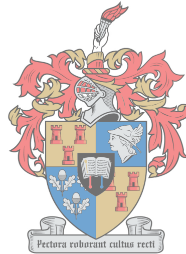
The host response to infection with pathogenic and non–pathogenic mycobacteria: a proteomics approach

by

Precious Zama Mahlobo



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Abstract

Tuberculosis (TB) continues to be a major health problem worldwide. In 2017, 1.6 million TB associated deaths were reported (WHO, 2017). The etiological agent of TB disease is Mycobacterium tuberculosis (*Mtb*), and is a highly successful pathogen due to its ability to persist in the host. The immune system uses the non-specific innate immunity as the first line of defence against invading pathogens. The interplay between macrophages and mycobacteria is not yet fully understood. Mass spectrometry is one of the most effective tools for identification and quantitation of proteins from complex mixtures of biological samples. It has been shown that mycobacteria cultured in detergent medium and detergent-free medium induce differential macrophage host response. Following on a study that identified differentially expressed genes using high-throughput RNA sequencing, we aimed to identify and quantify protein expression of murine bone marrow derived macrophages infected with non-pathogenic mycobacteria, *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG, and pathogenic mycobacteria, *Mycobacterium tuberculosis* H37Rv and *Mycobacterium tuberculosis* R179 grown in a detergent-free medium. The differential proteomes of C57Bl/6 cells in response to *Mtb* infection, were analysed at 12 hours post infection using liquid-chromatography-tandem mass spectrometry (LC-MS/MS). Four proteins MYH9, TLN1, AHNAK and GAL-3 were expressed by pathogenic mycobacteria. Moreover, corresponding genes (*Myh9, Tln1, Gal-3,* and *Ahnak*) of the differentially expressed proteins were quantified by using quantitative PCR (qPCR) to monitor and analyse gene expression at later time points, 12, 24 & 96 hours post-infection. At the later time points, *Myh9, Tln1* and *Ahnak,* were down-regulated indicating that these genes are only expressed at an early stage (up to 24 hours post-infection) of mycobacterial infection; while *Lgal-3* was up-regulated by all slow growers (BCG, H37Rv & R179) at 96 hours post-infection. Galectin 3 is a binding protein known to control the survival of *Mtb* during infection. The significance of this protein can be further investigated in TB patients and healthy controls.

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Abstrakte

Tuberkulose (TB) is steeds 'n wêreldwye gesondheidsprobleem. In 2017 is 1,6 miljoen TB-geassosieerde sterftes aangemeld (WGO, 2017). TB-siekte word veroorsaak deur Mycobacterium tuberculosis (Mtb) en is 'n hoogs suksesvolle patogeen weens die vermoë om te oorleef in die gasheer. Die immuunstelsel gebruik nie-spesifieke aangebore immuniteit as die eerste lyn van verdediging teen bakterieë. Die interaksie tussen makrofage en miobakterieë word nog nie heeltemal verstaan nie. Massaspectrometrie is een van die mees effektiewe gereedskap vir die identifisering en kwantifisering van proteïene uit komplekse mengsels van biologiese monsters. Ons poog om proteïenexpressie in beenmurgmakrofage te identifiseer en te kwantifiseer, geïnfekteer met nie-patogene miobakterieë (Mycobacterium smegmatis, Mycobacterium bovis BCG) en patogene mycobacteria (Mycobacterium tuberculosis H37Rv en Mycobacterium tuberculosis R179). Die differensiële proteïene van C57B1 / 6-selle in reaksie op Mtb-infeksie is 12 uur na infeksie geanaliseer met behulp van vloeibare chromatografie-tandem-massaspektrometrie (LC-MS / MS). Vier proteïene MYH9, TLN1, AHNAK en GAL-3 word uitgedruk deur patogene miobakterieë. Daarbenewens word die ooreenstemmende gene (Myh9, Tln1, Gal-3 en Ahnak) van die differensiaal-uitgedrukte proteïene gekwantifiseer met behulp van kwantitatiewe PCR (qPCR) om uitdrukkingsveranderinge te monitor op latere tydspunte, 12, 24 en 96 uur na infeksie. Die latere tydspunte het getoon dat Myh9, Tln1 en Ahnak slegs tydens vroeë ure van infeksies uitgespreek is; terwyl Lgal-3 opgegradeer word in makrofage geïnfekteer met BCG, H37Rv & R179 met 96 uur infeksie. Dit is bekend dat Galectin 3 'n bindende proteïen is wat bekend is om die oorlewing van Mtb tydens infeksie te beheer. Die rol van hierdie proteïene kan verder ondersoek word in TB-pasiënte en gesonde deelnemers. Dit sal ons in staat stel om die rol van hierdie proteïene tydens TB infeksie ten volle te verstaan.

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Abbreviations

°C	Degrees Celsius
µL	Microliter
B2M	Beta-2-microglobulin
BCG	Bacillus Calmette–Guérin
BMDM	Bone marrow derived macrophages
CAF	Central Analytical Facility
cDNA	Complimentary DNA
CFU	Colony forming unit
CO_2	Carbon dioxide
CPGR	Centre for Proteomic & Genomic Research
CSF-1	Colony-stimulating factor 1
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
FDR	False Discovery Rate
HCl	Hydrochloric acid
IFN-γ	Interferon gamma
IGg	Immunoglobulin G
kDA	Kilo Daltons
LC-MS/MS	Liquid-chromatography-tandem mass spectrometry
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
ml	Millilitre
MOI	Multiplicity of infection
MS	Mass spectrometry
NaCl	Sodium chloride
ng	Nanogram
NK	Natural killer
NLRs	NOD-like receptors
OADC	Oleic albumin dextrose catalase

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OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PILAM	Phosphoinositide-capped LAM
PRRs	Pattern recognition receptors
PSM	Peptide spectrum match
qPCR	Quantitative real-time polymerase chain reaction
RIN	RNA integrity number
RNA	Ribonucleic acid
RNAseq	Ribonucleic acid sequencing
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse-transcription polymerase chain reaction
s	Second
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSF	Syringing, settling and filtration
TB	Tuberculosis
TLRs	Toll-like receptors
Tm	Melting temperature
TNF	Tumour necrosis factor
Tris-HCl	Trisaminomethane-hydrochloride
UBC	Ubiquitin c
Mg	Microgram
MM	Micromolar

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Chapter 1:

1.1 Introduction

Tuberculosis (TB) continues to be a major health problem worldwide. In 2017, 1.6 million TB associated deaths were reported (WHO, 2017). The etiological agent of TB disease is *Mycobacterium tuberculosis* (*Mtb*), and is a highly successful pathogen due to its ability to persist in the host. The co-evolution of mycobacteria with humans, allowed it to acquire mechanisms of manipulating the host immune defence system (Gagneux, 2012). In order to survive inside the macrophage of the host, mycobacteria senses and responds to the changes in the immediate environment such as pH differences and other stress conditions that occur in the phagosome (Tan et al., 2013). Analysis of macrophage host response mechanism pathways during *Mtb* infection is very critical in understanding the pathogenesis and persistence of the bacilli inside the host environment. When macrophages are infected, inflammatory signals stimulate the influx of more macrophages and monocytes to the area of infection (Basu et al., 2012). Macrophage apoptosis is a cascade event used by macrophages against pathogens. Mycobacteria can replicate within macrophages and resist macrophage microbicidal mechanisms. The immune system uses the non-specific innate immunity as the first line of defence against invading pathogens (Basu et al., 2012; Muralidharan & Mandrekar, 2013). The interplay between macrophages and mycobacteria is not yet fully understood. A detailed understanding of macrophage response to *Mtb* infection is thus necessary to elucidate host components and pathways which are manipulated by *Mtb* for its own survival. With proper diagnosis and treatment, TB can be prevented and cured (WHO, 2017). The search for new TB drugs is mainly driven by the emergence of drug-resistant strains and poor health care. To develop better anti-TB drugs and TB vaccines; the early stages of infection are the most critical to elucidate pathogenicity of any infection (McDonough et al., 1993).

1.2 Literature Review

Pathogenicity of *Mtb* can be determined during the first few hours of infection. Macrophages use phagocytosis and phagosome maturation to kill most pathogens, *Mtb* is known to block this process (McDonough et al., 1993; Saunders & Britton, 2007). Macrophage serves as the first defence response of a host to *Mtb* infections (Neyrolles et al., 2015). Several membrane receptors, including Toll-like receptors (TLRs), complement receptors, mannose receptors, and scavenger receptors, are involved in the integration of with *Mtb* macrophage (Cambier et al.,

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2014). Uptake of *Mtb* by macrophage triggers a series of cell signalling pathways and initiation of an immune response. However, the mechanism of *Mtb* induced macrophage infection is different among species. *Mtb* often leads to arrest of phagosome maturation, anti-apoptosis response, and suppression of antibacterial response. Macrophages can recognize fungal, bacterial and viral pathogens, by identifying pathogen-associated molecular patterns (PAMPs) of the invading pathogen (Taube et al., 2015). The macrophage receptors which identify PAMPs are referred to as pattern recognition receptors (PRRs) (Taube et al., 2015). PRRs are classified based on the PAMP they recognise (Muralidharan & Mandrekar, 2013). The host response does not rely on one receptor for pathogen recognition. There is a variety of PRRs which recognise mycobacteria during infection (Takeda & Akira, 2005). Some of the PRRs that have been identified include Toll-Like Receptors (TLRs), NOD-like receptors (NLRs), C-type lectins and Dectin-1 (Kleinnijenhuis et al., 2011). An interplay between several receptors regulate changes in gene and protein expression triggered by bacterial infection (Kleinnijenhuis et al., 2011).

Toll-like receptors are highly conserved PRRs (Akira, 2006). Mycobacteria are recognised by TLR1, TLR2 and TLR9 (Akira, 2006; Basu et al., 2012). The expression of TLRs can either be extracellular (on the membrane surface of a macrophage) or intracellular (in the cytoplasm or organelles) (Akira, 2006). TLR 1 and 2 are extracellular expressed receptors while TLR9 is expressed in the intracellular compartments such as endosomes (Akira, 2006; Takeda & Akira, 2005). The expression of TLRs can also be strain dependent, mycobacterial cell membrane composition varies depending on strain type (Tsuji et al., 2000). *Mycobacterium tuberculosis* membrane is comprised of ManLam layer which is a potent anti-inflammatory activator (Akira, 2006; Gilleron et al., 2006). *Mycobacterium tuberculosis* has a 19 kDa lipoprotein which induces host response by interacting with TLR1 and TLR2 (Akira, 2006). The PRR TLR2 is required for the generation of reactive oxygen species, chemokine activation and MARK activation (Vujanovic, 2011). Macrophage receptor TLR2 can recognise the *Mtb* 19 kDa lipoprotein during infection and activate host response. *Mtb* also uses this TLR2-19 kDa recognition mechanism to inhibit host response activation pathways (Akira, 2006). The 19 kDa lipoprotein, interacts with TLR2 to inhibit IFN-$_{\gamma}$ production and MHC class II antigen processing activity (Akira, 2006). The cell membrane of non-pathogenic mycobacteria such as *M. smegmatis* contains PILAM layer which stimulates TLR2 (Akira, 2006). Host (macrophage) extracellular proteins are the first ones to encounter invading pathogens and initiate host response triggering signalling molecules which promote the production of specific antigen presenting cells and memory cells for development of acquired immunity. TLR9 recognises mycobacterial genomic DNA released

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during endosome and lysosome degradation (Akira, 2006). The presence of unmethylated CpG dinucleotides stimulates the expression of TLR9 (Akira, 2006; Dowling & Mansell, 2016). The CpG motif in mammalian species is highly methylated, enabling the macrophages to distinguish between self and non-self CpG (Akira, 2006).

When mycobacteria are cultured in detergent-free media, they spontaneously form pellicles, a biofilm-like structure (Segura-Cerda et al., 2018). Growth as pellicles in stationary cultures, is the method used by most manufacturers of *Mycobacterium bovis* BCG vaccine (Brennan, 2017). Mycobacterial biofilms promote drug tolerance, as well a specific *in vivo* immune response in infected guinea pigs (Kerns, 2014). It has been shown that mycobacteria cultured in detergent medium and detergent-free medium induce differential macrophage host response (Leisching et al., 2016). Different mycobacterial species contain different complex mixtures of mycolic acids that can provide a fingerprint. The development of an improved vaccine against tuberculosis is needed. Identification of novel host response genes or proteins would greatly facilitate the development of new vaccines. Host response associated mechanism of containing *Mtb* can provide an understanding of the protective mechanisms and lead to more targeted vaccine development (Walduck et al., 2004).

1.3 Macrophage cell signalling host response during mycobacterial infection

The interaction between live tubercle bacillus and the host immune response is still not well understood. Macrophage cells use innate defence mechanisms to kill invading pathogens. These mechanisms include generation of reactive oxygen and nitrogen intermediates, phagolysosomal fusion (the fusion of phagosome and lysosome), nutrient deficiency, and apoptosis. These defence mechanisms should limit pathogen growth or survival. The use of these defence mechanisms is ultimately mediated by changes in both the levels and activities of key proteins. With the introduction of the proteomics approaches, a number of proteomic studies of intracellular bacteria have been performed to gain insight into the bacterial adaptation to the host cell. A common feature among intracellular bacterial pathogens is the ability to sense the host cell environment and to adapt their metabolisms such as nutrient availability, pH, the osmotic pressure inside the cellular compartments and oxygen availability. Host response during *Mtb* infection is dependent on the interplay between T cells, macrophages and other leucocytes (Hasan et al., 2009).

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Tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and interleukin-12 (IL-12) are essential for host response (Hasan et al., 2009). The mechanism of macrophage innate host response does not change, it remains constant through species (mice/humans). Some mycobacterial species, *M. smegmatis*, are unable to survive the innate host response mechanisms yet some species such as *M. bovis* and *M. tuberculosis* are able to survive (Saunders & Britton, 2007). It is this, host-pathogen interplay that is not clear and most studies are directed towards identifying proteins expressed by mycobacteria during host infection (Stanley & Cox, 2013). Fewer studies have been conducted to identify host response proteins. The limitation of studying bacterial proteins arises when mycobacteria develop resistance against the drug, such as rifampicin resistance (Putim et al., 2017), driving the need for identification of new mycobacteria vaccine and drug targets (Sharma et al., 2015). Macrophages and dendritic cells are the first line of defence in recognizing various pathogens (Akira, 2006; Dowling & Mansell, 2016; Muralidharan & Mandrekar, 2013). The host response signalling pathways allow for the recognition and elimination of pathogens during macrophage infection (Akira, 2006; Muralidharan & Mandrekar, 2013). Macrophages escape phagocytosis and apoptosis by inhibiting phagolysosome and other cell signalling processes (Danelishvili et al., 2010; Rohde et al., 2007). Upon infection, mycobacteria use multiple pathways to ensure its survival inside the macrophage and have the ability to redirect macrophage apoptosis enabling the pathogen to kill the infected cell.

When the host cell is infected with a pathogenic strain of mycobacteria, the mycobacteria induces necrosis, a premature cell death, while macrophages infected with non-pathogenic strains on mycobacteria induce apoptosis, programmed cell death (Ashida et al., 2011). The apoptotic mechanism allows the macrophage to contain the infection in a localised area (Danelishvili et al., 2010; Rohde et al., 2007). IFN-γ and tumour necrosis factor (TNF) are secreted in patients who respond to *Mtb* from the beginning of infection leading to the disease (Jasenosky et al., 2015). IFN-γ secretion increases throughout infection in diseased patients. The presence of IFN-γ from the beginning of infection until critical stages of the disease suggest that IFN-γ is directly related to TB disease (Jasenosky et al., 2015). *Mycobacterium tuberculosis* can either replicate within the cell or is destroyed by the cell determined by its virulence. *Mtb* recognition by macrophages does not only result in the activation of innate immunity but also the development of antibodies for adaptive immunity. IFN-γ is a cytokine produced by macrophages at the early hours of infection, activates macrophages and the production of antigen-specific IFN-γ -producing T cells (Kulchavenya, 2013).

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1.4 The proteomics of infected macrophages

Several studies have employed proteome analysis for elucidating specific biomarkers that are expressed by *M. tuberculosis in vivo* (Kumar et al., 2011; Ryndak et al., 2015) because gene expression is not the exact correlate of the protein expression. *In vivo* proteomic characterization of bacteria during infection, can provide insight to which proteins are essential for intracellular survival of mycobacteria and how they manipulate host cell signalling (Gengenbacher et al., 2014). However, proteomic *in vivo* studies have lagged behind the genomic studies due to the requirement of eliminating host proteins for distinct visualization of bacterial proteins (Li and Lostumbo 2010). Expression analysis, as well as characterization of essential proteins of intracellular bacteria surviving and replicating within the host cells in *in vitro* conditions, can be used as a model for understanding TB infection. The intracellular proteome of many bacterial species such as *Listeria monocytogenes* (Van De Velde et al., 2009) *Coxiella burnetti* (Samoilis et al., 2010) and *Mycobacterium tuberculosis* (Monahan et al., 2001; Mattow et al., 2006; Singhal et al., 2012) have been analysed from phagosomes in the macrophages. Host protein response has also be studied in alveolar epithelial cells and pathogen-containing vacuoles (Agarwal et al., 2018; Hoffmann et al., 2018). A list of host proteins and pathways of macrophage mycobacterial infection performed on cellular extracts, phagosomes using mass spectrometry have been identified reported (Hoffmann et al., 2018).

The macrophage selectively applies stress on the mycobacteria, inducing the cell signalling essential for the host defence mechanisms while containing/killing mycobacteria within macrophages. MS studies have investigated mycobacteria containing vacuoles at different time points post infection, and elucidate the phagosome maturation block and other features of *Mtb* infection (Figure 1.1). The induction of survival mechanisms by mycobacteria, along with a range of host immunological effector molecules, emphasizes the complexity of the cross-talk that occurs between the macrophage and the mycobacterium. To characterize this cross-talk and to detail the changes which occur following the initial interaction between mycobacterium and macrophage we used mass spectrometry to identify mouse macrophage proteins induced by *Mtb* infection from mycobacterial species grown in medium without Tween-80. Mycobacterium tuberculosis, lose virulence with prolonged culture in artificial media supplemented with Tween (Leisching et al., 2016).

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Investigations revealed important information with regard to the morphological changes that mycobacteria undergo in a culture which could explain this loss in virulence. It has been reported that the bacilli of *Mycobacterium para-tuberculosis* and *Mycobacterium avium-Mycobacterium* intracellular Complex (MAC) became elongated and changed from a rough to a smooth appearance when Tween 80 was present in the growth medium as compared to excluding Tween 80 (Masaki, 1991). The authors suggest that the rough appearance of the bacilli is associated with the presence of glycopeptidolipids (GPLs) on the surface of the bacilli. It has been established that the mycobacterial capsule not only contributes to the virulent phenotype (Schwebach et al., 2001) but also enhances mycobacterium-macrophage interaction during the early stages of infection. There currently is no published study that used mass spectrometry to identify macrophage proteins induced by mycobacteria grown in a detergent-free medium.

