life Sciences, the biomarker market is likely to grow at an annual rate of 45% rising to £2.9 billion by 2008 in the UK alone. By far the largest use of biomarkers is in diagnostics. The biomarkers in diagnostics application are forecast to grow at an annual rate of 77% to $1.9 billion in 2008 and to date, the global diagnostics market is approximately £20 billion [1]. The world near-patient testing market currently generates revenues of US$3.3 billion and is expected to reach US$5.5 billion in 2009, with a compound annual growth rate of nearly 8%.

The major obstacle in the rising demand of biomarkers is the substantial initial investment required, in terms of both time and money. Biomarkers will increase the primary cost of clinical development. However, an appropriate strategy will be able to maximize the turnover and eventually reduce the financial cost involved. The cost and duration of clinical development will drop to acceptable limits with the integration of other drug discovery technologies and the development of larger biomarker portfolio in the near future [3].

However, the importance of biomarkers and its definition for molecular diagnostics market is gaining momentum, with Roche’s AmpliChip P450 (a biomarker to help physicians adjust drug dosing and selecting drugs) to be the first in receiving regulatory approval [3]. Test for other unmet diagnosis of clinical diseases, such as Alzheimer’s disease as well as the promising biomarker discovery e.g. for acute renal allograft rejection diagnosis are set to drive further growth in the biomarkers market.
4.1.2 Biomarkers Market Segments

The market segments for the biomarkers can be divided and summarized into the following five major areas:

a. Molecular diagnostics
   - Commercial molecular diagnostics biomarker test for infectious disease e.g. SARS, HIV1, HCV, HBV, STDs, etc uses standard instruments.
   - Biomarker test for major cancers i.e. breast, prostate, colon, ovarian, pancreatic, lung, leukemia.
   - Biomarker tests for cardiovascular, heart attack, stroke etc and other diseases e.g. Alzheimer’s.

b. Independent Clinical Molecular Diagnostics Services
   - In-vitro diagnostics (IVD) molecular diagnostics
   - Home Brew molecular diagnostics
   - Pharmacogenomic tests
   - Clinical proteomic tests
   - Doctor’s offices

c. Biotechnology, Pharmaceutical R&D and R&D Services
   - Discover new biomarkers using DNA chips, protein chips, cell arrays, Mass spectrometry, etc. and out-license
   - Commercialize useful molecular biomarkers as R&D test or clinical lab tests.
   - Biomarker discovery, validation and assay development.
   - Make new pharmacogenomic based drugs tied to genetic biomarker test that screen for responding patients.
   - Discover new biomarkers that screen for patients that respond to current drug products.
   - Target major diseases.

d. Academic and Government R&D
   - Basic research to discover biomarkers using DNA chips, protein chips, cells arrays, mass spectrometry, etc. and license to outsiders.
   - Public health applications – FDA, NIH, CDC etc.
   - Military applications.

e. Emerging Diagnostics
   - Near patient care
   - Emergency room
   - POC (Point of Care)

(Adapted from, Business Insight: www.globalbusinessinsights.com)
4.1.3 Biomarkers: Molecular & Noninvasive Diagnostic in Acute Renal Allograft Rejection Target Market

Establishment of biomarkers will be use in the screening of transplant patients for acute renal allograft rejection. Despite the escalating cost in renal transplantation, medication in immunosuppression therapy and various other clinical expenses, there are still no promises of successes in every transplant performed due to the risk of allograft rejection. Biomarker applications for noninvasive diagnostic will enable quick and accurate diagnosis as potential rejection can be identified at an early stage of post transplant. This will help reduce patient time spent in the clinic, improving patient’s lifestyle and reducing overall healthcare delivery costs.

Figure 4.1 worldwide renal transplants figures in different continents/regions, year 2000

* All numbers per million populations (pmp)
Source: US SRTR 2005 annual report [5].

Transplant rates vary worldwide and are dependent on cultural, legal, and socioeconomic factors. There are currently a total of over 95 per million populations of renal transplant performed in the year 2000 worldwide (Figure 4.1). Out of the breakdown to the four main world continents, over 50% of patients receiving renal transplant is from the USA (52 patients pmp), followed by Europe with 27 patients pmp (28%). Asia have the lowest rate of renal transplants of 3.2% (3 patients pmp) while as 13.7% of patients from Latin America (13 patients pmp).

In addition, there are also large populations of patients who are on the waiting list for renal transplants worldwide. For example in the Latin America, 45,000 are waiting for kidney transplant in the year 2000 while as less than 2 million patients need renal transplants in the China alone. As can be seen therefore, with the large population of renal transplants patients and on waiting list, potential biomarkers diagnostics in this space represents a significant market opportunity.
4.2 Primary Market Research - UK Target Market

4.2.1 Overview of UK Consumer Profile

*Causes of Death:*

<table>
<thead>
<tr>
<th>Natural Causes</th>
<th>Number of Deaths</th>
<th>% Change 2001-2002</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart and circulatory disease</td>
<td>237,850</td>
<td>-1.0</td>
</tr>
<tr>
<td>Cancer</td>
<td>159,331</td>
<td>0.6</td>
</tr>
<tr>
<td>Respiratory disease</td>
<td>78,589</td>
<td>3.7</td>
</tr>
<tr>
<td>Digestive-system disease</td>
<td>27,858</td>
<td>3.2</td>
</tr>
<tr>
<td>Mental and behavioral disorders</td>
<td>17,301</td>
<td>2.1</td>
</tr>
<tr>
<td>Diseases of the central nervous</td>
<td>16,644</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Genito-urinary disease</strong></td>
<td><strong>9,797</strong></td>
<td><strong>9.7</strong></td>
</tr>
<tr>
<td>Endocrine, nutritional and metabolic diseases</td>
<td>8,986</td>
<td>2.7</td>
</tr>
<tr>
<td>Other natural causes</td>
<td>28,544</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Total natural causes</strong></td>
<td><strong>584,900</strong></td>
<td><strong>0.7</strong></td>
</tr>
<tr>
<td>Other deaths</td>
<td>21,313</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>606,213</strong></td>
<td><strong>0.7</strong></td>
</tr>
</tbody>
</table>

Source: KeyNotes, the Pharmaceutical Industry, 2005

Genito-urinary disease (bolded) including kidney disease had been on significant growth over the recent years (data not shown) and showed highest level of growth as a cause of death in the 2002 (Table 4.1). Other cause of death in the UK includes the heart and circulatory disease which is the leading cause of all deaths in 2002 (39.2%). This was followed by the cancer, which account for 26.3% of all deaths in 2002.

Significant growth on the renal diseases shows the increasing demand for the renal replacement therapy including dialysis, renal transplantation, etc. On the other hand, there are also significant levels of growth shown by respiratory disease, diseases of the central nervous system and sense organs, and digestive-system disease.
4.2.2 UK Kidney Transplant

Figure 4.2 shows the summary of activity for cadaveric heartbeating (HB) and non-heartbeating donor kidney transplant and the transplant on the waiting list in the UK for the last ten years from April 1995 to March 2005. Data shown for cadaveric donor transplant as majority (over 70% in the year 2004-2005) of transplant are performed between cadaveric donor kidney transplants (Figure 4.3) than living donor transplants. The overall total number of cadaveric donor kidney transplant performed fell by 21% from 1582 in 1995-1996 to 1308 in 2004-2005.

**Figure 4.2** cadaveric kidney programs in the UK, 1 April 1995-31 March 2005, number of donors, transplants and patients on the active transplant list at 31 March

![Graph showing the number of donors, transplants, and patients on the active transplant list from 1995-1996 to 2004-2005.]

Source: NHS UK Transplant, 2005

Decreased number of transplants performed may due to various reasons included the decrease number of cadaveric kidney donor which as can be seen fell from 819 to 711 donors for the same period. However, the number of patients registered on the active transplant list at 31 March 2005 for a kidney transplant has increased by 32% since the last ten years, from 4113 to 5425 patients. The number of patients waiting for a kidney transplant represents 92 patients per million populations.

There are 19 kidney transplant centers in the UK including six alliances which includes North of England, South Thames, North Thames, Trent and South, Scotland, West and Wales. Among the total transplant carried out in the transplant centers in the UK, 81% of living donor kidney transplant, 87% of cadaveric HB and 87% of cadaveric NHB kidney transplant were performed by the alliances centers, while as 80% of the transplant on the waiting list comprised of patients from alliance centers (NHS UK Transplant, 2005).
Figure 4.3 Comparison between living donor transplant and cadaveric donor transplant (2004-2005) in the UK

Source: NHS UK Transplant, 2005

Figure 4.4 Long-term transplant survival in adult (≥18 years) recipients for (a) first cadaveric HB donor only & (b) living donor kidney transplants in the UK.