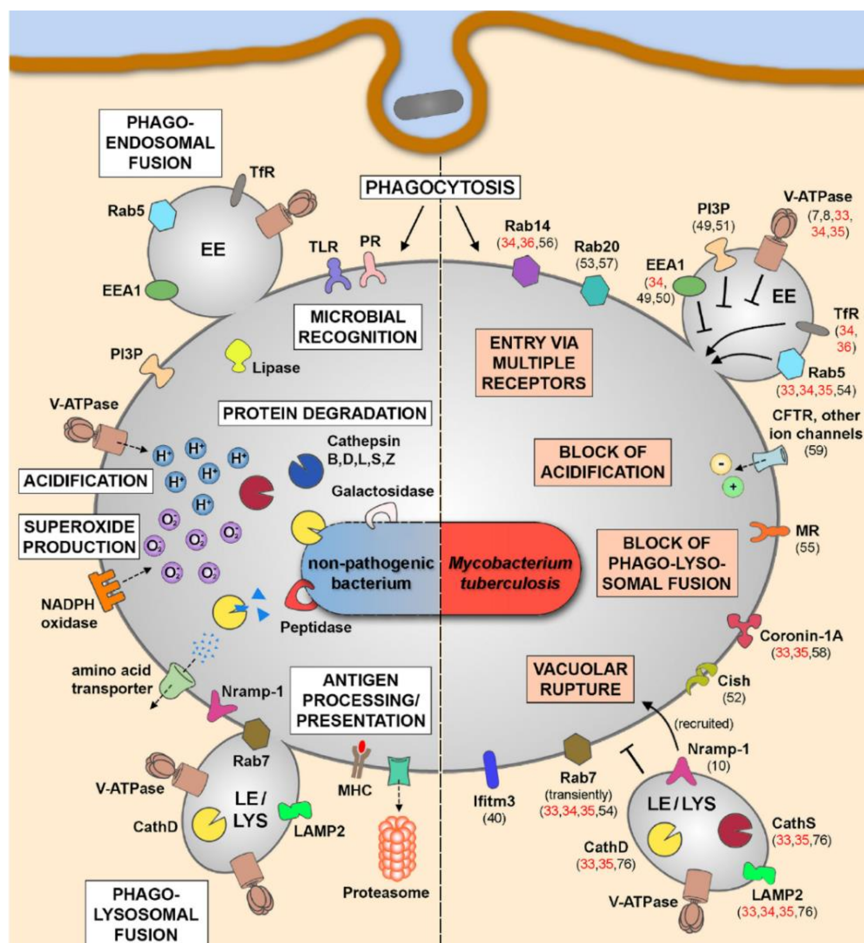

Figure 1.1: Phagosomal functions after internalization of non-pathogenic (left panel) and pathogenic mycobacterial infection (right panel). Schematic representation of the key players and main features after the uptake on non-pathogenic bacteria leading to the clearance of the internalized cargo (left panel). Upon *M. tb* infection, the pathogen is internalised in mycobacteria-containing vacuoles, which are delayed in phagosome maturation (right panel). Altered phagosomal functions are indicated with involved host molecules that were identified by mass spectrometry EEA1, early endosomal antigen 1; PI3P, phosphatidyl-inositol-3-phosphate; V-ATPase, vacuolar proton ATPase; TfR, transferrin receptor; Coronin-1A, CathD, Cathepsin D; CathS, Cathepsin S; and LAMP2, lysosome-associated membrane protein, (reproduced from Hoffman et al., 2018).

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1.5 Protein identification and quantification using mass spectrometry

The study of the proteome of the host response to mycobacteria infection can help to better understand the biology of macrophages and the complex interplay between mycobacteria and host cells. Mass spectrometry (MS) is an effective tool for proteome analysis, facilitating the identification and quantitation of proteins from complex mixtures (Barrera & Robinson, 2011). In addition to providing a list of proteins identified in a sample, quantitative proteomics has been fundamental in enhancing our understanding of protein expression and how protein abundance can change. There is a variety of innovative quantitative proteomic methods available. MS-based protein identification using LC-MS/MS is now widely adopted (Yates et al., 1995). Shotgun proteomics provides direct protein-level evidence for gene products by matching peptide tandem mass spectra, obtained by high-resolution tandem mass spectrometry, to predicted spectra from a proteome database or to entries from spectral reference libraries. Mass spectrometry-based proteomics often involves the analysis of complex mixtures of proteins derived from cell or tissue lysates or from body fluids, posing tremendous analytical challenges. After proteolytic digestion, the resulting peptide mixtures are separated by liquid chromatography and online electro-sprayed for mass spectrometric (MS) and tandem mass spectrometric (MS/MS) analysis (Figure 1.2). The information produced by the mass spectrometer, lists of peak intensities and mass-to-charge (m/z) values, can be manipulated and compared with lists generated from theoretical digestion of a protein or fragmentation of a peptide. Mass spectrometric identification of peptides and proteins is based on determining the mass of the peptides and then the mass of fragment ions derived from them (called tandem mass spectrometry or MS/MS). Proteins are mostly cleaved with sequence specific proteases (such as trypsin) so optimal size peptide can be generated and their masses (actually, their m/z values) are determined. In tandem mass spectrometry, each tryptic peptide ion whose m/z value is measured is then fragmented by collision with gas molecules to generate a series of product ions that are broken at various positions along the peptide backbone. The m/z value of the precursor ion and the set of masses corresponding to the resulting fragments is often sufficient to uniquely identify the sequence of the starting peptide, using database search algorithms.

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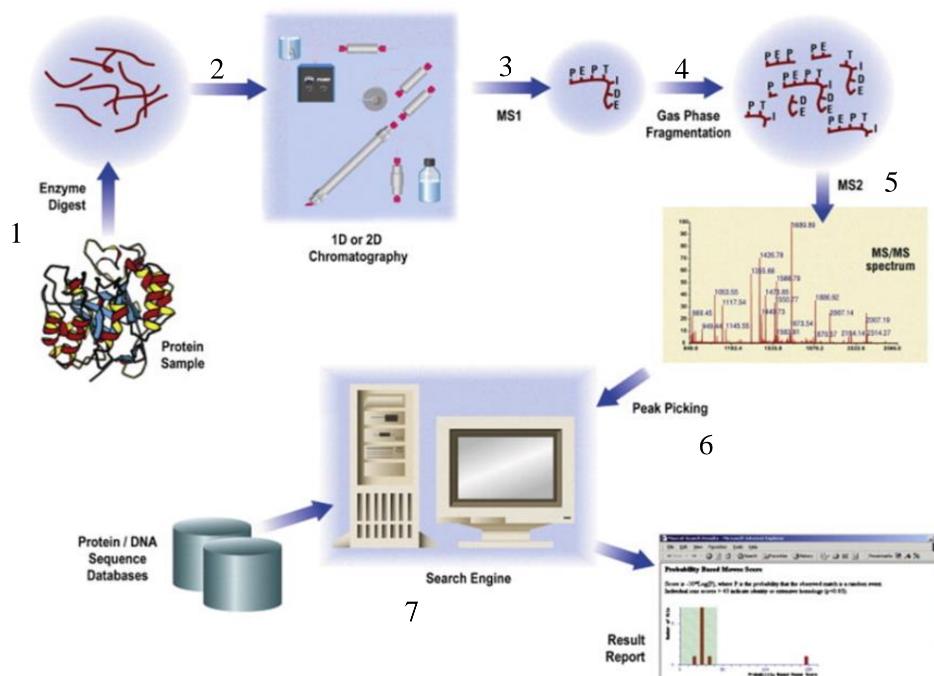

Figure 1.2: The workflow for proteomics analyses involves the enzyme, trypsin, is used to digest proteins to peptides (1). Chromatography is used to regulate the flow of peptides in the mass spectrometer (2). The peptides are selected one at a time using the first stage of mass analysis (3). Then, each isolated peptide is induced to fragment by collision (4), and the second step of the mass analysis is used to capture an MS/MS spectrum (5). For each MS/MS spectrum, the software is used to determine which sequence of peptides in a database of protein or nucleic acid sequences gives the best match (6). Each entry in the database is digested, in silico, using the known specificity of the enzyme, and the masses of the intact peptides are calculated. If the calculated mass of a peptide matches that of an observed peptide, the masses of the expected ions are calculated and compared with the experimental values. Some search engines also predict and compare the relative intensities of ion fragments (7) (reproduced from Cottrell, 2011).

A quantitative proteomics workflow consists of cellular lysis, protein separation and digestion followed by LC-MS analysis. Different protein identification methods can be used, (1) labelled quantification, most labelled quantitation approaches rely on the incorporation of a label into one or more of the samples to be analysed, (2) label-free quantitation (Cox et al., 2014). Mass spectrometric identification of protein peptides is based on determining the mass of the peptides and then the mass of fragment ions derived from them (known as tandem mass spectrometry or MS/MS) (Lennart et al., 2010). Liquid-chromatography MS/MS, proteins are fragmented by a protease (trypsin) before analysis, then the peptide mixture is fractionated by liquid chromatography (Aebersold & Mann, 2003). When combined with the mass spectrometric analysis, this is called LC-MS/MS. The LC-MS/MS approach is usually considered for identification of variant forms of proteins as it is not possible to determine which peptides occur together in a protein once the protein has been digested. However, the LC-MS/MS approach is

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experimentally simpler and sufficient if the objective is just to identify or quantitate proteins (Bruce et al., 2013). Discovery phase in proteomics is referred to as shotgun proteomics; this approaches identifies random fragments of a large sequence and assemble them by using prior knowledge about the full protein sequences (Lennart et al., 2010). There are many strategies used to quantify protein samples using a mass spectrometer. The systematic information on proteins is important because; proteins can interact with all other classes of molecular components, including other proteins. Protein mutations that affect posttranslational modifications (PTM) have been studied in most mycobacterium species and play a significant role in protein function.

Changes in protein abundance cannot be easily determined from DNA microarray data, because mRNA abundance is not directly related to protein abundance (Gstaiger & Aebersold, 2009). Improvements in sample manipulation, multiplexing, sensitivity, and reagent cost and time requirements are continuously being observed. Protein identification by mass spectrometry is still at its infancy. A standard analysis protocol has not yet been documented as it keeps being edited based on the data output from the mass spectrometry largely influenced by experimental design. Proteomics can be used for a number of different research purposes, biomarker identification (Chen et al., 2018; Hadizadeh et al., 2018), identification of novel metabolic pathways (Reddy et al., 2018), mycobacterial membranes (Gilleron et al., 2006), mycobacterial protein (Hoffmann et al., 2018), cancer cells, mass spec application is unlimited but comes with challenges. Quite a number of research studies have used MS to identify or characterise mycobacterial proteins expressed during macrophage infection (Infantes-Lorenzo et al., 2017, Li et al., 2017, Segura-Cerda et al., 2018), we have not seen a study which uses MS to identify macrophage proteins induced by mycobacteria grown in detergent-free medium.

1.6 Rationale of the study

The ability of *Mycobacterium tuberculosis* to sense environmental signals and implement adaptive changes is a key feature of living cells. It is essential to know which genes and proteins stimulate host response during early *Mtb* infection to help understand what defines TB disease. Previous studies in our laboratory, optimized a procedure for growing and processing detergent-free *Mtb* and assessed the response of bone marrow derived macrophages (BMDM) infected with multidrug-resistant *Mtb* (R179 Beijing 220 clinical isolate) using RNAseq. We compared the effects of the host response to *Mtb* cultured under standard laboratory conditions, Tween 80 containing medium, or in the detergent-free medium. RNAseq comparisons reveal 2651 differentially expressed genes in BMDMs infected with R179T *Mtb* vs. BMDMs infected with

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R179NT *Mtb* (Leisching et al., 2016). However, protein turn-over rate is not related to RNA transcription. It is, therefore, necessary that we identify and quantify protein regulation of macrophages infected with different mycobacterial strain grown in a detergent-free medium in order to validate previous studies in our laboratory performed using RNAseq. In addition, to our knowledge, this is the first study to use mass spectrometry to analyse macrophage proteins infected with *Mtb* grown in a detergent-free medium. We believe that results would be unique to previous studies that used detergent because it has been established that Tween-induced changes on the mycobacterial cell wall affect macrophage uptake and the immune response to *Mtb* (Sani et al., 2010).

Identification of differentially expressed genes and proteins could reveal cell signalling and metabolic pathways used by macrophages and elucidate host-pathogen interaction mechanisms. Current techniques of proteomics can be broadly divided into discovery and quantitative methods. Different mycobacterial strains were used to infect and analyse gene and protein expression changes in macrophages. On the other hand, we compared slow versus fast-growing mycobacteria because previous studies showed that slow-growing *Mtb* (BCG, H37Rv and R179) have a doubling time of 16-20 hours (Moriwaki et al., 2001) and fast-growing mycobacteria such as *M. smegmatis* have a doubling time of 3-4 hours (Lu et al., 2001). *M. tuberculosis* H37Rv and R179 are identified as pathogenic because they cause disease inside the host. Although BCG survives in macrophages it is still non-pathogenic because it is an attenuated mycobacterial species. *M. smegmatis* is also non-pathogenic to humans but is killed in macrophages within 24 hours of infection (Moriwaki et al., 2001).

To advance the understanding of macrophage response during infection, the reproducible quantification of the proteins that catalyse and control cell signalling is critically important. A basic proteomics study compares protein expression under homeostatic conditions to the abundance of the same proteins in response to an extracellular stimulus. Which is why we tried to minimize this difference by using mycobacteria grown in a detergent-free (Tween 80) medium. The aim of the study was to utilise mass spectrometry to establish a protocol for protein identification and quantification in mouse macrophages infected with mycobacteria grown from detergent-free medium. Mouse models are not lucrative enough to study the host-pathogen interaction of human infectious diseases. Establishment of this protocol could be useful for protein extraction in human monocyte-derived macrophages.

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1.7 Aims

1.7.1 To perform proteomic analysis on mouse bone marrow derived macrophages infected with pathogenic and non-pathogenic mycobacterial species to identify proteins that are differentially induced at an early infection time-point.

- To isolate BMDMs and infected with mycobacteria, *M tuberculosis* H37Rv, *M. tuberculosis* R179, *M. bovis* BCG and *M. smegmatis*, cultured in detergent (Tween 80) free medium.
- To extract macrophage protein after 12 hours of infection and analyse using mass spectrometry (LC-MS/MS).

1.7.2 To employ qPCR to determine whether the differentially induced proteins identified in Aim 1 are due to changes in their gene expression, and analyse how their expression changes at later time-points during the course of the infection.

- To isolate BMDMs and infected with mycobacteria, *M tuberculosis* H37Rv, *M. tuberculosis* R179, *M. bovis* BCG and *M. smegmatis*, cultured in detergent (Tween 80) free medium.
- To extract macrophage total RNA after 12, 24 and 96 hours of infection. To analyse gene expression of selected genes using qPCR.

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Chapter 2: Methods and Materials

2.1 Mycobacterial strains

Four mycobacterial stains were used, *Mycobacterium tuberculosis* H37Rv, *Mycobacterium tuberculosis* R179, *Mycobacterium bovis* BCG and *Mycobacterium smegmatis*. Mycobacterial species used in this study were obtained from the Division of Molecular Biology and Human Genetics in Stellenbosch University, South Africa.

2.2 Culture conditions

All mycobacterial species were grown in a detergent-free medium (the absence of Tween 80). All experiments performed in a biosafety level 2 cabinet inside a biosafety level 3 laboratory. Tween 80 was not used in growing bacterial cultures because it has been shown that the addition of Tween changes some membrane proteins on the mycobacterial surface (Wang et al 2011; Sani et al. 2010). Only T25 flasks were used because it was previously observed by a former student that more clumping occurs in larger (T75) flasks.

2.2.1 Culture conditions for slow-growers

In a BSL-3 cabinet inside a BSL-3 laboratory, *M. tuberculosis* H37Rv and *M. tuberculosis* R179 were cultured under same conditions because they are all slow-growing mycobacteria. *Mycobacterium bovis* BCG was cultured in a BSL-2 cabinet outside the BSL-3 laboratory. All species were grown in T25 flasks without shaking. Middlebrook 7H9 medium (Difco, Becton Dickinson, USA) supplemented with 10% oleic acid albumin-dextrose-catalase (OADC, Becton Dickinson, USA) and 0.5% glycerol (Merck Millipore, Germany) (detergent-free) was prepared. A stock vial of *Mtb* and *M. bovis* that was previously grown in the presence of Tween 80 was used in order to start with little to no clumps and minimize clumping in the starter culture. The bacteria was thawed and then passed 10× through a G25 (Becton Dickinson, USA) needle before seeding. The starter culture was grown to an OD_{600} of 0.2 – 0.3, higher OD produced more bacterial clumps. A 1ml starter culture was diluted in 9 ml detergent-free 7H9 medium and grown to an OD_{600} of 0.3 – 0.4. The flasks were kept in airtight boxes and incubated in a 37˚C walk-in incubator. When an OD_{600} of 0.3 had been reached each flask was sub-cultured into 5 T25 flasks (10 flasks in total). Each flask was split into 2 x T25 flasks where 5 ml culture was added to 5 ml Tween-less 7H9 medium (20 flasks in total) and grown to an OD_{600} of 0.4 to minimize

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clumping, cultures grown past this OD were observed to clump exponentially, consequently resulting in a significantly lower yield of single-celled bacteria.

2.2.2 Culture conditions for fast-grower

A frozen starter stock culture of *M. smegmatis* kept in a -80˚C freezer was used to grow new *M. smegmatis* cultures. The starter culture was grown in a 100 ml Erlenmeyer flask overnight in a 37˚C incubator with constant shaking without exceeding an OD_{600} of 0.4. After 24 hours the bacteria was sub-cultured into a 500 ml Erlenmeyer flask and stocks were made the following day.

2.2.3 Stocks preparation

Mycobacterial stocks for all species were prepared as described previously (Leisching et al., 2016). Cultures were combined into 4 X 50 ml Falcon tubes (Becton Dickinson, USA) and let the clumps settle for 10 minutes. The top 45ml from each falcon tube was transferred into a new falcon tube and centrifuged the tubes at 1500 rpm for 5 minutes to minimize the presence of bigger clumps. The supernatant was discarded. The pellet was resuspended in 5 ml of 7H9 with no Tween-80, giving a total of 20 ml. All pellets were combined, resuspended and allowed to settle for 10 minutes. Only the top 17 ml was used to make 1 ml aliquots into 1 ml cryovials (Merk, USA) and frozen at minus 80˚C.

2.2.4 Determination of colony forming units (CFU) counts

The concentration of frozen bacteria in each 1ml vial was determined by titration. A total of 3 vials were used for *Mtb* stock titration. Each vial was syringed 10× with a G25 needle and the bigger clumps were allowed to settle. *M. smegmatis* settled for 1 minute, BCG for 30 seconds, *M. tuberculosis* H37Rv for 10 minutes and *M. tuberculosis* R179 for 1 minute. The top 750 µl of the bacteria was added into 4.3 ml of 7H9 medium and filtered through a 5.0 micron filter to ensure only single bacteria are obtained. Four dilutions were made, $10^{-1} – 10^{-4}$, and plated 50 µl on 7H11 agar plates. The plates were kept in a sealed box and incubated in a 37˚C walk-in incubator. The colonies were counted after 3 days for *M. smegmatis* and 4–6 weeks for *Mycobacterium bovis* BCG, *M. tuberculosis* H37Rv and *M. tuberculosis* R179.

Colony forming units (CFU) counts were done as shown follows;

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Dilutions	Counts (colonies)
10^{-3}	64 colonies
	64×10^{3} (20) = 1.28×10^{6} bacteria per 1 ml
10^{-4}	23 colonies
	23×10^{4} (20) = 4.6×10^{6} bacteria per 1 ml

2.3 Mouse bone marrow extraction

Animal housing and ethics statement

Animals were housed (3 per cage) in a temperature controlled room with a 12-hour light-dark cycle and had free access to food and water. This research study was approved by the Stellenbosch University Animal Ethics committee on Animal Care and use and complies with the South African Animal Protection Act (Act no 71, 1962). Animal Ethics No. N07/09/195; 1GH_PIE01.

A 6-8-weeks-old black C57Bl/6 female mice were used for bone marrow extraction. Mouse euthanasia was performed by using the cervical dislocation method. After the euthanasia, the mouse was dissected, separated the femur and tibia from which the bone marrow was extracted (Carbone et al., 2012).

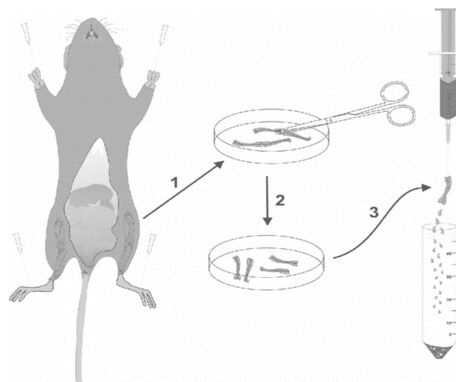

Figure 2.1: Schematic overview of the protocol for isolation of bone marrow cells. The back and front legs were fixed, the skin of the legs was removed to the ankle, and the long bones of the legs of the fixed mouse are exposed by removing the muscle tissue (1). The legs were detached from the body at the hip joint and the ankle. The tibia and femur of each leg were separated at the knee joint (2), the bone ends were chopped off, and the bone marrow rinsed out with a syringe filled with BMMC medium (3) and collected in a Falcon tube.

The mice were pinned to a dissecting board and alcohol sterilized to minimize contamination (Figure 2.1). The skin of the abdomen and upper leg were cut open and pinned the skin down to minimize hair contact. The knee was located using the white fibrous tissue of the tendon as guide; using a forceps the leg was gripped perpendicularly and firmly a few millimetres below the knee joint and inserted the scissors into the leg’s flesh alongside and in the middle of the femur; the

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scissors was opened up against the forceps to dislocate the knee joint. This allowed the protruding femur to be pulled away from the flesh, leaving some flesh and the hip joint to be cut free with a scalpel. The bone was briefly dipped in 70% alcohol and placed in a 50 ml falcon tube with 10 ml RPMI 1640. This was all done in a biosafety cabinet at the animal facility, the bones were then transported on ice to the tissue culture laboratory for bone marrow extraction. Opposite ends of the bone were cut open at the supracondylar line and pectineal line. Bone marrow was extracted by inserting a G25 needle at one end and flushed out the bone marrow with RPMI 1640. The bone marrow was flushed out (10×) on each end until all the marrow was extracted. A G25 needle was used to break up the clumps.

2.3.1 Bone marrow culture conditions and cell differentiation

The precursor cells were plated at a concentration of 2×10^5, as starter concentration, the cells multiply to a concentration of 2×10^6 after 5 days. An aliquot of the cells was counted on a haemocytometer while the remaining cells were left on ice. The aliquot was diluted with 2% acetic acid to lyse the red blood cells and counted the small shiny precursor cells. Cells were diluted to 2×10^5 cells per ml in complete medium consisting of RPMI 1640 (with L-glutamine, BE12-702F, Lonza) + 10% FBS (Foetal Bovine Serum, Biochrom, Germany) + 10% L-929 conditioned medium (source of colony stimulating factor-1 (CSF-1)). To obtain a monolayer, 2.5 ml of cells per well in a 6-well plate and 1.5 ml per well in a 12-well plate (Nunc, Thermo Scientific, USA) was added. The plates were incubated at 37˚C in 5% CO_2 incubator (Esco Technologies, USA). The day from which the cells were seeded was counted as day 1, cells were washed on day 5 to remove undifferentiated/ unattached cells and debris. The cells were washed twice with warm RPMI 1640 only and re-incubate with fresh complete medium. The medium was changed after 3 days. The matured macrophages are spindle shaped and more round when densely packed. The medium was replenished every two days. Differentiated cells were ready for infection after 7 days of seeding. Cells were monitored and visualised daily for contamination and to ensure there is no nutrient deficiency.

2.3.2 Infection conditions

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Mycobacteria stock vials were thawed and clumps were disrupted by passing through a 1 ml tip 10 times followed by syringing 10 times (20 passes) through a G25 needle. Major clumps were allowed up to 10 minutes to settle, where after the top 750 µl was added to 4.25 ml growth medium RPMI 1640 with 10% CSF-1 (colony stimulating factor-1). The 5 ml bacterial suspension was filtered immediately through a 5.0 µm pore size filter (Merck Millipore, Germany) and 10% FBS added. The required volume (depending on titration and MOI) was then added to bone marrow derived macrophages (BMDMs) in complete medium. BMDMs were infected with either *M. tuberculosis* H37Rv, *M. tuberculosis* R179, *M. bovis* BCG or *M. smegmatis* at an MOI of 1 – 2 (1 – 2 mycobacteria per macrophage) and allowed 4 hours for uptake. The cells were then washed 3 times with warm phosphate buffered saline (PBS), and incubated for an additional 8 hours, 20 hours and 92 hours in complete medium (12 hours, 24 hours and 96 hours in total). No antibiotics were added during the washing, antibiotics can be added to ensure the removal and killing of any extracellular bacteria. Uninfected BMDMs served as the control.

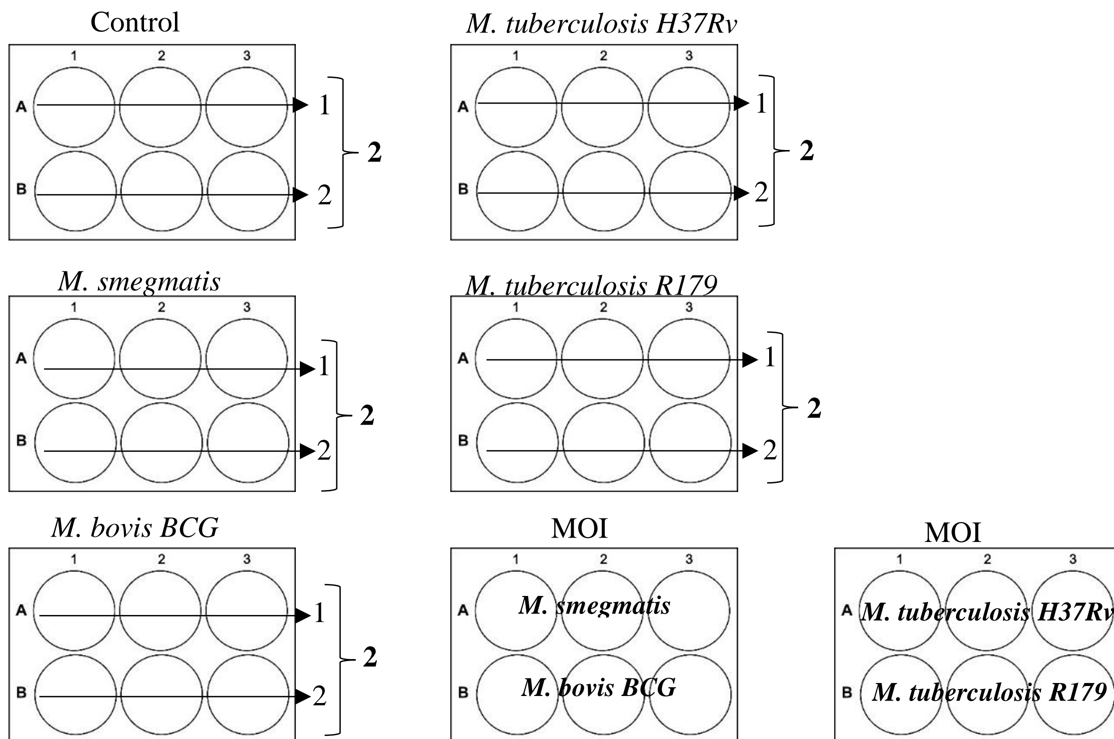

Figure 2.2: Protein extraction experiment set-up: 12-hour infection experiments were performed in 6 well plates. After 12 hours of infection with mycobacteria, the cell lysate were pooled together (A1 + A2 + A3 = sample 1; B1 + B2 + B3 = sample 2) to make one sample, therefore there were 2 technical replicates for every plate infected with mycobacteria. A total of 10 protein samples (2 samples from each 6 well plate) were generated in every infection experiment. Each experiment required one mouse to seed macrophages in seven 6-well plates. The “MOI” plates were used to determine bacterial uptake, 3 wells for each species.

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2.4 Determination of bacterial uptake by macrophages

After 4 hours of infection, bone marrow derived macrophages were lysed with sterile 0.1% Triton X-100 releasing internalised mycobacteria. This was done to determine how many single mycobacterial cells were internalised by each macrophage. Four dilutions were made, $10^{-1} – 10^{-4}$, and 50 µl was plated on 7H11 agar plates (all dilutions were plated). The plates were kept in a sealed box and incubated in a 37˚C walk-in incubator. The colonies were counted after 3 days for *M. smegmatis* and 4–6 weeks for *M. bovis* BCG, *M. tuberculosis* H37Rv and *M. tuberculosis* R179. The number of colonies counted and calculated, determined how many mycobacteria were internalised by the macrophages after infection. The final multiplicity of infection that was obtained was 1 bacteria per macrophage.

Multiplicity of infection (MOI) calculation.

Macrophages per well	: 2×10^6
Internalised mycobacteria/ CFU	: 96×10^3 (20) = 1.92×10^6 bacteria per 1 ml
MOI	: Macrophages ÷ CFU = 2 ÷ 1.92 = 1.04

2.5 Protein extraction

After 12 hours of infection, lysis buffer (20mM Tris-HCl, 137mM NaCl, 2mM EDTA, 0.1 % Triton X-100 and 10% glycerol), with 1× of protease inhibitor (Roche, Switzerland) was added to each well. Complete cell lysis was achieved by scraping the cells using a cell scraper and pipetting 5 – 10 times with a 1 ml pipette pit. Cells were left on ice for 10 minutes followed by centrifugation at 200×g for 10 minutes at 4°C, to achieve at least 80% cell lysis. The supernatant was collected and centrifuged at 8000×g for 30 minutes, the post-nuclear supernatant (PNS) was collected, filtered through a 0.2 µm acrodisc syringe filter (PVDF, SIGMA-ALDRICH, USA) into 1.5 ml eppendorf tubes and stored at -80˚C. Protein quantity and quality was assessed by using the Bradford assay and SDS-PAGE. Bradford assay: a standard curve was generated by using 10% Bovine serum albumin, Cat No. HD14-4 (BSA, QIAGEN, USA). A working stock of 1 mg/ml was made, BSA concentrations used to generate the standard curve were 2 µg, 4 µg, 8 µg, 12 µg, 16 µg and 20 µg, 900 µl of 1× Bradford dye reagent, Cat No. 500-0205 (Bio-Rad, USA) was added. Protein samples were kept on ice. 5 µl of the sample, 95 µl distilled water and 900 µl of 1× Bradford dye reagent were mixed to make 1 ml and absorbance taken at OD_{595} on a spectrophotometer (MRCLAB Spectro UV-16, Israel). A control sample contained water and Bradford reagent.

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SDS-PAGE; 10 µg of each protein sample was used. The sample was mixed with 1× XT sample buffer (Bio-Rad, USA) in an eppendorf, heated at 90˚C on a heating block for 2 minutes. The sample was loaded in SDS-PAGE gel, Criterion XT Bis-Tris Gels, (Bio-Rad, USA), in 1× running buffer XT MOPS (Bio-Rad, USA) run at 90 Volts for 30 minutes. The gel was stained with Aqua Stain, Cat. No. AS001000 (VACUTEC, SA) and viewed using the Bio-Rad gel-documentation system.

Sample transportation conditions:

Protein samples in 1.5 ml eppendorf were placed on ice in a polystyrene ice-box for transportation and sent to the Centre for Proteomic and Genomic Research (CPGR, Observatory, Cape Town, South Africa). Preparation for mass spectrometry was done by CPGR using trypsin to digest protein samples.

2.6 Mass spectrometry

2.6.1 Sample detergent removal and digestion

Detergents present in the sample extraction buffer were removed using detergent removal columns (Pierce 8777) according to the manufacturer's instructions. Samples were then dried by vacuum centrifugation and re-suspended in 50 µl of 50 mM triethylammonium bicarbonate (TEAB, Sigma T7408). The protein was then reduced by the addition of 0.1 volume of 100 mM tris (2-carboxyethyl) phosphine (TCEP, Sigma 646547) to each sample followed by incubation at 60°C for 1 hour. Alkylation was accomplished through addition of 0.1 volume of 100 mM methyl methanethiosulphonate (MMT, Sigma 208795) made up in isopropanol (Sigma 34965) to each sample and incubated at room temperature for 15 minutes. Proteins were digested by adding trypsin (Promega PRV5111) made up in 50 mM TEAB to a final protein: trypsin ratio of 20:1, and incubation was carried out overnight at 37°C. Samples were dried and resuspended in 0.1% trifluoroacetic acid (TFA, Sigma T6508) prior to clean-up by Zip-Tip (as per manufacturer's instructions, Sigma Z7200070). Samples were then resuspended in a final volume of 12 µl loading buffer (0.1% FA, 2% CAN in LC water).

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2.6.2 Liquid chromatography mass spectrometry

Liquid chromatography mass spectrometry (LC-MS) analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fischer Scientific, USA) coupled with a Dionex Ultimate 3000 nano-HPLC system. Peptides were dissolved in 0.1% Formic Acid (FA; Sigma 56302), 2% Acetonitrile (ANC; BJLC015CS, Burdick & Jackson, USA) and loaded on a PepAcclaim C18 trap column (300 µm × 5 mm × 5 µm). Chromatographic separation was performed with a PepAcclaim C18 trap column (75 µm × 25 mm × 1.7 µm). The solvent system employed was solvent A: LC water (Burdick & Jackson BJLC365); 0.1% FA and solvent B: ACN, 0.1% FA. The multi-step gradient for peptide separation was generated at 300 nL/min as follows: time change 5 min, gradient change: 2 – 5% Solvent B; time change 40 min, gradient change: 5 – 18% Solvent B; time change 10 min, gradient change: 18 – 30% Solvent B, time change 2 min, gradient change: 30 – 80% Solvent B. the gradient was then held at 80% solvent B for 10 minutes before returning to 2% solvent B for 15 min to condition the column. Data was acquired until the 65th minute. The mass spectrometer was operated in positive ion mode with a capillary temperature of 320˚C. The applied electrospray voltage was 1.95 kV. The mass spectrometer data were analysed, mass range of 350–2000 mass/charge (*m*/*z*), and the survey scans were acquired at a resolution of 70000, with a maximum of 2 missed cleavage, precursor tolerance set at 10.00 ppm. Spectral counts per protein were required to meet an increasing threshold until an empirical protein false discovery rate (FDR) of <1% was achieved. Proteins were considered only when they contained a minimum of two distinct peptides.

Table 2.1: Data acquisition for liquid chromatography mass spectrometry.

Full Scan	
Resolution	70,000 (@ m/z 200)
AGC target value	3e6
Scan range	350–2000
Maximum injection time (ms)	100
Data-dependant MS/MS	
Inclusion	Off
Resolution	17,500
AGC target value	1e5
Maximum injection time (ms)	50

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Loop count	15
Isolation window width (Da)	3
NCE (%)	27
Data-dependent settings	
Underfill ratio (%)	1
Charge exclusion	1, 7, 8, >8
Peptide match	Preferred
Exclusion isotopes	On
Dynamic exclusion	60

Table 2.2: Details of search parameters for protein identification.

Num.	Rule	Value
0	Protein database	E:/Databases/Mouse/Mus_musculus_10090_23112016_ref.fasta
1	Spectrum-level FDR	Auto cut
2	Cleavage residues	RK
3	Digest cutter	C-terminal cutter
4	Peptide termini	Fully specific
5	Maximum number of missed cleavages	2
6	Fragmentation type	CID low energy
7	Precursor tolerance	10.0 ppm
8	Fragment tolerance	20.0 ppm
9	Charges applied to charge-unassigned spectra:	1, 2, 3
10	Precursor mass max	10000
11	N-glycan search	None
12	O-glycan search	None
13	Skip bad spectrum	TRUE
14	Off by x isotopes	-2, -1, 0, +1, +2
15	Contaminants added	FALSE
16	Decoys added	TRUE
17	% Additional parameters:	
18	Disulfide Enable	FALSE

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19	Trisulfide Enable	FALSE
20	DSS Crosslink Enable	FALSE
21	Custom Crosslink Enable	FALSE
22	Wildcard Enable	FALSE
23	Combyne cut off score	Auto
24	Protein FDR cutoff	1%
25	Focused DB created	FALSE
26	Export mzldentML	TRUE
27	Score version	2
28	Precursor_assignment_flags	2
29	po_NumberMonosReturn	2
30	%Modification searches:	
31	Common_modification_max	1
32	Rare_modification_max	1
33	% Fixed and variable modifications:	
34	Oxidation / +15.994915 @ M |	Common2
35	Deamidated / +0.984016 @ N, Q |	Commom2
36	Methylthio / +45.987721 @ C |	Fixed
37	Product version	PMI-Byonic-Com:v2.6.46

2.6.3 Proteomic data analysis

Additional statistical analysis was performed. Raw data files were obtained from CPGR and processed using MSGF+ for peptide identification software (Kim & Pevzner, 2014) . The raw data was searched against a mouse database (Mus_musculus_10090) and *M. tuberculosis* H37Rv reference proteome UP000001584 (UniProt-proteome).

To determine which experiments had better quality, only well-attested proteins were retained (for example, requiring each protein to be supported by at least three spectra across the LC-MS/MS experiments), determined by enquiring whether the number of identified spectra were above 800 or not. If the file had spectra below 800 it was rejected. To eliminate proteins with sparse evidence, all the proteins that had fewer than five spectra were eliminated. For each protein group a contingency table was constructed containing the number of spectra matched to a protein group in experiment 1 and the number of spectra matched to this protein group in experiment 2. A Fisher Exact Test p-value for this contingency table was computed containing the p-value, proteins containing p-values <0.05 were considered as regulated proteins.

2.7 RNA extraction

Figure 2.3 describes the experiment design for the extraction of macrophage total RNA infected with mycobacteria for 12, 24 and 96 hours. The later time-points assess how the host response changes from 12 hours to 96 hours after infection with mycobacteria.