Source: NHS UK Transplant, 2005

Figure 4.4 (a) shows the long-term transplant survival in adult recipients for first cadaveric heartbeating donor kidney only transplants and Figure 4.4 (b) shows the long-term transplant survival in adult recipient for living donor transplants in the UK. Both results show improvement in overall transplant survival over the years. In pediatric recipients (graft not shown), long-term transplant survival shows there are improvement in one and two tear survival over the years but not in the five year survival estimates for first cadaveric heartbeating donor kidney only transplant in the UK. While as long term survival in pediatric recipient for living donor kidney transplant in the UK (graft not shown) shows there are no statistically significant differences in transplant survival across the year group (NHS UK Transplant, 2005).
4.3 Competitors Analysis

4.3.1 Direct Competitors

Current major player from the US and UK in the biomarkers research and development industry includes:

- BioMarker Pharmaceuticals, Inc.
- SurroMed, Inc.
- DakoCytomation A/S
- Diagnostics Products Corporation
- Roche Diagnostics
- Paradigm Genetics, Inc.
- Genaissance Pharmaceuticals, Inc.

As mention, the success of Roche in becoming the first FDA-cleared test for AmpliChip P450 as mention shows that the biomarker discovery is gaining worldwide recognition and importance. The AmpliChip is now available in most important market include US and UK market.

4.3.1 Indirect Competitors

The tissue needle core biopsies of renal tissue and secondly the measurement of clinical presentation in the diagnosis of acute renal allograft rejection are indirect competitors to the molecular and noninvasive diagnosis methods with biomarkers. The standard test for the diagnosis of acute renal allograft rejection currently remain the tissue biopsies but this and other diagnosis methods have certain drawbacks compared to potential biomarkers diagnostics:

- Clinical Presentation

Biochemical parameters e.g. the rise in the level of serum creatinine may be use as the implication of allograft rejection or dysfunction but the reasons can be elusive. This is as renal function may not always correlate with histologic improvement, thus rise in the level of serum creatinine may not reflect early changes in the allograft due to rejection (Li et al., 2001; Roberti & Reisman, 2001).

- Tissue Biopsies

Tissue biopsies provide only a limited sample of the organ; it is expensive, invasive and has a diagnostic sensitivity of only 79%-88%. Biopsy is also associated with 2%-7% risk of complications such as pain, bleeding and shock, anuria, haematuria, arterio-venous fistulas, graft loss and patient death (Kolb et al, 1994; Racusen et al, 1999). Sampling errors and lack of sensitive histologic patterns leads to subsequent disparities between clinical and microscopic findings (Roberti & Reisman, 2001). Also, biopsies do not allow for frequent or serial monitoring of immune response and allograft function as su-
clinical rejection may develop in allograft before evidence of graft dysfunction (Shapiro et al., 2001).

In conclusion, with tissue biopsies and clinical presentation, the outcome of renal transplantation and the occurrence of acute rejection is still difficult to predict. The necessarily late application of anti-rejection therapy often results in only partial restoration of renal transplant function and associated with morbidity and mortality.

4.4 SWOT Analysis

- **Strengths**
  - The advent of biomarkers discovery will improve results by allowing biomarkers pattern profiling rather than reliance on a single target promises from clinical diagnosis to drug discovery.
  - Biomarkers allowed noninvasive diagnosis with easily acquired body fluids e.g. saliva, urine, serum and eliminate surgical which brought to complications and pains.

- **Weaknesses**
  - Biomarker discovery and validation have been regarded as expensive and time consuming and requires a lot of labor work.
  - This weakness also adds to a further level of complexity to the biomarkers in clinical development phase.
  - The study is good applied research with a definite clinical end point that may save time, money and lives. However, even if a test is delivered and works it may not be a commercial success in terms of profitability.

- **Opportunities**
  - Researchers can integrate biomarker identification with the drug development and pre-clinical testing process for sustainable growth in the biomarkers market.
  - Researchers can also develop biomarkers for use in long-term diagnostics, which may have a separate market once the drug is commercialized.