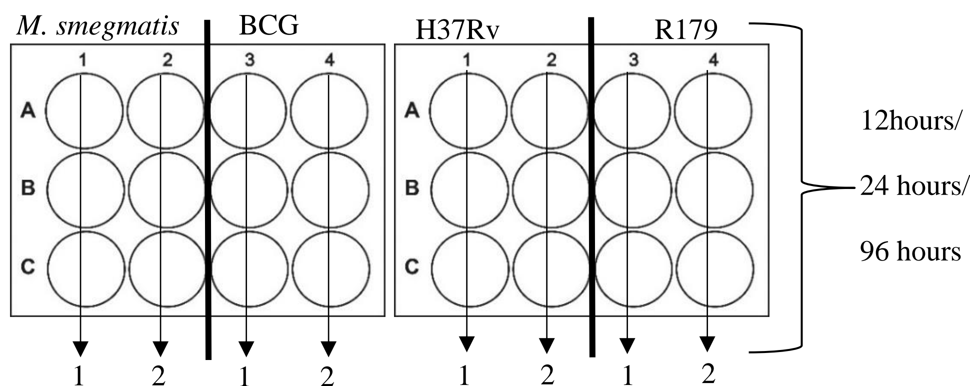

Figure 2.3: The 12, 24 and 96 hours infection experiments for RNA extraction. After 12, 24 and 96 hours of infection, the cell lysates were pooled together (plate 1:A1 + B1 + C1 = *M. smegmatis* sample 1; A2 + B2 + C2 = *M. smegmatis* sample 2; A3 + B3 + C3 = BCG sample 1; A4 + B4 + C4 = BCG sample 2) to make one sample. A total of 8 RNA samples were generated for every time point (12, 24 and 96 hours). The 12, 24 and 96 hours experiments were performed 3 times.

BMDM RNA was extracted using the RNeasy Plus Mini Kit, Cat. No. 74134 (Qiagen, Limburg, Netherlands) after the infection period, 12, 24 and 96 hours, (Figure 2.3)**.** Cells were lysed and harvested by adding RLT Plus and vortexed for 30 s, transferred the lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube. The gDNA Eliminator spin column was centrifuged for 30 s at ≥8000 x *g*, discard the column, and saved the flow-through. One volume of 70% ethanol to the flow-through, and mixed well by pipetting. 700 µl of the sample, to an RNeasy

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spin column placed in a 2 ml collection tube and centrifuge for 15 s at ≥8000 x g. Discard the flow-through. Added 700 µl Buffer RW1 to the RNeasy Mini spin column, centrifuged for 15 s at ≥8000 x *g* and discarded the flow-through. Added 500 µl Buffer RPE to the RNeasy spin column and centrifuged for 15 s at ≥8000 x *g*, discard the flow-through. Added 500 µl Buffer RPE to the RNeasy spin column and centrifuged for 2 min at ≥8000 x g. RNeasy spin column was placed in a new 1.5 ml collection tube (supplied). Added 30–50 µl RNase-free water directly to the spin column membrane, and centrifuge for 1 min at ≥8000 x *g* to elute the RNA. . RNA was frozen immediately at -80°C until reverse-transcription PCR and qPCR was performed. RNA quality and quantity was assessed using the Agilent 2100 Bioanalyser at the Central Analytical Facility (CAF), Stellenbosch University, Cape Town, South Africa. RNA samples with RNA integrity Number (RIN) above 9.0 were used for qPCR. Three biological replicates for qPCR were used and each biological replicate was run in duplicate.

2.7.1 cDNA synthesis and quantitative PCR

For cDNA synthesis, 0.5 µg RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit, Cat. No. 205311 (Qiagen, Limburg, Netherlands). Thawed gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water at room temperature (15–25°C). Prepared the genomic DNA elimination reaction on ice, incubated for 2 min at 42°C on a light-cycler machine then placed immediately on ice. Prepared the reverse-transcription master mix on ice (Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix), added template RNA. Incubated for 15 min at 42°C, the cDNA was incubated for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. The reverse-transcription reactions were kept on ice and proceeded directly with real-time PCR, or stored reverse-transcription reactions at –20°C for long-term storage. For qPCR, reverse-transcription reactions were diluted 5× with molecular water (20 µl of cDNA + 80 µl H_2O).

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The qPCR amplification was performed in 96-well plates and run on a LightCycler96 system (Roche, Germany). LightCycler1480 SYBR Green Master, Cat. No. 27122300 (Roche, Germany) was used with the following QuantiTect primer assays (Qiagen, Limburg, Netherlands) at a reaction volume of 20 µl: *Ahnak* Mm_Ahnak_1_SG QuantiTect Primer Assay, Cat No. QT01758078 (Qiagen), *Lgal3* Mm_Lgal3_1_SG QuantiTect Primer Assay, Cat No. QT00152558 (Qiagen), *Myh9* Mm_Myh9_1_SG QuantiTect Primer Assay, Cat No. QT000107408 (Qiagen, *Tln1* Mm_Tln1_1_SG QuantiTect Primer Assay, Cat No. QT00142107 (Qiagen). Reference genes used were *Ubiquitin C* (Mus musculus), Mm_Ubc_1_SG QuantiTect Primer Assay, Cat No. QT00245189 (Qiagen), *Beta-2 microglobulin* (Mus musculus), Mm_B2m_2_SG QuantiTect Primer Assay, Cat No. QT01149547 (Qiagen).

The amplification procedure entailed 45 cycles of 95°C for 10 s followed by 60°C for 10s and finally 72°C for 10s. Relative expression analysis was performed using the equation $N = N0 \times 2^{Cp}$ (LightCycler196 software, Roche), normalizing against the above mentioned reference genes. All samples were run in triplicate with a positive control and a non-reverse transcription control in accordance with the MIQE guidelines.

2.7.2 RNA Statistical analysis

Statistical significance was performed with GraphPad Prism v.5 software. ANOVA was used for comparisons involving 3 or more groups. All values expressed as means ± SEM with a $p < 0.05$ were considered as significant.

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Chapter 3: Results

3.1 Protein analysis

All protein samples were quantified using the Bradford assay (Table 3.1) and assessed protein quality using SDS-PAGE (Figure 3.2). Two lysis buffers were tested for efficiency in cells lysis for protein extraction (Figure 3.3) and used PCA plot to clearly distinguish biological variation introduced by the use of different buffers (Figure 3.4).

Table 3.1: Protein sample concentration obtained from a minimum of 4 infection experiments. The minimum protein mass required for analysis was 50 µg/ml.

Biological replicate 1	**Mass µg**	**Biological replicate 2**	**Mass µg**
M. bovis BCG1	51.8	*M. bovis* BCG1	24
M. bovis BCG2	54.6	*M. bovis* BCG2	33
M. smegmatis1	50.4	*M. smegmatis1*	13
*M. smegmatis*2	47.6	*M. smegmatis*2	22.5
H37Rv1	58.8	H37Rv1	33
H37Rv2	54.6	H37Rv2	33
R179	51.8	R179	37.5
R179	53.2	R179	20
Uninfected	49	Uninfected	42
Uninfected	44.8	Uninfected	24
Biological replicate 3	**Mass µg**	**Biological replicate 4**	**Mass µg**
M. bovis BCG1	18.08	*M. bovis* BCG1	31.5
M. bovis BCG2	33.28	*M. bovis* BCG2	33.3
M. smegmatis1	28.72	*M. smegmatis1*	60
*M. smegmatis*2	26.48	*M. smegmatis*2	55.2
H37Rv1	30.24	H37Rv1	46.6
H37Rv2	43.92	H37Rv2	38.3
R179	43.12	R179	35.1
R179	42.91	R179	60.6
Uninfected	40.16	Uninfected	59.5
Uninfected	52.32	Uninfected	61

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After 12 hours of infection and protein extraction, proteins were quantified using the Bradford assay as previously described in section 2.5. The recommended protein mass for analysis at CPGR was 50 µg/ml. Only 13 protein samples contained the required minimum protein mass. Replicate 1, 2 and 3 contained protein samples extracted using extraction buffer with glycerol. Replicate 4 samples were run 6 months after the first 3 replicates and was extracted using non-glycerol buffer.

3.1.1 SDS-PAGE

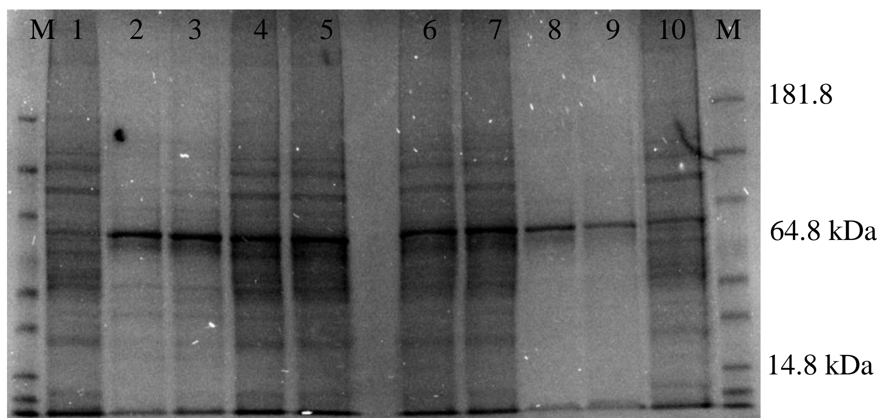

Figure 3.1: Protein samples extracted from *M. tb* infected mouse macrophages. Lanes: M – protein ladder, 1 control (uninfected), lane 2&3 *M. smegmatis*, lane 4&5 BCG; lane 6&7 H37Rv, lane 8&9 R179; lane 10 control.

Protein quality was assessed by SDS-PAGE (as described in 2.5) from macrophages infected with *M. smegmatis*, *M. bovis* BCG, *M. tuberculosis* H37Rv, *M. tuberculosis* R179 and uninfected macrophages. In Figure 3.1, protein samples represented where from replicate 1. We also used SDS-PAGE to see if there was a visible difference between samples extracted with glycerol buffer and those extracted with a non-glycerol buffer (Figure 3.2). The band intensity observed showed variation between samples, a greater band intensity was observed in samples from replicate 1 (glycerol) as compared to replicate 4 (Figure 3.2).

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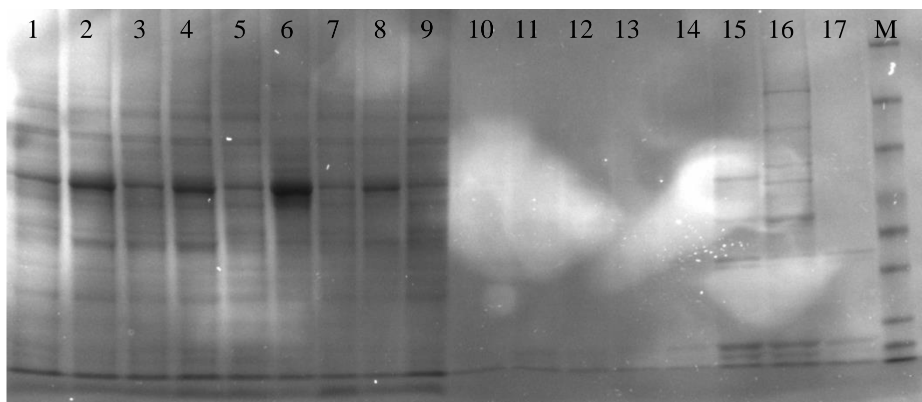

Figure 3.2: Macrophage protein samples extracted from a glycerol and non-glycerol extraction buffer. Lanes 1 – 9, protein samples extracted using a lysis buffer containing glycerol, lanes 10 – 17, protein samples extracted using a lysis buffer without glycerol. Lane 1&2 *M. smegmatis*, lane 3&4 BCG, lane 5&6 H37Rv, lane 7&8 R179, lane 9 control (uninfected), M – protein ladder. Lane 10&11 *M. smegmatis*, lane 12&13 BCG, lane 14&15 H37Rv, lane 16&17 R179, M – protein ladder.

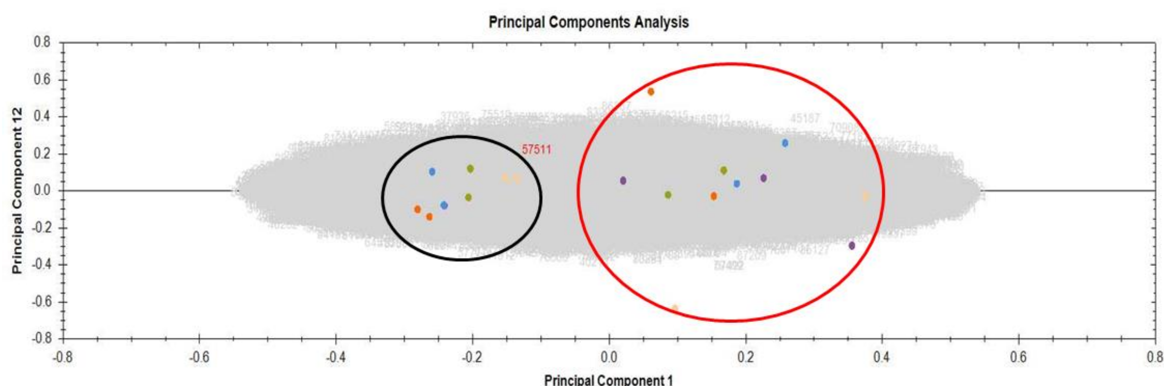

Figure 3.3: The PCA plot (for pilot test) shows variation between macrophage protein samples extracted using a glycerol buffer (highlighted in a black circle) and samples extracted using a non-glycerol buffer (highlighted in a red circle). These groups represent 2 biological replicates (glycerol versus non-glycerol) and 2 technical replicates for each condition, that is, 2 uninfected (control, yellow), 2 *M. smegmatis* (orange), 2 *M. bovis* (blue), 2 *M. mycobacterium* (H37Rv, green) and 2 *M. mycobacterium* R179 (purple). We observed that replicates had a high degree of biological variation between the 2 groups, where samples belonging to the same condition did not cluster in close proximity to each other.

Figure 3.3 shows biological variation observed in a subset of samples extracted with two different extraction buffers, glycerol and non-glycerol. To proceed with the study we used the original extraction buffer which contained glycerol based on the results and the budget for mass spectrometry analysis increased because the column had to be thrown away after running samples that contained glycerol (glycerol contamination). It showed that the non-glycerol extraction buffer produced samples of high variation, meaning samples were not associated with another even when they were extracted under the same conditions as with the non-glycerol buffer.

3.1.2 Liquid chromatography and mass spectrometry

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The following processing steps were used to analyse the mass spectrometry data; (1) which experimental files (mzML) have higher quality, (2) from the files with good quality, which proteins contain minimum spectra, (3) are these proteins expressed across all experiment, (4) proteins that are expressed across all files, are they up or down regulated? MS-GF+ (Kim & Pevzner, 2014) was used to accomplish peptide identification by scoring MS/MS spectra against peptides derived from a protein sequence database, UniProt sourced Mus musculus reference proteome(10090).

The differences in protein replicates were observed by analysing charge distribution across all batches (Figure 3.4).

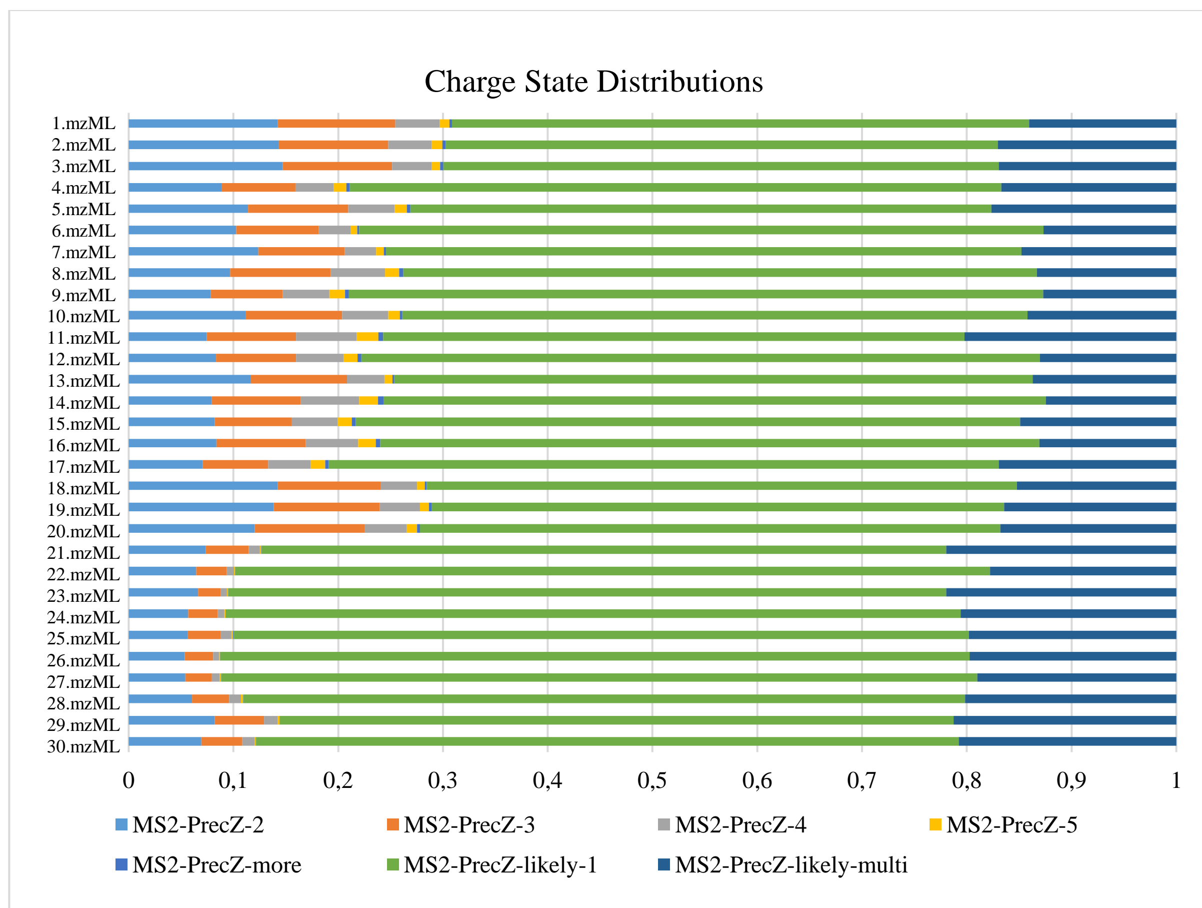

Figure 3.4: Z Distribution table showing precursor distribution in each mzML experiment file. The MS/MS fraction shown are, MS2-PrecZ-2 (fraction of MS/MS is doubly charged), MS2-PrecZ-3 (fraction of MS/MS is triply charged), MS2-PrecZ-4 (fraction of MS/MS is quadruply charged), MS2-PrecZ-5 (fraction of MS/MS is quintuply charged), MS2-PrecZ-more (fraction of MS/MS is higher than 5 charged), MS2-PrecZ-likely-1 (fraction of MS/MS lack known charge but look like + 1s), MS2-PrecZ- likely-multi (fraction of MS/MS lack known charge but look like > + 1s). Two separate batches are represented by the files, batch 1 (1.mzML – 20.mzML) and batch 2 (21.mzML – 30.mzML). Batch 1 had a large fraction of +2 and +3 precursors compared to batch 2.

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Protein samples were generated from 3 biological replicates of mouse macrophages infected with different mycobacterial species, 6 *M. smegmatis* (1, 2, 11, 12, 21 and 22), 6 *M. bovis* (3, 4, 13, 14, 23 and 24), 6 *Mtb* H37Rv (5, 6, 15, 16, 25 and 26), 6 *Mtb* R179 (7, 8, 17, 18, 29 and 30), 6 uninfected/ control (9, 10, 19, 20, 27 and 28). A total of 30 mzML files (quality metrics produced directly from the mass spectrometry data), LC-MS/MS experiments, were analysed. The peptide quality fractions, determined by charge state distribution, from 30 samples analysed in a mass spectrometry is represented as mzML files (Figure 3.4). The figure shows what fraction of the MS/MS scans were labelled with each charge state. Good quality fractions of the MS/MS scans are called +2 or +3 precursors, relating to doubly charged ions and triply charged ions respectively. Files labelled 1–20 (batch 1) had a larger fraction of +2 and +3 precursors compared to the files 21–30 (batch 2). Differences in charge distribution were observed among files from the different batches. The distribution of +2 and +3 charges in batch 1 (1.mzML – 20.mzML) was larger than that observed in batch 2 (21.mzML – 30.mzML), indicating variation in groups. Batch 1 and batch 2 were run on the mass spectrometry 6 months apart, which resulted in batch effect. Batch effect is the difference observed in samples as a result of time difference and sample handling.

Data processing was limited by retaining high-quality mzML files. The high quality files were determined by the number of peptide spectrum matches (PSMs) which is the total number of identified peptide spectra matched to each protein group. To eliminate files with sparse evidence, all files with PSMs below 800 were rejected. Only 11 mzML experimental files remained (Table S1) and 19 mzML files were rejected (Table S2). To further analyse well-attested proteins from the remaining experimental files, all the proteins that had fewer than five spectra were eliminated; 403 proteins retained. For each remaining experiment, a pairwise comparison was conducted. A contingency table was constructed containing the number of spectra matched to one protein group in experiment X, the number of spectra matched to this protein group in experiment Y, the number of spectra matched to something other than this protein in X, and the number of spectra matched to something other than this protein in Y. A Fisher Exact Test p-value for this contingency table was computed represented by the HYPGEOM.DIST function call.

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Protein abundance was determined by the total number of peptide spectrum matches (PSMs), high PSMs is directly proportional to high spectral counts as seen in Table 3.2. Data processing and statistical analyses revealed that only four macrophage proteins, Myosin 9 (MYH9), Talin-1 (TLN1), Galectin-3 (GAL-3), Neuroblast differentiation-associated protein (AHNAK), were regulated after 12 hours of infection (Table 3.2).

Table 3.2: Regulated proteins identified in bone marrow derived mouse macrophages after 12 hours of infection with mycobacteria (*M. bovis* BCG, *M. tuberculosis* H37Rv, *M. tuberculosis* R179 and control). Proteins expressed by infected macrophages were identified using the MS-GF+ method.

						BCG1	BCG2	BCG3	Control1	Control2	Control3	H37Rv1	H37Rv2	R179	*smegmatis*	*smegmatis*
Accession	**Protein name**	**Sequence Coverage**	**Distinct Peptides**	**Distinct Matches**	**Total PSMs**	**1237**	**1164**	**1146**	**908**	**1125**	**1120**	**1742**	**1587**	**1259**	**825**	**1170**
NP_071855.2	MYH9	29.08	48	60	181	18	7	7	5	24	3	36	39	21	17	4
NP_035732.2	TLN1	21.14	35	44	158	17	3	6	24	15	8	23	21	27	11	3
NP_001139425.1	GAL-3	40.53	12	24	74	9	5	3	0	7	0	16	14	10	8	2
NP_033773.1	AHNAK	25.42	34	39	59	6	0	0	7	0	0	17	18	9	2	0

Spectral counts

Four differentially expressed proteins were identified. Protein abundance of the identified proteins, MYH9, TLN1, GAL-3, and AHNAK, was higher in macrophages infected with pathogenic mycobacteria (H37Rv/R179) represented by the spectral counts (Table 3.3).

Table 3.3: Protein abundance and significance observed in BMDMs after infection with mycobacteria (*M. bovis* BCG, *M. tuberculosis* H37Rv, *M. tuberculosis* R179 and control).

a) Myosin 9 (Myh9)

Spectral counts		18	7	7
		BCG1	**BCG2**	**BCG3**
36	**H37Rv1**	0.1367	0.0006	0.0007
39	**H37Rv2**	0.0391	7.20E-05	8.8 E-05
21	**R 179**	0.3949	0.0106	0.0118

Spectral counts		5	24	3
		Control1	**Control2**	**Control3**
36	**H37Rv1**	0.0012	0.4742	9.60E-06
39	**H37Rv2**	0.0001	0.2254	6.90E-07
21	**R 179**	0.0128	0.7562	0.0003

b) Talin-1 (Tln1)

Spectral counts		17	3	6
		BCG1	**BCG2**	**BCG3**
23	**H37Rv1**	0.6161	0.0015	0.0247
21	**H37Rv2**	0.6129	0.0017	0.0264
10	**R 179**	0.0947	9.50E-06	0.0003

c) Galectin-3 (GAL-3)

Spectral counts		0	7	0
		Control1	**Control2**	**Control3**
16	**H37Rv1**	0.0011	0.1993	0.0003
14	**H37Rv2**	0.0017	0.2351	0.0005
10	**R 179**	0.0043	0.3345	0.0016

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d) Neuroblast differentiation-associated protein AHNAK (Ahnak)

Spectral counts		6	0	0
		BCG1	BCG2	BCG3
17	H37Rv1	0.0954	0.0001	0.0001
18	H37Rv2	0.046	4.80E-05	5.40E-05
9	R 179	0.3153	0.0027	0.0029

Spectral counts		7	0	0
		Control1	Control2	Control3
17	H37Rv1	0.3855	0.0001	9.60E-06
18	H37Rv2	0.256	3.20E-05	6.40E-05
9	R 179	0.6607	0.0021	0.0032

The pathogenic mycobacteria only consisted of 2 H37Rv group and 1 R179 group, these 2 groups were designated the pathogenic group for the purpose of analysis. From a total of 403 detected proteins (Table S1), which contained a minimum of 5 spectra, a threshold p-value (< 0.05), was used to identify significant proteins, highlighted in grey are p-values less than 0.05. This meant only 5% of the detected proteins were expected to have a p-value below 0.05 by random chance. Proteins that contained at least 66% (6/9) significant p-values within the group comparison, i.e. BCG versus H37Rv and control versus H37Rv (Table 3.3), were considered as regulated proteins/ proteins induced by mycobacterial infection. After using the p-values as a measure of significance, we observed that these proteins were only regulated in macrophages infected with BCG, H37Rv, and R179 (Table 3.3). A fairly low protein expression was observed in the uninfected and BCG infected macrophages. Therefore MYH9, TLN1, GAL-3, and AHNAK were upregulated by the pathogenic mycobacterial species.

3.2 Quantitative PCR

The proteins identified from the mass spectrometry data, code for *Ahnak, lgal3, Myh9 and Tln1*.The 4 protein coding genes of the detected proteins were quantified by quantitative real-time PCR (qPCR) to examine their transcriptional change in infected macrophages relative to the uninfected macrophages. Macrophage gene expression was evaluated after 12, 24 and 96 hours of infection with mycobacteria (Figure 3.5 – 3.8). Only two RNA extraction time points were used for macrophages infected *M. smegmatis*, 12 hours and 24 hours post-infection, because *M. smegmatis* does not survive in macrophages beyond 24 hours and has a high rate of extracellular growth (Anes et al., 2006). Statistical significance was performed with GraphPad Prism software version 5. ONE WAY ANOVA for group comparison. All values expressed as means ± SEM with a $p < 0.05$ considered as significant.

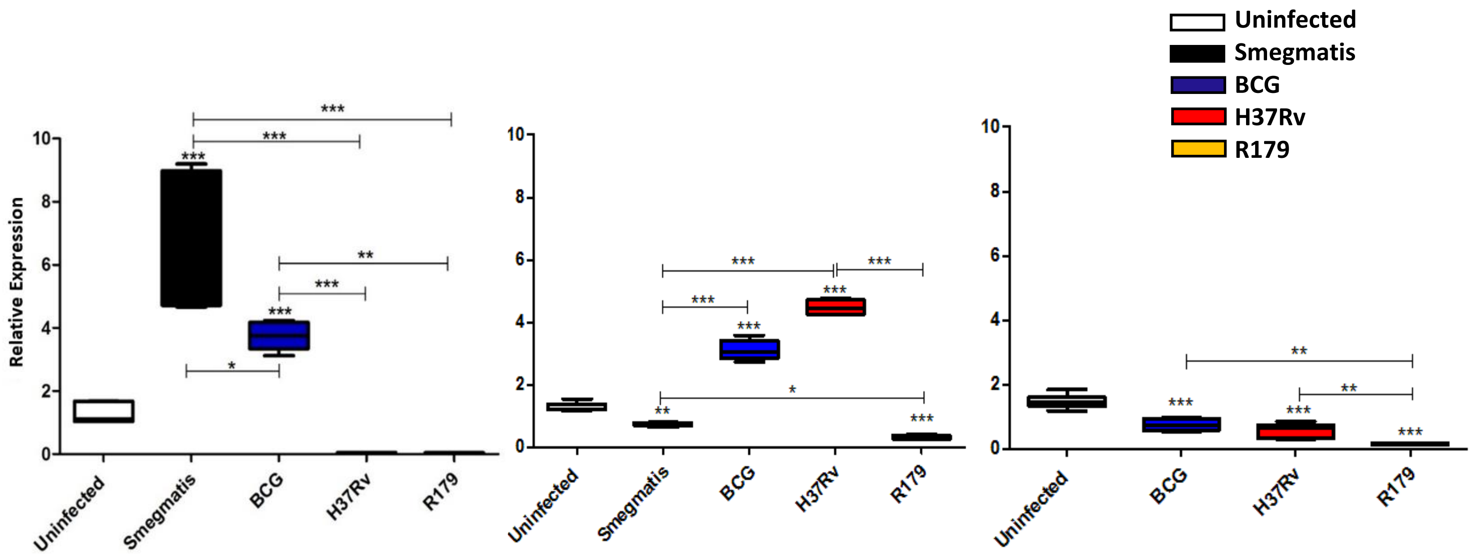

Figure 3.5: Neuroblast differentiation-associated protein (*Ahnak*) host gene expression of infected BMDMs. The changes in gene expression of mycobacteria infected BMDMs was assessed at 12, 24 and 96 hours post-infection. Host gene response was assessed from 5 different groups of BMDMs: uninfected macrophages (white) used as a control, *M. smegmatis* (black), *M. bovis* BCG (blue), *M. tuberculosis* H37Rv (red), *M. tuberculosis* R179 (yellow). *Ahnak* was analysed for expression after 12 (a), 24 (b) and 96 (c) hours of infection using qPCR and GrapPad Prism. The stars represent the level of significance, p-value: < 0.05 = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****

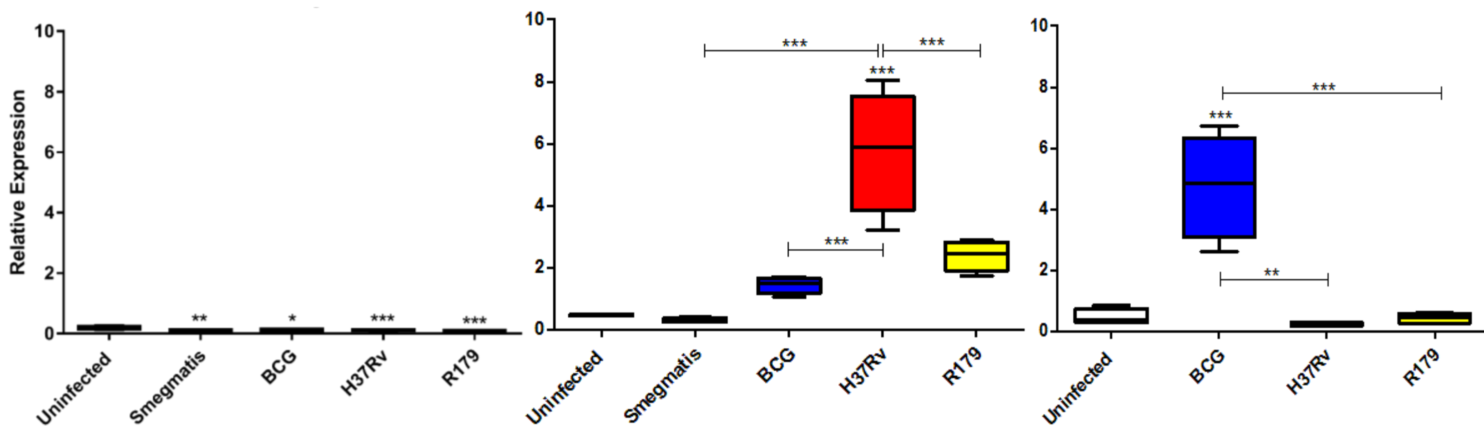

Figure 3.6: Myosin-9 *(Myh9)* host gene expression of infected BMDMs. The changes in gene expression of mycobacteria infected BMDMs was assessed at 12, 24 and 96 hours post-infection. Host gene response was assessed from 5 different groups of BMDMs: uninfected macrophages (white) used as a control, *M. smegmatis* (black), *M. bovis* BCG (blue), *M. tuberculosis* H37Rv (red), *M. tuberculosis* R179 (yellow). *Myh9* was analysed for expression after 12 (a), 24 (b) and 96 (c) hours of infection using qPCR and GrapPad Prism. The stars represent the level of significance, p-value: < 0.05 = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****

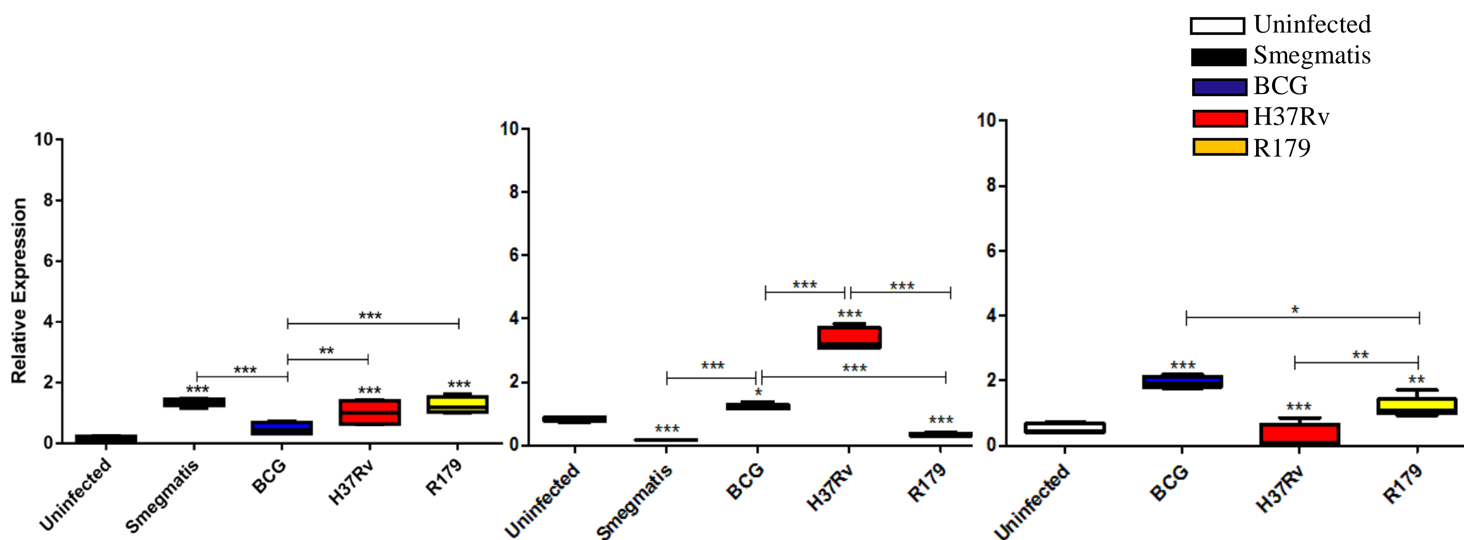

Figure 3.7: Talin1 (*Tln1*) host gene expression of infected BMDMs. The changes in gene expression of mycobacteria infected BMDMs was assessed at 12, 24 and 96 hours post-infection. Host gene response was assessed from 5 different groups of BMDMs: uninfected macrophages (white) used as a control, *M. smegmatis* (black), *M. bovis* BCG (blue), *M. tuberculosis* H37Rv (red), *M. tuberculosis* R179 (yellow). *Tln1* was analysed for expression after 12 (a), 24 (b) and 96 (c) hours of infection using qPCR and GrapPad Prism. The stars represent the level of significance, p-value: < 0.05 = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****

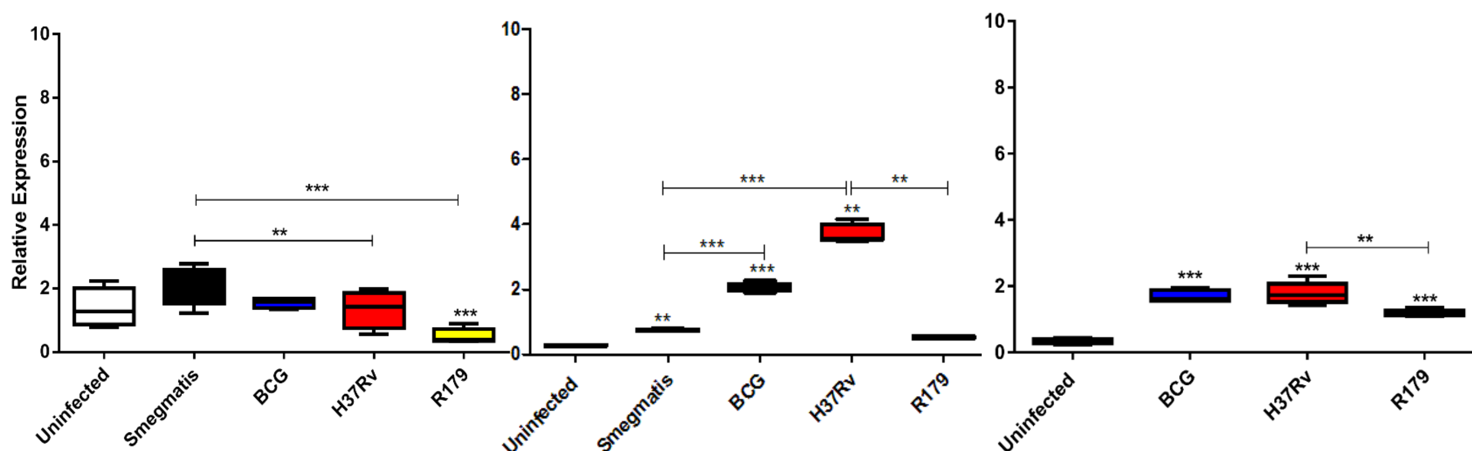

Figure 3.8: Galectin3 *(Lgal3)* host gene expression of infected BMDMs. The changes in gene expression of mycobacteria infected BMDMs was assessed at 12, 24 and 96 hours post-infection. Host gene response was assessed from 5 different groups of BMDMs: uninfected macrophages (white) used as a control, *M. smegmatis* (black), *M. bovis* BCG (blue), *M. tuberculosis* H37Rv (red), *M. tuberculosis* R179 (yellow). Macrophage gene, galectin-3 (*Lgal3*), was analysed for expression after 12 (a), 24 (b) and 96 (c) hours of infection. The stars represent the level of significance, p-value: < 0.05 = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****

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Ahnak gene expression, 12 hours post infection, was upregulated by the non-pathogenic *M. smegmatis* and *M. bovis* BCG. There was a significant difference between the 2 species, fast-grower *M. smegmatis* and the slow-grower *M. bovis* BCG, the expression of *Ahnak* was higher in *M. smegmatis* infected macrophages. After 24 hours of infection, BCG and H37Rv significantly induces *Ahnak* expression in macrophages and is downregulated by all species after 96 hours of infection. There was no significant increase in *Lgal3* gene expression but was significantly downregulated by R179, 12 hours post-infection. After 24 hours of infection Lgal3 was upregulated by BCG and H37Rv. The expression of *Lgal3* was significantly upregulated 96 hours post-infection by all species, BCG and H37Rv and R179. *Myh9* is downregulated in all mycobacteria infected macrophages at 12 hours post-infection. After 24 hours of infection it is only upregulated by the pathogenic *Mtb* H37Rv, and after 96 hours of infection *Myh9* is only upregulated in *M. bovis* BCG infected macrophages. The expression of *Tln1* is significantly upregulated by all species at 12, 24 and 96 hours post infection.