- **Threats**
  - There is a presumption that biomarker tests are being unreliable.
4.5 Financial Statement

The research in the biomarkers discovery with proteomic analysis for the acute rejection of renal allograft will be carried out in the University of Sunderland. With the establishment of the novel biomarkers product, real-time PCR assay will be used with transplant patient’s urine samples in the clinical diagnostics of acute renal allograft rejection. Certain assumptions were made for the development of financial projections which include the budget for the research of biomarkers discovery phase (R&D) for three years, ten years of product life and VAT at 17.5%. The laboratory in the University of Sunderland is equipped with standard laboratory utilities for the biomarkers discovery phases and no expenses will be made for the purchase of the basic laboratory equipment for example micropipettes, centrifuges and spectrophotometer.

However, some specific laboratory equipment and research operational cost for example proteomics and real-time PCR are included in the account of investment as in Table 4.2. The prices for the various utilities needed were based on the process of the relevant product in the order catalogue and there is a 10% discount in the orders through the university. The balance sheet for real-time PCR assay in the clinical screening of acute renal allograft rejection in transplant patient is given in Table 4.3. Urine samples to be diagnosed will be run in duplicate in the 32 wells real-time PCR and thus the maximum production rate of 16 urine samples per day. Screening of acute renal allograft rejection will be made in the clinical laboratory or pathology lab of hospital units or transplant centers. Thus, no full time technicians will be needed for the specific task of diagnosis with biomarkers for acute rejection with real time PCR. The work will be taken over and routinely as part of the original technicians or clinicians work in the pathology lab and clinical laboratory and thus the reduced operational cost for the labor or staff.

The biomarkers spreadsheet is given in Table 4.4 for ten years product life cycle. Selling price per product of diagnostic with biomarkers is £43.34. Although not really profitable, the biomarkers will be use to screen patient straight after renal transplantation as acute rejection normally occurs early after transplantation. Frequent screening will be performed in the early phase i.e. in the first 6 months after transplantation for the serial monitoring of immune responses in the graft followed by 6 months interval after early phase frequent screening. Although the frequent screening will increase the transplant patients medical cost, but there will have cost saving for the patients in the long run with the early detection acute rejection which allowed the proper medical measurements to be performed.

Frequent screening will also have reduced incidence of graft dysfunction due to acute rejection. This can be seen from the study by (Harper et al., 2000) where about 20% of the patients in the US who are on the waiting list are those with a failed graft due to acute rejection and about 15% of the procedures performed are repeated transplantations. Graft dysfunctions not only have impact on patients’ health but also added to the escalating medical cost in transplant and also for the repeat transplantation required due to graft dysfunction.
## Table 4.2 Investment in research and development (R&D)

<table>
<thead>
<tr>
<th>Equipments</th>
<th>List Price (£)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xcise Robot (Shidmadzu)</td>
<td>110,000</td>
</tr>
<tr>
<td>SameSpots Progenesis Software (Nonlinear Dynamic)</td>
<td>10,000</td>
</tr>
<tr>
<td>Progenesis PG220 Software (Nonlinear Dynamic)</td>
<td>15,000</td>
</tr>
<tr>
<td>MALDI-TOF Mass Spectrometry</td>
<td>80,000</td>
</tr>
<tr>
<td>Bio Rad IEF Cell</td>
<td>4,000</td>
</tr>
<tr>
<td>Rotogene 3000, real-time cycler (Corbett Research)</td>
<td>21,000</td>
</tr>
<tr>
<td><strong>Sub-total:</strong></td>
<td>240,000</td>
</tr>
</tbody>
</table>

### Operational Cost (3 years)

**a) Proteomics:**
- 60 X Bio Rad Ready Gel @ £9 each                                      540
- 60 X Bio Rad IPG Strips 7cm @ £43 for 12                               215
- 2 X MALDI Calibrants @ £200 each                                      400
- 2 X MALDI Target Plates @ £140 each                                    280
- 3 X SYPRO Ruby Protein Gel Stain (1L) @ £187 each                      561
- General consumables (E.g. IEF trays, buffers, enzyme trypsin etc.)    1500

**b) Real-time PCR**
- 20 X TaqMan Probe @ £204 each                                         4080
- 5 X Taq Immomix @ £80 each                                             400
- 5 X RT-PCR Kit @ £200 each                                             1000
- General consumables (E.g. buffers, filter tips etc.)                   500

**c) Sample Preparation**
- PARIS™ Kit/Reagent (250 reactions)                                    1025
- 3X Roche Protease Inhibitors Cocktail @ £15.50                          47
- 10 X Bio Rad Chromatography Column @ £60 each                           600
- General lab consumables (E.g. plastic ware, tips, etc.)               1500

**Sub-total:**                                                           12,648

### Others (including 30% from equipments maintenance and 3 years operational cost) 75,790

### Total Investment in R&D 328,442
### 4.5.1 Balance Sheet

Production rate = 16 urine samples/day, 2 workers, and 48 working weeks per year, → 1536 urine samples/year

**Table 4.3** The balance sheet: Real-time PCR assay (32 wells) in the screening of acute renal allograft rejection in transplant patient.