Overall we observed that the expression of these genes is only upregulated by the non-pathogenic mycobacteria at 12 hours post infection and downregulated in macrophages infected with pathogenic strains. Gene expression increased after 24 and 96 hour post infection in pathogenic strains.

Chapter 4: Discussion and Conclusion

4.1 Discussion

Recently intracellular transcriptome of *Mtb* has been reported in BMDMs (Leisching et al., 2016), however, gene expression analysis may not accurately determine the protein expression and turnover in the cells. Mass spectrometry has been widely used for the identification of macrophage proteins that have not been predicted by genome analysis. This approach was used in the present study for the comparison of intracellular *Mtb*-infected macrophage proteins. More efficient intervention measures are urgently needed for better TB control, particularly with respect to the increasing prevalence of drug-resistant strains. However, drug development is hindered by the incomplete understanding of the molecular mechanisms underlying infection, survival, and persistence of *Mtb* in the human host. Using mass spectrometry, four macrophage proteins were detected to be expressed after 12 hours of infection with *Mtb*, but RNA expression was only induced by *M. smegmatis*. It has been well documented that although mRNA transcripts get translated to form their respective protein products, the gene-protein expression correlation

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is always not absolute particularly due to various post-transcriptional and post-translational factors (Maier, Güell and Serrano 2009; Liu, Beyer and Aebersold 2016). The use of mass spectrometry to identify proteins will enable us to extend our understanding of macrophage-*Mtb* to fight against TB infection. A typical LC-MS experiment consists of four phases: (i) sample preparation, including proteolytic digestion of the proteins; (ii) peptide separation through liquid chromatography; (iii) mass spectrometry analysis; and (iv) computational data interpretation. All these steps can introduce significant variability that needs to be controlled in order to obtain reproducible results. More work needs to be done on sample preparation for this study.

The expression of *AHNAK* is associated with macrophage proliferation and calcium signalling (Trimble & Grinstein, 2007). It has been discovered that mice with the *Ahnak* gene deletion develop severe lesions (Matza et al., 2008). We observed that the expression of *Ahnak* was induced by *M. smegmatis*, BCG and H37Rv, confirming its association with mycobacterial infection. *Mtb* blocks calcium regulation which plays a significant role in the production of pro-inflammatory responses and regulate mycobacterial survival in macrophages. Calcium dependent phagosome maturation is involved in the inhibition of mycobacteria sphingosine kinases, which enables *Mtb* survival within macrophages (Trimble & Grinstein, 2007). Sphingosine kinase-1 is known to mediate *M. smegmatis* induced inflammatory responses in macrophages.

Macrophages are composed of intracellular and extracellular proteins. Macrophage membrane receptors, integrin proteins, binds to actin intracellular proteins facilitated by Talin-1 (Manso, 2017). Integrin proteins facilitate cell adhesion and activate signal transduction. Actin proteins are important for muscle contraction and cell movement (Dominguez & Holmes, 2011). Muscle contraction and movement in macrophage infection is related to phagocytosis. Mycobacteria induce the expression of *Tln1* in macrophages and the expression of this gene could be associated with pathogen recognition.

Myosins are actin-based motors constituting a large super-family of more than 15 members. Myosins contain ATPase motor domain which binds actin and drives movement (Berg et al., 2001). The association of *Myh* and mycobacterial infection has not been studied. Different classes of the myosin family are involved in the uptake of different pathogens. The uptake of *Listeria monocytogenes* requires myosin VIIA (Sousa et al., 2004), while *Shigella flexneri* dependent upon myosin II (Rathman et al., 2000). Our study shows that mycobacterium infection is associated with *Myh9*.

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Galectins-3 (GAL-3), carbohydrate-binding proteins, bind *Mtb* glycan on the cell surface. Galectins are involved in a variety of biological processes, including tumour cell adhesion and progression, inflammation and wound healing (Kavanaugh et al., 2013, Loimaranta et al., 2018) and play a role during innate immune response (Vasta, 2009). *Lgal3* expression was significantly induced in *Mtb* infected macrophages. GAL-3 protein might have a role in innate immunity as it has been shown that activation of IFN-α2b led to an increase in expression and release of GAL-3 into cell culture medium of breast cancer cell line and serum of breast cancer patients (Iacobelli et al., 1988). During macrophage inflammatory response GAL-3 expression is induced by the activation of IFN-α, IFN-ɤ, IFN-β and TNF (Loimaranta et al., 2018). Galectins are β-galactoside-binding animal lectins that have been reported to participate in various physiological and pathological processes, including immunity, inflammation and cancer progression (Loimaranta et al., 2018). *Lgal3* is also a key regulator to Wnt/ β-catenin signalling pathway (Shimura et al., 2005). Wnt/ β-catenin signalling modulates cell proliferation, mobility and survival (Clevers & Nusse, 2012; Kohn & Moon, 2005; Korkmaz et al., 2016). The Wnt is also involved in the regulation of the inflammatory response in murine macrophages and is significantly reduced in lungs of *Mtb* infected mice (Schaale et al., 2011). We observed that expression of *Lgal3* is induced by *M. smegmatis* 12 hours post infection, while *M. bovis* and *Mtb* only induced the expression of this gene 24 and 96 hours post infection. Similarly it has been shown that *Lgal3* expression is up-regulated in response to a mycobacterial infection (Loimaranta et al., 2018). One study suggests that the regulation of Gal-3 is controlled by mycobacterial tyrosine kinase because *ptkA* deficient mycobacteria did not survive in macrophages and there were high levels of Gal-3 (Jaiswal, & Srivastava, 2018).

The intracellular survival of slow growing mycobacteria BCG, H37Rv and R179 and the killing of fast growing mycobacteria *M. smegmatis* within macrophages led us to examine the protein and gene expression profiles of macrophages infected with actively replicating mycobacteria. Our finding shows that mycobacterium species differentially regulate gene expression in murine macrophages. A study by Pheiffer et al. 2005, compared 3 *Mtb* isolates, Beijing, F23 and F11 and found that there was variation in antigen composition among the 3 strains. These observations are similar to the findings observed in our study after infection of macrophages with mycobacteria. BCG, an attenuated strain of *M. bovis*, is similar to *Mtb* (H37Rv and R179) in how they influence macrophage host response. Both BCG and H37Rv are slow growing mycobacteria, they also inhibit the fusion between lysosomes and phagosomes thus inhibit the maturation of the phagosomes (Rao et al., 2009). It is well known that different mycobacterial

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species have a variation in their structural composition and protein expression. But it is still not clear how the infected host responds to different mycobacteria.

4.2 Conclusion

Elucidation of how macrophages respond to different mycobacterial species is fundamental to our understanding of mycobacterial pathogenicity and TB disease. Proteomics experiments generate large amounts of data that need to be processed, first to identify proteins with confidence, then to quantify them within a reasonable error rate. In experiments that compare multiple conditions, one often needs to know whether the observed change in expression of a particular protein is statistically significant. The proper statistical treatment of these tasks is critical in reaching the correct biological insights. The expression profiles observed in mouse macrophages do not necessarily reflect macrophage response in infected humans. We doubt that the same results would be obtained in humans *in vivo*. The only methods that have been used to study human infections *in vivo* are mouse models and guinea pigs. The other method that is more related to human cell lines is the use of THP-1 as an *in vitro* model of infection. For future studies, we would like to use mass spectrometry to identify proteins expressed in human derived blood macrophages when infected with mycobacteria. This will be achieved by isolating PBMCs from human blood, to isolate macrophages and infect them with mycobacteria. The macrophage protein will be extracted and analysed using mass spectrometry. Knock-down/ knock-up experiments can be used to analyse protein expression changes in the host and analyse bacterial survival during infection.

4.3 Limitations

During the study, we encountered a few obstacles. The protein extraction protocol we used was adopted and optimised from Shinji et al., 1994. The glycerol in the buffer was feared to cause column contamination during mass spectrometry analysis according to the CPGR. We had to remove glycerol in the buffer and optimized protein extraction using a non-glycerol extraction buffer. The protocol did not work, CPGR then recommended we used phosphate buffer with 0.2% SDS instead of Triton because they have also encountered a problem from samples containing Triton X-100. The SDS-phosphate buffer did not work at all, we tried to optimize different SDS concentrations (0.1, 0.15, 0.2, 0.25, 0.3 and 0.35). CPGR was informed about the difficulties in using phosphate buffer. Protein samples extracted using two different extraction

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buffers was sent to CPGR for a preliminary analysis to see if the presence or absence of glycerol made any difference.

Another limitation was the low sample protein concentrations obtained during infection experiments. This was due to the low percentage of macrophages lysed for protein extraction. We did eleven macrophage infection experiments which amounted to 110 protein samples. Thirty protein samples were initially sent to CPGR. Majority of the samples had protein mass below the recommended 50 µg/ml. After a thorough analysis by CPGR, it was picked up that one of the biological replicates had a very high degree of variation and could not be used for statistical analysis and therefore an additional batch was required. This was after 6 months of the initial run of batches sent for analysis, hence the quality of the study was affected by the batch effect.

CPGR also carried out statistical analysis of the identified proteins. We asked CPGR to identify proteins that were upregulated and downregulated in macrophages. Results received concerning the analysis was not satisfactory because it was not clear how the results were obtained and no one from CPGR was willing to explain. Professor David Tabb assisted with analysis by using MS RAW file data from CPGR to reanalyse data. When the statistical analysis was done the Benjamini-Hochberg FDR to estimate q-values for each protein group was not computed. However, we are aware that the p-values obtained from these experiments were not an indication of protein significance as most of these proteins would mostly fall-off if the false discovery rate was applied.

Another limitation which may have led to poor data quality is that the signal to noise ratio was not processed, although we believe filtering signal from noise would not have made a difference because of poor data quality. The discovery phase involves testablishment of experimental protocols that result in the identification of sample proteins from plasma, cytosol etc. When the proteins of interest are likely to be present in low abundance, the probability of their detection may be increased by carrying out fractionation steps that either enrich for the protein class of interest (e.g., DNA affinity chromatography to enrich for transcription factors) or that deplete highly abundant proteins (e.g. immuno-affinity chromatography). Protein fractionation such as centrifugation can also be used, although it requires a very high number of cells (macrophage concentration).

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Supplementary data

Table S1: The table below shows 11 experiments that were accepted for analyses, with the PSM (explained in the results section) above the minimum threshold of 800 PSMs. Experiments that passed the standard criterion for regulated proteins. Proteins with the minimum spectra of 5 (highlighted above the red line), were accepted and further analysed for significance determined by a p-value < 0.05.

				Total-PSMs	**1237**	**1164**	**1146**	**908**	**1225**	**1120**	**1742**	**1587**	**1259**	**825**	**1170**	
				Status	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	
Accession	**Coverage**	**Distinct Peptides**	**Distinct Matches**	**Filtered Spectra**	**BCG1**	**BCG2**	**BCG3**	**Cntrl1**	**Cntrl2**	**Cntrl3**	**H37Rv1**	**H37Rv2**	**R179**	**Smeg1**	**Smeg2**	**Total spectra**
NP_031419.1	69.3	29	63	614	52	41	33	19	32	30	46	43	19	25	41	381
NP_001234913.1	56.1	43	71	584	36	22	22	35	39	22	37	29	27	24	24	317
NP_033738.1	42.4	24	58	482	42	29	22	9	26	24	47	32	16	23	32	302
NP_112442.2	54.6	40	78	509	17	31	32	26	23	30	34	24	20	9	35	281
NP_034357.2	28.4	53	63	356	20	22	23	16	23	16	35	25	20	13	27	240
NP_033803.1	77.4	32	52	447	15	25	22	15	20	22	22	20	22	15	33	231
NP_033529.3	49.0	39	54	307	23	9	14	9	25	15	30	21	28	24	15	213
NP_031978.2	54.7	32	40	376	17	17	21	24	21	24	24	17	10	14	18	207
NP_001020559.1	67.7	30	49	302	19	20	26	16	12	4	26	27	18	15	20	203
NP_035164.1	49.7	22	35	346	18	20	20	13	12	19	19	23	11	10	22	187
NP_071855.2	29.1	48	60	206	18	7	7	5	24	3	36	39	21	17	4	181
NP_034963.2	64.3	43	62	272	20	14	16	12	24	15	26	13	15	7	16	178
XP_006526669.1	43.2	15	30	282	26	14	15	6	17	14	25	17	9	12	14	169
NP_035785.1	49.8	19	34	274	24	4	6	15	22	4	20	31	19	21	1	167
NP_034860.2	56.4	24	35	310	15	13	19	9	14	11	19	18	16	14	18	166
NP_035732.2	21.1	35	44	214	17	3	6	24	15	8	23	21	27	11	3	158
NP_032933.1	54.9	19	33	300	17	14	11	16	14	18	14	16	15	10	12	157
NP_001156906.1	51.5	35	47	279	11	18	11	19	16	22	14	13	8	7	16	155
NP_001170778.1	57.8	24	38	238	17	12	11	6	11	14	24	18	10	9	14	146

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NP_032328.2	37.2	30	39	186	9	15	15	13	7	13	19	15	11	9	11	137
NP_033414.1	47.5	26	42	219	16	11	8	12	13	10	19	15	11	9	11	135
NP_035229.2	52.7	27	36	204	9	13	17	7	12	2	22	18	16	8	8	132
NP_067248.1	56.7	15	25	209	12	11	11	6	20	12	15	15	12	11	7	132
NP_001240812.1	48.8	25	32	194	9	12	14	7	10	3	21	17	14	7	9	123
NP_035202.1	57.9	9	14	226	11	10	14	13	9	13	13	12	9	10	7	121
NP_001035999.1	44.1	16	25	192	19	9	6	9	11	10	14	14	8	7	12	119
NP_666228.1	48.8	17	26	189	15	3	4	13	13	3	15	24	9	15	0	114
NP_033476.1	40.0	13	23	190	17	3	4	12	13	3	13	21	13	13	0	112
NP_598917.1	34.4	25	34	177	12	10	8	11	11	12	10	12	7	8	10	111
NP_001129557.1	32.9	21	29	150	14	6	13	5	8	9	13	17	9	8	9	111
NP_031933.1	31.2	22	31	174	9	8	6	6	9	11	16	14	13	9	7	108
XP_006498053.1	38.4	22	29	160	9	12	8	6	13	10	13	7	10	6	12	106
NP_032854.2	53.5	17	23	150	8	7	14	4	9	8	10	12	7	10	15	104
NP_036151.1	47.1	22	27	206	6	9	8	12	8	10	13	10	10	6	11	103
NP_001287996.1	55.1	17	29	159	8	7	5	6	14	14	16	12	9	6	6	103
NP_035162.1	45.8	23	28	179	5	12	10	9	7	14	10	4	7	4	18	100
NP_082347.1	54.2	18	24	213	12	9	9	6	10	14	7	9	5	5	14	100
NP_033441.2	42.5	16	21	171	11	8	8	6	13	5	15	9	10	5	10	100
NP_032138.3	43.8	17	23	140	8	9	9	3	9	10	12	12	10	8	6	96
NP_031611.1	43.1	16	20	150	9	11	13	5	9	5	11	9	6	4	13	95
NP_068695.1	33.5	24	28	145	8	9	8	6	12	10	8	12	5	6	8	92
NP_001276460.1	51.6	21	28	120	8	8	7	3	9	8	13	12	9	5	7	89
NP_848713.1	58.8	15	22	158	10	6	5	9	10	7	10	10	10	5	6	88
NP_032521.1	43.0	14	28	131	6	7	3	5	6	16	15	10	7	5	8	88
NP_780706.1	30.9	9	21	135	10	12	4	2	9	4	12	13	7	6	9	88
NP_034894.2	25.3	19	26	113	8	5	10	2	11	9	13	12	6	6	3	85
NP_001103681.1	33.0	17	26	165	7	6	8	6	10	6	8	8	8	6	11	84
XP_006540317.1	31.9	23	26	129	7	7	7	6	11	8	8	11	4	5	7	81
NP_001254624.2	28.9	13	16	147	9	6	5	3	8	15	8	10	7	5	5	81
NP_034610.1	27.6	19	23	111	2	9	13	7	5	9	8	7	6	2	10	78
NP_001071173.1	20.1	12	16	111	5	7	3	5	10	8	10	9	9	4	8	78

NP_001300863.1	44.7	9	19	124	6	10	8	6	9	5	8	7	5	5	9	78
NP_031713.1	48.8	11	20	122	8	4	6	5	9	2	16	9	8	6	4	77
NP_035761.1	33.3	23	29	101	6	3	2	8	12	4	9	14	7	8	2	75
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NP_001074743.1	35.4	15	19	101	10	10	7	1	4	4	8	9	4	5	9	71
NP_057930.2	15.0	20	24	95	5	9	7	1	5	4	10	8	6	4	10	69
NP_032181.2	30.5	11	19	125	7	4	3	6	8	3	11	10	8	4	5	69
NP_031617.1	56.3	21	25	129	6	6	3	8	7	5	8	9	5	2	7	66
NP_067448.1	46.8	15	17	105	7	9	5	2	4	7	7	7	3	5	10	66
NP_079923.3	45.4	11	16	110	8	3	3	4	6	4	8	6	9	9	5	65
NP_033536.2	22.0	14	16	104	5	7	7	5	4	8	6	7	6	3	6	64
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NP_038660.1	50.2	11	17	111	7	3	7	8	6	2	8	8	6	5	4	64
NP_659172.1	72.8	11	16	116	3	3	4	5	11	4	9	11	5	5	4	64
NP_080471.2	19.6	9	11	108	4	8	5	4	6	5	8	4	5	5	8	62
NP_033773.1	25.4	34	39	75	6	0	0	7	0	0	17	18	9	2	0	59
NP_038498.2	40.2	17	20	108	7	3	7	1	6	5	8	4	6	5	6	58
NP_001098086.1	21.4	14	16	99	4	8	7	4	4	8	5	4	6	3	5	58
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NP_035564.1	52.6	9	12	122	6	5	6	6	6	7	5	5	4	1	7	58
NP_080749.2	29.3	11	15	97	9	2	1	5	5	2	7	14	5	7	0	57
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NP_034113.1	37.3	12	19	72	5	5	5	0	5	7	3	9	6	0	4	49
NP_001276655.1	41.6	11	20	77	5	5	5	4	4	3	6	9	5	1	2	49

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NP_031479.1	57.1	12	15	78	6	6	3	1	3	1	7	6	6	5	4	48
NP_035658.1	31.5	12	17	80	3	7	2	0	3	7	8	4	4	3	6	47
NP_067253.1	86.4	10	14	80	6	4	4	4	10	0	7	4	2	1	5	47
NP_035010.3	26.6	18	18	71	2	4	3	2	5	9	6	4	5	0	6	46
NP_034454.2	25.7	9	10	75	6	1	4	2	5	4	6	5	5	5	3	46
NP_001304146.1	25.4	5	9	67	5	2	2	3	5	2	8	6	7	4	1	45
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NP_659207.1	29.3	9	15	63	4	9	5	0	5	5	6	4	1	2	3	44
NP_001304314.1	42.4	8	9	66	4	2	4	5	7	3	5	4	3	4	3	44
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NP_001033088.1	46.3	7	13	65	1	9	3	1	2	7	3	4	3	2	8	43
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NP_001028465.1	25.8	10	12	62	2	5	2	2	5	2	5	4	5	2	6	40
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NP_062798.1	13.5	2	5	69	5	2	4	4	2	8	3	3	2	3	2	38
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NP_034552.1	21.3	11	13	59	1	4	4	1	3	3	3	5	3	3	3	33
NP_849242.1	18.5	10	11	45	1	3	1	3	4	6	4	6	2	2	1	33
NP_001268950.1	18.6	6	11	65	7	0	0	10	1	1	5	4	2	3	0	33
NP_001259026.1	26.8	5	13	63	3	3	2	5	0	3	3	1	3	5	5	33

NP_001240734.1	41.6	10	13	52	2	4	5	2	2	4	5	1	3	0	4	32
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NP_254279.1	35.3	8	10	50	5	2	4	1	2	3	3	4	2	4	2	32
NP_001303604.1	14.5	5	8	51	3	3	2	1	3	4	3	4	3	2	4	32
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NP_722490.1	9.3	4	8	37	5	1	2	2	3	2	4	4	3	1	1	28
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NP_035193.1	31.5	4	6	44	3	3	1	2	2	3	3	2	4	1	3	27
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NP_079672.1	38.1	7	7	36	7	2	1	2	0	0	6	4	0	2	2	26
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NP_031819.1	81.6	8	9	35	1	5	1	1	2	3	2	2	0	2	6	25
NP_034712.2	8.6	7	7	36	3	3	2	1	3	1	4	3	2	2	1	25
NP_080405.1	25.6	7	8	46	2	1	1	2	5	2	6	2	2	0	2	25
NP_062615.1	20.8	4	5	52	2	0	1	3	4	5	3	2	1	3	1	25
NP_075891.1	39.5	5	6	31	2	2	1	1	5	1	2	3	4	1	2	24
NP_001139429.1	8.4	5	6	49	2	2	3	3	1	3	2	1	3	2	2	24
NP_034818.1	21.2	4	5	29	2	2	2	3	3	1	5	3	2	0	1	24

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NP_659095.1	16.4	4	6	34	2	4	4	1	3	1	2	0	3	2	2	24
NP_033991.1	30.4	6	6	34	3	2	2	1	0	1	5	1	5	0	3	23
NP_700472.1	11.1	4	4	26	3	1	2	0	3	3	2	3	3	2	1	23
NP_001272360.1	32.5	8	9	36	2	3	1	2	2	0	5	3	2	2	0	22
NP_061346.2	41.7	7	7	42	0	3	3	0	2	2	4	2	3	0	3	22
NP_033562.3	30.6	7	10	27	0	5	2	2	0	5	2	0	2	0	4	22
NP_001140179.1	17.1	7	8	24	1	1	1	0	3	2	5	4	3	2	0	22
NP_001272418.1	21.3	7	7	22	2	1	2	1	3	3	4	3	2	1	0	22
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NP_001153096.1	12.9	7	8	25	3	3	6	1	1	1	1	1	1	0	2	20
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NP_080270.2	42.9	9	11	41	1	1	1	3	1	1	4	5	2	0	0	19
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NP_080828.1	19.6	4	4	28	0	4	3	0	0	3	3	0	2	0	1	16
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NP_035007.1	5.0	2	2	27	2	2	1	1	1	2	1	1	1	2	2	16
NP_001074896.1	2.0	6	6	26	1	2	1	0	1	2	2	2	1	1	2	15
NP_001136422.1	10.4	6	6	16	2	2	2	0	0	2	2	2	0	0	3	15
NP_033966.1	14.7	5	5	20	1	2	2	1	0	2	1	3	1	0	2	15
NP_001165571.1	13.3	5	6	16	2	3	1	0	1	0	3	2	2	1	0	15
NP_082045.1	8.1	4	4	18	1	0	1	0	3	1	2	2	3	0	2	15
NP_071306.2	27.5	4	4	18	1	1	1	2	1	0	5	2	1	0	1	15
NP_031534.2	7.9	4	5	21	3	0	0	0	1	1	3	4	1	2	0	15
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NP_032733.1	7.5	3	3	25	1	1	2	1	0	1	1	2	2	2	2	15
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NP_080218.1	9.3	3	3	24	0	0	3	1	1	1	3	1	2	2	1	15
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NP_001073853.1	21.6	3	5	19	1	1	1	1	2	1	2	5	0	0	1	15
NP_031447.1	7.2	2	3	17	3	1	1	0	1	0	3	2	2	1	1	15
YP_009359666.1	2.2	2	3	18	1	2	1	1	0	2	2	2	1	1	2	15
NP_032124.1	33.9	5	6	21	0	3	1	0	2	0	3	2	1	0	2	14
NP_075631.2	13.7	4	4	24	1	4	2	0	1	3	0	0	0	0	3	14
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NP_742146.1	7.4	2	3	23	1	0	1	4	2	1	1	2	1	1	0	14
NP_061223.2	28.0	7	9	18	1	2	1	1	1	3	2	1	0	0	1	13
NP_058017.1	13.3	6	7	19	2	0	2	2	1	0	0	2	2	2	0	13
NP_035868.1	23.2	6	8	24	1	3	2	2	0	3	0	0	0	0	2	13
NP_780469.1	2.8	5	5	14	0	2	1	0	0	0	4	3	1	2	0	13
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NP_598911.1	19.6	4	5	19	2	0	1	0	0	2	0	1	3	2	2	13
NP_542126.1	32.3	4	8	16	1	1	1	1	0	1	3	1	2	0	2	13
NP_035305.1	6.1	4	4	15	1	0	0	0	5	0	2	4	1	0	0	13

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NP_666231.1	7.9	4	4	16	0	2	1	0	0	2	3	3	0	0	2	13
NP_001191908.1	5.9	3	3	22	0	2	2	1	0	2	1	1	2	0	2	13
NP_032846.2	7.7	3	4	24	0	1	2	0	3	1	1	1	3	0	1	13
NP_032329.1	35.3	3	4	27	1	2	2	0	1	3	0	0	1	0	3	13
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NP_033138.1	31.3	3	5	18	3	1	0	1	0	0	3	2	1	1	0	12
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NP_077777.1	7.6	2	3	25	1	0	0	2	2	1	1	2	1	2	0	12
NP_001278037.1	28.0	2	2	14	2	0	0	2	1	1	2	2	1	1	0	12
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NP_001300902.1	12.1	3	3	14	1	0	0	1	1	2	2	1	1	1	1	11
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NP_033118.1	36.3	3	5	12	5	0	0	0	0	0	0	2	4	0	0	11
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NP_733467.1	6.3	3	3	13	0	1	3	0	1	2	0	1	1	1	1	11
NP_444338.2	11.2	2	3	22	0	1	1	1	1	3	1	1	1	0	1	11
NP_598487.1	14.3	5	5	16	1	0	0	2	0	0	2	3	1	1	0	10

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NP_034403.1	13.4	5	6	12	0	2	3	0	1	0	2	0	1	0	1	10
NP_036160.1	14.9	4	4	11	0	2	1	0	0	1	1	2	1	0	2	10
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NP_035430.1	18.6	2	3	12	2	0	0	0	0	0	2	2	1	2	0	9

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NP_001289006.1	21.3	2	2	9	2	1	1	0	1	0	2	1	0	0	1	9
NP_705727.1	4.4	2	2	12	0	0	1	1	1	0	2	1	2	1	0	9
NP_034253.3	2.5	2	3	11	2	0	0	1	0	0	1	1	3	1	0	9
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NP_945175.1	17.9	2	2	9	2	1	0	0	2	0	2	1	0	0	1	9
NP_035831.2	28.5	8	11	22	0	1	1	2	0	0	0	1	2	0	1	8
NP_075622.1	35.2	5	5	9	1	0	0	0	0	0	1	3	1	2	0	8
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NP_032543.2	7.7	4	4	8	1	1	2	0	0	0	1	1	0	0	1	7
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NP_997406.1	23.1	3	3	7	1	1	0	0	0	0	2	2	0	0	1	7
NP_766523.2	8.1	2	2	8	0	0	1	0	1	1	1	1	0	2	0	7
NP_056550.1	3.5	2	3	23	0	0	0	2	1	0	1	0	2	1	0	7
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NP_032387.1	5.9	2	2	7	3	1	1	0	0	0	1	0	0	0	1	7
NP_031536.2	11.9	2	2	8	0	0	1	0	1	1	1	2	1	0	0	7
NP_001297611.1	3.2	2	2	7	1	0	0	1	0	0	2	2	1	0	0	7
NP_032695.3	7.4	2	2	8	0	2	3	0	0	0	1	0	0	0	1	7
NP_077135.1	11.9	2	2	7	2	0	0	0	1	0	2	2	0	0	0	7
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NP_038615.2	6.7	2	2	7	0	0	1	0	2	0	2	1	0	0	0	6
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NP_035470.3	7.7	2	2	10	2	0	0	1	0	0	1	1	0	0	0	5
NP_084514.2	0.7	2	2	5	1	0	0	0	0	0	1	2	1	0	0	5
NP_067485.1	7.5	2	3	7	1	0	0	0	1	0	0	2	1	0	0	5
NP_113553.1	4.2	2	2	5	0	0	0	1	0	0	1	1	1	1	0	5
NP_038497.2	5.6	2	2	6	0	1	0	0	0	0	1	1	1	0	1	5

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NP_032997.3	9.5	2	3	5	0	1	1	0	0	1	0	0	2	0	0	5
NP_032186.2	15.4	2	2	5	0	2	2	0	0	0	0	0	1	0	0	5
NP_001103709.1	12.7	2	2	5	0	0	2	0	1	1	0	0	0	0	1	5
NP_035426.1	7.2	2	2	7	1	0	0	0	0	0	0	3	1	0	0	5
NP_001275507.1	13.3	2	3	7	1	1	0	1	0	0	1	0	0	0	1	5
MINIMUM OF FIVE SPECTRA																
NP_570959.1	18.7	5	5	7	0	1	0	0	1	0	0	0	2	0	0	4
NP_035443.1	25.8	4	4	8	0	0	1	0	1	0	2	0	0	0	0	4
NP_033139.2	24.4	4	4	8	0	1	0	0	0	1	1	0	0	0	1	4
NP_057886.1	22.3	4	5	5	0	2	0	0	0	0	2	0	0	0	0	4
NP_058676.1	14.2	3	3	4	1	0	0	0	0	0	1	1	1	0	0	4
NP_001240680.1	10.4	3	3	6	0	0	1	0	0	0	1	1	0	1	0	4
NP_001297469.1	2.9	3	3	4	0	0	1	0	0	2	0	0	0	0	1	4
NP_001029037.1	40.1	3	3	10	1	0	1	1	0	0	1	0	0	0	0	4
NP_079824.2	14.4	3	3	5	1	0	1	0	0	0	0	0	0	1	1	4
NP_033971.1	11.7	3	3	5	0	1	1	0	0	0	0	0	0	0	2	4
NP_001153711.1	9.0	3	3	7	0	1	2	0	0	0	0	0	0	0	1	4
NP_084011.1	3.1	3	3	5	0	1	0	0	0	1	1	1	0	0	0	4
NP_032474.2	4.9	3	4	4	0	0	0	1	0	0	0	1	2	0	0	4
NP_001157308.1	5.6	3	3	4	0	1	0	0	0	0	1	2	0	0	0	4
NP_598599.2	6.5	3	3	4	0	0	0	0	0	0	0	4	0	0	0	4
NP_001070733.1	10.8	3	3	4	0	1	0	1	1	1	0	0	0	0	0	4
NP_001001932.1	3.3	3	3	4	0	0	0	1	0	1	1	0	1	0	0	4
NP_038714.2	9.7	3	3	4	1	0	0	0	0	0	0	3	0	0	0	4
NP_079561.1	15.1	3	4	8	0	0	1	0	1	1	0	0	0	1	0	4
NP_001153189.1	16.8	3	3	7	2	0	0	1	0	0	0	0	1	0	0	4
NP_001157287.1	5.5	3	3	6	0	2	1	0	0	0	0	0	0	0	1	4
NP_001295379.1	7.5	3	3	8	0	1	0	0	1	1	0	0	0	0	1	4
NP_061352.2	7.3	2	2	7	0	0	1	0	0	2	1	0	0	0	0	4
NP_001152823.1	15.4	2	2	7	0	1	1	0	0	1	0	0	0	0	1	4
NP_775539.1	4.4	2	2	9	1	1	0	1	0	0	0	0	0	0	1	4

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NP_033937.2	4.0	2	2	5	0	1	1	0	0	1	0	0	0	0	1	4
NP_035960.2	3.4	2	2	6	0	0	0	1	0	0	0	0	2	1	0	4
NP_033967.1	6.1	2	2	4	1	1	0	0	0	0	0	2	0	0	0	4
NP_001159899.1	8.2	2	3	7	1	0	0	0	1	0	0	1	1	0	0	4
NP_620087.3	18.4	2	3	5	0	0	0	0	2	0	0	0	2	0	0	4
NP_032973.1	8.0	2	2	4	0	0	0	0	1	0	1	1	1	0	0	4
NP_032155.1	11.3	2	2	4	1	0	0	0	0	0	1	2	0	0	0	4
NP_075860.1	20.6	2	2	5	0	0	1	0	0	0	1	0	2	0	0	4
NP_473398.1	7.8	2	2	4	0	0	1	0	1	1	0	0	1	0	0	4
NP_057932.2	8.4	2	2	4	0	0	0	0	1	0	2	0	0	0	1	4
NP_001192259.1	2.4	2	2	4	0	0	2	0	0	1	0	0	0	0	1	4
NP_080239.1	12.7	2	2	5	0	0	1	0	0	0	2	1	0	0	0	4
NP_035488.1	11.3	2	2	5	1	1	0	0	0	0	1	1	0	0	0	4
NP_570934.1	8.0	2	2	5	2	0	1	0	0	0	0	1	0	0	0	4
NP_001158149.1	3.9	2	2	4	0	1	0	0	0	1	0	0	1	0	1	4
NP_997044.2	17.9	2	2	6	0	1	0	0	0	0	1	1	0	0	1	4
XXX_YP_177962 .1		3	3	3	0	1	0	1	0	0	0	0	0	0	1	3
NP_001077424.1	16.9	3	3	4	0	1	0	0	0	0	1	1	0	0	0	3
NP_766635.1	11.9	3	3	4	0	1	1	0	0	0	1	0	0	0	0	3
NP_081245.1	3.1	3	3	3	1	0	0	0	0	1	0	1	0	0	0	3
NP_067499.2	14.3	3	3	3	0	0	0	0	0	0	1	2	0	0	0	3
NP_058655.3	11.7	3	3	3	1	0	0	0	0	0	0	1	1	0	0	3
NP_032968.2	3.6	3	3	4	0	1	0	0	0	0	0	0	0	0	2	3
NP_075898.1	13.4	2	2	3	0	0	1	0	1	1	0	0	0	0	0	3
NP_001017959.1	4.3	2	2	12	0	1	1	0	0	0	0	0	0	0	1	3
NP_001034481.1	47.7	2	3	5	0	0	0	0	1	0	0	0	0	1	1	3
NP_036096.1	7.3	2	2	6	0	0	1	0	0	0	0	0	0	0	2	3
NP_058086.2	5.1	2	2	6	0	0	0	0	1	0	0	0	2	0	0	3
NP_938045.2	3.3	2	2	3	0	0	0	0	0	0	1	2	0	0	0	3
NP_038574.3,	15.1	2	2	3	0	0	0	0	0	0	1	2	0	0	0	3
NP_080112.1	7.3	2	2	3	0	0	0	1	0	0	1	1	0	0	0	3

NP_033121.2	10.8	2	2	3	1	0	0	0	0	0	0	1	1	0	0	3
NP_001186194.1	3.3	2	2	3	0	0	0	0	0	1	1	1	0	0	0	3
NP_077753.1	7.4	2	2	3	1	0	0	0	0	0	2	0	0	0	0	3
NP_035318.1	5.5	2	2	3	1	0	0	0	0	0	1	1	0	0	0	3
NP_666338.2	4.4	2	2	3	0	0	0	0	0	0	1	0	0	0	2	3
NP_001139374.1	7.0	2	2	3	1	0	0	0	0	0	0	0	1	1	0	3
NP_001104301.1	4.9	2	2	3	1	0	0	0	0	0	1	0	0	1	0	3
NP_035945.1	3.6	2	2	3	1	0	0	1	0	0	0	0	1	0	0	3
NP_034390.1	4.2	2	2	4	0	2	0	0	0	0	0	0	0	0	1	3
NP_033908.2	3.2	4	4	5	0	0	1	0	0	0	0	0	0	0	1	2
NP_031570.2	31.3	3	3	3	0	0	0	1	0	0	1	0	0	0	0	2
NP_001300865.1	13.4	3	3	3	0	0	0	0	0	1	0	0	1	0	0	2
NP_077754.3	7.7	3	3	4	0	0	1	0	0	1	0	0	0	0	0	2
NP_033842.3	6.9	3	3	7	0	1	0	0	1	0	0	0	0	0	0	2
NP_038668.2	18.3	3	3	9	0	0	1	0	1	0	0	0	0	0	0	2
NP_598912.4	7.4	3	3	3	1	0	0	0	0	0	1	0	0	0	0	2
NP_065625.2	25.8	2	2	2	0	0	0	0	0	0	1	1	0	0	0	2
NP_035863.1	8.1	2	2	2	0	0	0	0	0	0	1	1	0	0	0	2
NP_080170.1	5.7	2	2	2	0	0	0	0	0	0	1	1	0	0	0	2
NP_058572.2	14.1	2	2	2	0	0	0	0	0	0	1	0	1	0	0	2
NP_031646.2	7.9	2	2	3	0	0	0	0	0	0	1	0	0	0	1	2
NP_001242984.1	9.9	2	2	4	0	0	0	0	0	1	0	0	0	1	0	2
NP_031650.3	13.2	2	2	6	0	0	0	1	0	0	0	1	0	0	0	2
NP_033822.2	4.2	2	2	5	0	1	0	0	0	0	0	0	0	0	1	2
NP_001020609.1	4.3	2	2	2	0	0	0	0	1	0	1	0	0	0	0	2
NP_058066.2	13.5	2	2	3	0	0	0	0	1	1	0	0	0	0	0	2
NP_001303251.1	7.1	2	2	2	0	0	0	0	1	0	1	0	0	0	0	2
NP_032240.3	5.5	2	2	2	0	0	0	0	0	0	1	0	0	0	1	2
NP_034507.2	7.0	2	2	3	0	1	0	0	0	0	0	1	0	0	0	2
NP_032040.1	4.8	2	2	8	0	0	0	0	0	2	0	0	0	0	0	2
NP_033173.2	3.5	2	2	2	0	0	0	0	0	0	0	1	1	0	0	2
NP_001292748.1	9.0	2	2	2	0	0	0	1	0	0	0	1	0	0	0	2