<table>
<thead>
<tr>
<th>INVESTMENT</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>R&amp;D</strong></td>
<td>£252,648</td>
<td></td>
</tr>
<tr>
<td>Others (30% of R&amp;D)</td>
<td>£75,790</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>£328,442</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OPERATIONAL COST</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maintenance (3% of investment)</strong></td>
<td>£9,853</td>
<td></td>
</tr>
<tr>
<td><strong>Labor (2 staff)</strong></td>
<td>£10,000</td>
<td></td>
</tr>
<tr>
<td><strong>Utilities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sample Preparation:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARIS™ reagent (50 reactions X4)</td>
<td>£512</td>
<td></td>
</tr>
<tr>
<td>Other (e.g. PBS, buffers etc.)</td>
<td>£400</td>
<td></td>
</tr>
<tr>
<td><strong>Real Time PCR:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TaqMan Probe (200 reactions)</td>
<td>£1,360</td>
<td></td>
</tr>
<tr>
<td>Taq Immomix (200 reactions)</td>
<td>£125</td>
<td></td>
</tr>
<tr>
<td>RT-PCR Kit (50 reactions X4)</td>
<td>£250</td>
<td></td>
</tr>
<tr>
<td>Laptop</td>
<td>£710</td>
<td></td>
</tr>
<tr>
<td>Incremental overhead (50% maintenance and labor)</td>
<td>£9,927</td>
<td></td>
</tr>
<tr>
<td>Taxes and Insurances (2.5% of investment)</td>
<td>£8,211</td>
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<tr>
<td><strong>Total Identified Operating Costs</strong></td>
<td></td>
<td>£41,348</td>
</tr>
<tr>
<td><strong>Contingency Allowance (15% of identified operating cost)</strong></td>
<td></td>
<td>£6,202</td>
</tr>
<tr>
<td><strong>Total Manufacturing Costs</strong></td>
<td></td>
<td>£47,550</td>
</tr>
</tbody>
</table>
4.5.2 The Spread Sheet

Production rate = 1536 urine samples per year
Investment in R&D plus Others = £328,442
Total Manufacturing Cost = £47,550
Manufacturing Cost (Order Price) = £30.96 per sample / 47554.56
Working capital = £40,000
Selling price (Production Rate) = 40% addition to manufacturing/order price = £43.34 Per sample / 66576.38 per year
Sales Expenses = (50% of order price, £? Per sample) = £ per year
Tax = 17.5%
Product Life = 10 years

Table 4.4 The Acute Renal Allograft Rejection Biomarkers Spreadsheet

<table>
<thead>
<tr>
<th>Year</th>
<th>Capital Cost</th>
<th>Gross Income</th>
<th>Manuf. Cost £</th>
<th>Taxes</th>
<th>Net Income £</th>
<th>Present Value Factor</th>
<th>Present Value</th>
<th>Cumulative Value</th>
<th>Present Value Factor</th>
<th>Present Value</th>
<th>Cumulative Value</th>
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<td>1</td>
<td>252,648</td>
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<td>0.80000</td>
<td>(12,000)</td>
<td>(12,000)</td>
<td>0.76920</td>
<td>(11,538)</td>
<td>(11,538)</td>
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<tr>
<td>2</td>
<td>75,790</td>
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<td></td>
<td></td>
<td></td>
<td>0.64000</td>
<td>(3,360)</td>
<td>(15,360)</td>
<td>0.59170</td>
<td>(3,106)</td>
<td>(14,444)</td>
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<tr>
<td>3</td>
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<td>60,515</td>
<td>47,269</td>
<td>236.3</td>
<td>8,584.8</td>
<td>0.51200</td>
<td>680.60</td>
<td>(14,679)</td>
<td>0.45520</td>
<td>(605.1)</td>
<td>(14,039)</td>
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<td>0.40960</td>
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<td>2,969.4</td>
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<td>47,269</td>
<td>3,528.9</td>
<td>5,293.4</td>
<td>0.16780</td>
<td>1,172.0</td>
<td>190.7</td>
<td>0.12260</td>
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<td>(2,126)</td>
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<td>5,293.4</td>
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<td>937.30</td>
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<td>658.6</td>
<td>(1,467)</td>
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<td>750.10</td>
<td>1,878.2</td>
<td>0.07250</td>
<td>506.4</td>
<td>(961)</td>
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</tr>
</tbody>
</table>
PART 5

DISCUSSIONS
5.1 Methods Optimization

U937 lymphocytes cell line stimulated with cytokines INF-\(\gamma\) and TNF-\(\alpha\) was treated as pathogenic state while as those not treated with cytokines acted as normal control. Cytokines INF-\(\gamma\) and TNF-\(\alpha\) were use in this study to stimulate U937 lymphocytes cell line as these molecules are pro-inflammatory and would simulate an event such as acute rejection in organ transplantation. Cells were brought to protein extraction followed by protein sample concentration via lyophilizing. The resulting protein sample visualization in 1D gels have observed vertical streaking due to protein degradation.

Protein samples were investigated by adding enzyme protease inhibitors. The following 1D gel showed better resolution than protein samples without enzyme protease inhibitors, suggesting increased enzyme protease activities in protein samples during lyophilizing. Protein samples with enzyme protease activities were then brought to the visualization in 2D PAGE but the resulting 2D gels shown both horizontal and vertical streaking problems. While sample preparation in IEF focusing were optimized according to the Bio Rad instructions manual, the streaking problems were presume due to the occurrence of interfering compound in protein sample. To rule out the possibility of the occurrence of interfering compounds, protein samples desalting were performed and the resulting 2D gel images gave better resolution than 2D gel images without desalting.

Principles of 2D PAGE lies in isoelectric focusing (IEF) of proteins according to their isoelectric points (pIs) in first dimension protein separation followed by second dimensional protein separation with SDS polyacrylamide gel according to protein size (molecular weight). IEF is carried out in an immobilized pH gradient (IPG) under denaturing and reducing conditions with the use of chaotropic agents, detergents, reducing agents, buffers, and ampholytes to solubilized proteins. SDS (sodium dodecyl sulfate) which is required to fully solubilized proteins cannot be used in 2D PAGE due to incompatibility with IEF. As a result, problems occur with the prevalence of wide diversity of protein abundance, molecular weight, charge, isoelectric point (pl) and solubility. Due to the principles of 2D PAGE it is biased in detecting abundant and water-soluble proteins (Speicher, 2004) and may inhibit biomarker detection. For instance, highly hydrophobic proteins such as membrane proteins are difficult to solubilize. Under such situations, the membrane proteins will fail to enter the IEF gel in the first dimension of 2D PAGE.

The most common interfering agents in 2D PAGE are salts (Speicher, 2004). The influence of salts within protein samples to the 2D PAGE was experienced with the optimization of protein samples with IPG strips with protein samples with and without desalting treatment (Figure 3.6). The IPG strip with desalting treatment gave good results with fine divided protein bands along the IPG strip while as IPG strip without desalting have the observed protein precipitation on the both end of the strip due to uneven electrophoretic separation. Therefore, possible occurrence of interfering substance in 2D PAGE will influence protein visualization in 2D PAGE and have to be inactivated or removed during sample preparation.
In addition, to reinforce the possibilities of the occurrence of salts in protein samples, visualization of protein samples with desalting in 2D gels was made. In comparison between 2D gel images with samples with and without desalting (Figure 3.8), there are clear observation of streaking and fewer protein spots than expected in protein without desalting while as samples after desalting gave better resolution with no occurrence of protein precipitation.

Other important interfering compounds in 2D PAGE include proteases, lipids, polysaccharides and phenols compounds (Speicher, 2004). However, optimization for the occurrence and influence of protease in 2D PAGE in this experiment cannot be observed quite significantly as protein samples visualization for the both the 2D gels in Figure 3.8 and Figure 3.4 were treated with enzyme protease inhibitors but with desalting and without desalting respectively. To see the effect of protease in 2D gels, comparison have to be made between protein samples both with desalting treatments but with and without enzyme protease inhibitors. However, inactivation of protease in sample preparation for 2D PAGE is very important. Present of proteases within the protein samples will cause protein degradation and the present of artifactual spots in the subsequent 2D gels visualization (Speicher, 2004). Most importantly, presents of protease within samples may degrade the proteins you want to study.

High-throughput 1D polyacrylamide gel electrophoresis can be a good downstream analysis method for proteomics and also as a means of initial evaluation for IEF step in 2D PAGE. Optimization with 1D PAGE will help in the detection of very large, very basic, and very acidic proteins which can readily detected on 1D gel but can poorly detected or missed when analyzed on 2D gels (Speicher, 2004). In this study, comparison of protein samples with and without lyophilisation (Figure 3.1), and with and without protease inhibitors (Figure 3.2) were first visualized in 1D gels. The resulting better resolutions in 1D gel of lyophilized protein sample with enzyme protease inhibitors gave an indication that there was occurrence of proteases activities and sample proteins need to be optimized with proteases inhibitors for 2D PAGE. However, occurrence of other contaminants in protein sample e.g. DNA still can not be dismissed with this indication.

**Figure 5.1** Low abundance proteins in 2DE of U937 cell line protein lysate in non-stimulated sample after desalting. First dimension: 7cm IPG strips pH 4-9. Second dimension: vertical SDS-PAGE (4-16%). Red fluorescent dye SYPRO-Ruby stains.
As mentioned, 2D PAGE is also known for it bias characteristic towards high abundance proteins rather than low abundance proteins (e.g. low copy number protein). Problem may rise with the low abundance protein which is relatively important as it can play critical regulatory roles in a given cell lysate or tissue. Example of the low abundance proteins of U937 cells in this study were given in Figure 5.1 with the visualization of low intensity protein spots in the 2D gel. Regional horizontal streaking problems in the gel images in Figure 3.7 were due to the probable cause of sample overloading. Protein sample overload can be solved by sample dilution. However, dilution of protein sample for lower concentration of sample loaded into 2D PAGE will cause the diminished of the low abundance protein. For this reason, sample preparation can be optimized via prefractonation of protein sample. Approaches in prefractonation of protein samples included chromatographic techniques, subcellular fractionation and differential extraction. Prefractonation is essential primarily in higher eukaryotic tissues and will give more comprehensive coverage of proteins in a given tissues or cells by reducing the complexity of protein sample or via enrichment and visibility of low abundance protein.

In this study, the problems with the occurrence streaking and probable cause of interfering compounds were reduced with the samples treatment with proteases inhibitors and desalting. Optimization of both 1D and 2D gels with desalting and protease inhibitors of concentrated protein samples have succeed in achieving better resolution of proteins visualization in both the 1D and 2D gels and on IPG strips. Problems remain with the proper concentration of U937 protein sample need to be loaded into the 2D PAGE given the occurrence of the regional horizontal streaking in the 2D gel images as in Figure 3.7.

5.2 Computer Aided 2D Gel Image Analysis Algorithms

Acquisitions of 2D digital images will followed by the analysis of the parallel 2D gels with computer aided 2D image analysis. Other than Progenesis (Nonlinear Dynamic), other software packages for the analysis of 2D gels images include Delta2D, Melanie-III, Z3 and Gellab II. In the 2D gel analysis algorithms, gel distortions are mathematically treat to allow gel images warped and gel matching. Steps involved in between the analysis include image alignment, spot detection, background subtraction, artifact removal, contrast enhancement, quantification, and finally gel comparison to allow the visualization of spots which is unique to one gel or differential express spot or spot pattern in between the compared gels.

Although currently there is no available software that can provide totally hand-free analysis with high accuracy, manual spot editing can be done to correct some errors from automatic task although manual spot editing may be time consuming. Analysis of the stimulated and non-stimulated 2D gels in Figure 3.7 in this study shows that computer aided 2D gel image analysis (Progenesis, Nonlinear Dynamic) is useful in the findings of differential expressed proteins in the parallel 2D gels. With the computer software, some down-regulated or up-regulated proteins were found (examples were shown in Figure 3.15 and 3.17). Data were constructed (Figure 3.16) allowing investigations and statistical analysis. However, due to the regional horizontal streaking in both of the
stimulated and non-stimulated 2D gel images (Figure 3.7), spots investigation and “spot picking” were limited to the region of the 2D gels with better resolution and divided protein spots.

5.3 Future Studies

1D and 2D PAGE, known for its availability and simplicity can be relatively high throughputs and a very good technique in downstream proteomic analysis. However, sample preparation for gel based proteomic studies need to be optimized for individual projects due to the intrinsic variability of the proteome diversities in high and low abundance proteins in given specimens or tissue, and occurrence of interference compounds. Urine can be exploited in the proteome studies of renal diseases due to its availability and non-surgical. Nonetheless, urine is known for its complexity, with prevalence of high abundant protein and high salts concentration which may complicate the detection of relatively low-abundance proteins.

Long term aim of this project is to use urine sample to detect 10-15 suitable biomarker for the acute rejection of renal allograft with proteomics analysis. Potential biomarkers will then bring to develop PCR methods for the detection of the acute rejection biomarkers. Thus, urine sample preparation needs to be optimized for the urinary proteomics of the project long term aims. As what have been achieved in this study, protein sample preparation can be optimized with desalting and enzyme protease inhibitors treatment to give a better 2D gel images. Treating of protein samples with protease inhibitors have reduce protein degradation on 1D gels while as desalting have claimed better resolution in the IPG strip and 2D gels visualizations. Therefore, treating of protein sample with protease inhibitors and desalting may be applicable for the sample preparation with urine samples. Prefractionation of urine sample may also be carried out to reduce the complexity of urine proteome.

Recent development in the 2D computer aided software algorithms although fully automatic is not always perfect due to limitation in detection or matching standard. Editing of incomplete data set after spot detection is always required. There are also problems in missing values and the subsequent difficulties in statistic analysis. Occurrence of interfering compounds will also distort the 2D proteome images and the subsequent difficulties in computer analysis. Future study with urine sample preparation would need to be optimized and to be run in replicate gels to produce 2D gel images with high reproducibility and in order to do a proper statistical analysis. Replicate gels will also help in reduce detection of false positive and false negative through gel to gel variation. Urine sample may also need to be optimized for proper staining in order to decrease the gel to gel variation and increase the higher output from the computer aided analysis.

5.4 Conclusions

Stimulated and non stimulated U937 cell line in this study were brought for protein extraction and concentrated via lyophilisation. Lyophilized samples shown vertical
streaking in 1D gel due to protein degradation and were optimized with protease inhibitors. Better protein bands visualization was achieved in the following 1D gel. Protein sample with protease inhibitors however have observed streaking problems on 2D gels. Protein samples were then desalted together with protease inhibitors and succeeded in the following visualization with IPG strip and 2D gel images with better resolution compared to IPG strip and 2D gels without desalting. Subsequent 2D gels were brought for computer-aided 2D gel images analysis (with Progenesis and SameSpots from Nonlinear Dynamic) for differential protein expression in between the stimulated and non-stimulated U937 protein samples. Following the software analysis, there are detected both up and down regulated protein expression between the parallel gels.

Results for the study shown sample preparation for proteomics can be optimized for individual study and methods were here developed for the urinary proteomic analysis of acute rejection in renal allograft and biomarkers discovery. Other considerations in the sample preparation for example protein sample prefractation and replicating gels can be applied for the future study with urine sample for generating reproducible and high-resolution protein separations.

With the establishment of suitable biomarkers for acute rejection from proteomics analysis however, the proteomics assay alone is impractical for everyday screening of recipients. The advance of polymerase chain reaction (PCR) can be used in the correlative study with proteomics. The potential biomarkers identified from urinary proteomics can be assess for their suitability as prognostic indicator of acute rejection with real time PCR and a PCR based methods for the detection of the acute rejection will be established. PCR assay for the biomarkers of acute renal allograft rejection will be used in the screening of renal transplant patient non-invasively with urine samples.

Information derived from market research demonstrates that biomarkers is an emerging market with great commercial potential in various market segments from clinical applications to drug discovery. Importance of acute renal allograft rejection to the success in renal transplantation added by complications with immunosuppressants proves the need in the improvement of diagnostics. Biomarkers discovery for the diagnosis of acute renal allograft rejection will be timely and have great commercial potential with the prevalence of worldwide high renal transplant rate. In the UK alone, there is shown significant increase in renal diseases in recent years which prompt to increase need in renal transplants. Patients on the renal transplantation waiting list are also in the rise.

Altogether, proteomic analysis has the potential in the biomarkers discovery for acute renal allograft rejection. Establishment of biomarkers in the screening of renal transplant patient will be able to monitor recipient’s immune response to allograft as a means to adjust immunosuppressants doses. Screening of patients will reduce immunosuppressants associated complications and detect acute rejection before it is clinically apparent. The subsequent diagnosis with potential acute rejection biomarkers will help reduce the burden of the disease and thus enable healthier patient lifestyle, more cost-effective healthcare delivery and target care.
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