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NP_001272966.1	7.1	2	2	2	0	0	0	0	0	1	0	1	0	0	0	2
NP_613069.3	3.3	2	2	2	0	0	0	0	0	1	0	1	0	0	0	2
NP_001103662.1	2.4	2	2	2	0	0	0	0	0	0	0	0	2	0	0	2
NP_001278126.1	22.4	2	2	2	0	0	0	0	0	0	1	0	1	0	0	2
NP_077228.1	16.8	2	2	2	0	0	0	0	0	0	1	0	1	0	0	2
NP_919444.2	0.8	2	2	2	0	0	0	0	0	0	1	0	1	0	0	2
NP_080097.1	10.1	2	2	3	0	0	1	0	0	1	0	0	0	0	0	2
NP_001161980.1	6.1	2	2	2	0	0	1	0	0	0	0	1	0	0	0	2
NP_001278115.1	3.4	2	2	2	0	0	0	0	0	1	1	0	0	0	0	2
NP_001033789.1	6.9	2	2	2	2	0	0	0	0	0	0	0	0	0	0	2
NP_033298.1	7.9	2	2	4	0	1	0	0	0	1	0	0	0	0	0	2
NP_031664.3	6.3	2	2	2	0	1	0	0	0	0	0	1	0	0	0	2
NP_001278158.1	2.1	2	2	2	0	0	0	1	0	0	0	0	0	0	0	1
NP_031412.2	2.9	2	2	6	0	1	0	0	0	0	0	0	0	0	0	1
NP_032433.2	2.5	2	2	3	0	1	0	0	0	0	0	0	0	0	0	1
NP_038653.1	5.4	2	2	3	0	0	0	0	1	0	0	0	0	0	0	1
NP_001073598.1	8.6	2	2	3	0	0	0	0	0	1	0	0	0	0	0	1
XXX_NP_001156 610.1		2	2	2	0	0	0	0	0	1	0	0	0	0	0	1
NP_001153222.1	9.2	2	2	4	0	0	0	1	0	0	0	0	0	0	0	1
NP_034455.1	8.4	2	2	3	0	0	0	0	0	0	0	0	0	0	1	1
NP_032430.2	4.8	2	2	7	0	0	0	1	0	0	0	0	0	0	0	1
NP_001033320.1	42.7	2	3	6	0	0	0	0	1	0	0	0	0	0	0	1
NP_035800.2	17.5	2	2	7	0	0	0	0	0	0	1	0	0	0	0	1
NP_001077407.1	13.6	2	2	2	0	0	0	0	0	0	0	0	1	0	0	1
NP_058587.1	4.2	2	2	2	0	0	0	0	0	1	0	0	0	0	0	1
NP_659074.2	2.2	2	2	2	0	0	0	0	0	1	0	0	0	0	0	1
NP_001095874.1	9.5	2	2	4	0	0	0	0	0	0	0	0	1	0	0	1
NP_034369.1	11.5	2	2	2	0	1	0	0	0	0	0	0	0	0	0	1
NP_058054.2	18.0	5	7	7	0	0	0	0	0	0	0	0	0	0	0	0
NP_001182350.1	51.5	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0
NP_001157134.1	8.7	3	3	4	0	0	0	0	0	0	0	0	0	0	0	0

NP_034607.3	10.3	3	3	3	0	0	0	0	0	0	0	0	0	0	0	0
NP_001171080.1	14.6	2	2	3	0	0	0	0	0	0	0	0	0	0	0	0
NP_077198.1	11.2	2	2	4	0	0	0	0	0	0	0	0	0	0	0	0
NP_001076429.1	4.0	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0

Table S2: The table below shows 19 experiments that were rejected from analyses, the PSM (explained in the results section) was lower than the minimum threshold of 800 PSMs.

Accession	277	352	455	534	326	546	704	598	550	658	499	244	28	398	352	75	485	113	660
	Reject	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**	**Reject**
	BCG4	**BCG5**	**BCG6**	**Control4**	**Control5**	**Control6**	**H37Rv3**	**H37Rv4**	**H37Rv5**	**H37Rv6**	**R179-2**	**R179-3**	**R179-4**	**R179-5**	**R179-6**	**Smeg3**	**Smeg4**	**Smeg5**	**Smeg6**
NP_031419.1	3	10	11	11	8	23	14	15	21	24	8	13	0	15	13	4	12	2	26
NP_001234913.1	21	7	15	15	16	18	27	22	11	12	21	9	2	9	15	3	22	8	14
NP_033738.1	2	4	9	8	8	22	12	16	16	18	5	15	0	9	8	4	7	1	16
NP_112442.2	12	7	10	9	7	18	20	14	18	27	16	3	3	12	12	0	17	6	17
NP_034357.2	5	0	8	7	6	6	9	6	10	11	10	4	0	1	3	1	8	0	21
NP_033803.1	12	6	12	12	6	17	17	16	20	20	12	7	1	15	9	1	9	4	20
NP_033529.3	0	3	5	14	3	8	6	7	8	8	6	3	0	3	5	1	6	0	8
NP_031978.2	8	8	11	14	12	13	9	13	13	14	7	1	1	10	5	2	12	4	12
NP_001020559.1	0	5	4	1	0	2	5	5	13	15	4	5	0	10	13	0	2	0	15
NP_035164.1	9	4	14	8	9	14	9	14	11	8	6	6	0	6	4	3	15	2	17
NP_071855.2	0	5	0	4	0	3	4	0	2	1	0	4	0	0	0	0	0	0	2
NP_034963.2	3	1	2	8	2	6	11	6	9	7	9	4	0	6	4	1	5	0	10
XP_006526669.1	2	2	6	5	4	18	6	8	11	13	3	7	0	5	4	2	5	1	11
NP_035785.1	0	11	9	12	6	3	20	9	0	0	13	8	0	0	0	6	10	0	0
NP_034860.2	4	6	7	8	6	7	11	9	14	14	8	8	1	9	8	3	9	2	10
NP_035732.2	1	1	5	1	2	3	13	13	0	0	8	2	0	0	0	1	4	0	2
NP_032933.1	8	3	14	9	7	7	8	12	10	9	10	5	3	5	5	2	12	6	8
NP_001156906.1	3	7	7	3	6	10	10	6	9	13	8	1	1	15	7	1	5	4	8
NP_001170778.1	2	1	5	10	2	8	6	5	11	8	2	3	0	8	8	2	2	0	9
NP_032328.2	0	2	3	4	0	6	4	5	3	4	3	1	0	2	2	0	3	0	7

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NP_033414.1	4	2	4	9	4	4	7	8	5	4	8	3	2	1	3	2	6	2	6
NP_035229.2	1	5	2	3	3	0	11	7	4	7	5	6	1	1	3	2	3	0	8
NP_067248.1	3	2	6	9	4	7	4	8	5	5	2	0	0	5	3	1	4	1	8
NP_001240812.1	1	5	2	2	3	1	11	7	4	6	5	6	1	1	3	2	3	0	8
NP_035202.1	5	4	8	7	6	10	8	4	10	8	4	3	1	5	4	1	5	2	10
NP_001035999.1	4	4	6	6	1	3	5	5	5	4	4	5	0	2	4	2	8	0	5
NP_666228.1	0	8	7	9	3	3	16	5	0	0	8	3	0	0	0	4	9	0	0
NP_033476.1	0	8	7	9	5	3	14	7	0	0	8	3	0	0	0	5	9	0	0
NP_598917.1	4	1	2	7	3	6	11	6	3	5	4	1	0	2	1	0	3	2	5
NP_001129557.1	1	0	0	4	2	1	5	5	1	6	4	0	0	1	1	0	3	0	5
NP_031933.1	4	1	6	4	4	5	9	7	3	3	5	4	0	1	0	1	3	1	5
XP_006498053.1	2	3	4	5	3	6	3	4	4	6	3	1	0	2	1	0	3	0	4
NP_032854.2	1	0	2	2	1	2	4	3	6	7	0	2	0	2	4	2	1	1	6
NP_036151.1	9	4	7	4	7	5	5	8	8	6	6	3	2	6	1	5	8	5	4
NP_001287996.1	0	2	2	7	2	3	5	3	4	7	2	3	0	4	5	2	2	0	3
NP_035162.1	3	2	2	3	4	8	6	1	10	10	6	1	0	7	5	0	1	0	10
NP_082347.1	8	3	6	5	8	8	7	8	9	6	6	6	1	5	6	0	8	5	8
NP_033441.2	3	2	3	4	3	6	4	6	5	8	5	2	1	4	3	1	4	1	6
NP_032138.3	0	1	2	6	1	4	5	1	2	3	1	2	0	4	5	2	0	0	5
NP_031611.1	0	2	3	5	3	1	5	4	5	7	2	2	0	1	4	0	4	0	7
NP_068695.1	4	1	1	4	3	4	5	4	4	3	4	1	0	2	0	0	3	2	8
NP_001276460.1	0	0	0	2	0	2	3	3	3	4	0	0	0	3	4	0	2	0	5
NP_848713.1	4	2	8	2	5	3	6	6	7	5	4	2	1	1	2	0	6	1	5
NP_032521.1	2	0	3	0	2	6	4	4	6	2	2	1	0	4	0	1	4	0	2
NP_780706.1	1	2	1	3	1	7	3	2	3	6	2	1	0	4	1	1	2	1	6
NP_034894.2	0	2	0	4	1	8	2	0	3	2	2	2	0	0	0	1	0	0	1
NP_001103681.1	7	2	5	4	5	4	5	6	5	7	3	0	0	7	5	0	7	2	7
XP_006540317.1	4	1	1	4	3	2	4	4	4	3	3	1	0	2	0	0	3	2	7
NP_001254624.2	2	2	2	6	4	11	4	4	8	5	2	1	1	3	3	1	3	0	4
NP_034610.1	0	2	0	3	0	5	3	2	4	3	0	0	0	1	1	0	2	0	7
NP_001071173.1	1	0	0	3	1	2	2	0	3	4	2	1	0	2	6	1	1	0	4
NP_001300863.1	1	3	0	1	2	1	4	4	4	9	4	1	0	2	2	0	2	0	6

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NP_031713.1	2	2	5	1	1	3	4	5	0	4	2	3	0	2	2	0	5	1	3
NP_035761.1	0	4	3	2	0	1	1	2	2	3	0	0	0	4	1	1	1	0	1
NP_598557.3	0	0	2	4	1	2	3	1	6	4	2	0	0	2	3	0	1	0	2
NP_001240667.1	2	0	0	0	2	4	1	2	5	4	4	1	0	4	6	0	1	1	8
NP_001139425.1	0	1	0	2	0	0	0	0	0	0	0	3	0	0	0	1	0	0	0
NP_001258693.1	2	0	0	0	2	5	1	2	6	3	4	1	0	3	5	0	1	1	6
NP_001003908.1	0	0	0	2	0	4	0	0	0	1	0	1	0	0	1	0	0	0	4
NP_001074743.1	0	0	0	3	0	1	1	0	4	7	0	1	0	4	4	0	1	0	4
NP_057930.2	1	3	1	2	1	1	4	1	1	3	3	2	0	0	0	0	1	0	2
NP_032181.2	1	0	3	4	3	2	10	8	2	2	6	3	0	1	3	2	4	1	1
NP_031617.1	6	5	5	5	3	3	3	5	4	3	2	1	0	4	1	1	5	2	5
NP_067448.1	1	0	1	2	0	3	3	2	4	4	4	0	0	5	5	1	1	0	3
NP_079923.3	3	0	3	3	0	4	3	1	4	5	2	0	0	5	4	0	2	0	6
NP_033536.2	1	1	1	3	1	2	3	2	5	6	2	1	0	4	3	0	1	0	4
NP_032088.1	0	0	0	2	0	1	3	0	0	1	0	1	0	1	2	0	0	0	2
NP_038660.1	3	1	4	7	2	0	6	6	0	2	8	1	0	1	2	0	2	2	0
NP_659172.1	3	2	4	5	3	2	7	7	2	1	6	1	0	2	1	0	3	3	0
NP_080471.2	3	0	3	1	1	1	6	4	2	5	6	3	0	2	0	0	5	0	4
NP_033773.1	0	0	3	1	0	0	7	2	0	0	0	1	0	0	0	0	2	0	0
NP_038498.2	3	2	2	4	1	4	4	2	2	4	3	1	0	4	3	2	3	1	5
NP_001098086.1	1	1	1	3	1	3	4	2	4	5	3	1	0	4	3	0	1	0	4
NP_001288230.1	4	4	6	5	4	2	6	6	2	1	3	2	0	1	1	0	5	2	1
NP_035564.1	4	3	3	3	1	5	3	5	4	6	2	0	1	6	5	0	3	5	5
NP_080749.2	0	6	4	5	1	2	10	1	0	0	4	2	0	0	0	2	3	0	0
NP_035783.1	2	7	2	6	2	3	3	3	0	0	1	3	1	0	0	1	3	1	1
NP_075907.2	2	1	3	3	2	0	0	3	4	4	5	2	0	4	2	1	4	0	3
NP_033473.1	0	6	0	6	1	1	3	1	0	0	1	3	0	0	0	0	3	0	1
NP_033784.2	3	3	2	2	8	1	2	2	4	3	1	1	1	2	4	0	1	1	4
NP_031824.1	0	0	0	3	0	4	0	0	4	5	1	0	0	4	1	0	0	0	4
NP_034113.1	1	4	0	2	0	2	1	0	2	5	0	0	0	3	3	0	0	0	0
NP_001276655.1	0	2	3	2	1	0	7	4	0	0	4	1	0	0	1	0	3	0	0
NP_031479.1	0	1	2	2	0	1	3	4	3	2	3	2	0	0	1	0	4	0	2

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NP_035658.1	0	1	0	5	0	3	3	1	4	3	2	2	0	3	1	0	1	1	3
NP_067253.1	1	2	1	2	5	1	0	2	0	2	4	2	0	5	0	0	3	2	1
NP_035010.3	0	6	0	3	0	3	4	1	0	1	5	0	0	0	0	0	0	0	2
NP_034454.2	0	0	2	1	2	1	3	4	0	2	3	1	0	1	2	0	3	0	4
NP_001304146.1	1	4	2	1	1	2	3	2	2	0	1	0	0	1	0	0	1	0	1
NP_034498.1	0	0	0	5	0	3	1	2	4	5	0	0	0	2	2	0	0	0	4
NP_659207.1	0	1	0	0	0	3	1	0	1	4	0	1	0	3	3	0	0	0	2
NP_001304314.1	1	0	1	1	1	3	5	1	2	2	2	0	0	1	0	0	1	0	1
NP_001313523.1	0	1	0	1	0	1	3	1	0	0	0	1	0	0	0	0	1	0	0
NP_001033088.1	2	1	1	1	1	2	0	1	1	4	0	1	0	1	1	0	2	0	3
NP_001129541.2	0	0	0	2	0	1	1	0	3	3	0	0	0	1	2	0	0	0	2
NP_082235.1	2	1	1	2	2	3	3	3	1	0	2	1	0	1	0	0	2	1	2
NP_808383.3	4	4	2	2	3	4	4	1	5	5	3	2	0	2	3	0	3	1	4
NP_001028465.1	0	0	1	2	0	2	3	1	1	2	1	1	0	2	2	0	1	0	3
NP_034488.1	0	0	0	1	0	3	1	1	2	3	1	0	0	0	1	0	0	0	3
NP_038499.2	1	0	1	2	2	0	3	2	0	2	3	1	0	1	1	0	1	0	3
NP_034236.2	1	2	1	0	1	1	2	4	1	0	2	1	0	0	0	2	3	0	3
NP_062798.1	0	1	3	0	3	4	1	3	2	2	2	3	0	1	1	1	3	0	1
NP_035441.1	1	1	0	0	3	4	2	3	4	3	0	0	0	1	1	0	2	1	5
NP_032709.1	0	2	4	3	2	2	3	2	3	2	2	0	0	2	1	0	1	1	2
NP_001035978.1	3	2	3	2	3	1	3	3	1	4	3	3	0	2	3	1	1	1	2
NP_034085.2	1	0	0	0	1	0	1	1	3	2	1	0	0	0	0	0	1	0	2
NP_038584.2	0	0	0	2	0	2	1	0	3	4	1	1	0	3	3	0	0	0	1
NP_034112.3	0	0	0	1	0	1	3	1	1	1	3	0	0	1	1	0	0	0	1
NP_033455.1	5	2	6	2	2	1	2	2	1	2	4	0	2	2	0	2	5	4	1
NP_001300640.1	1	0	0	1	0	2	2	0	2	1	1	0	0	2	1	0	1	0	2
NP_032251.2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
NP_034552.1	1	1	1	3	1	4	1	1	2	2	1	0	0	2	2	0	1	1	2
NP_849242.1	0	0	1	1	2	2	1	1	0	1	1	0	0	1	0	0	0	0	1
NP_001268950.1	2	1	4	0	5	1	5	6	0	0	1	2	0	0	0	0	4	1	0
NP_001259026.1	2	0	5	0	3	1	3	2	2	1	3	0	0	0	0	0	6	0	2
NP_001240734.1	0	1	1	1	0	1	2	2	1	2	1	0	0	3	2	0	1	0	2

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NP_001159863.1	0	0	0	1	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_254279.1	0	0	0	2	1	1	3	1	1	4	0	1	0	0	2	0	0	0	2
NP_001303604.1	1	0	0	1	0	2	3	1	2	2	3	0	0	1	1	0	0	0	2
NP_032730.1	0	0	0	2	1	0	2	1	2	3	2	0	0	2	2	0	2	0	3
NP_038586.2	1	0	0	1	3	1	1	1	2	3	1	0	0	4	4	0	0	0	2
NP_032326.3	1	1	1	2	1	1	1	2	0	0	2	0	0	0	0	0	1	0	0
NP_666355.1	0	0	0	0	0	1	1	0	1	3	0	1	0	2	2	0	0	0	3
NP_001160061.1	1	0	0	1	0	3	3	1	2	0	1	0	0	1	1	0	1	0	2
NP_001192314.1	0	0	0	2	0	1	1	1	1	2	1	3	0	2	2	0	0	0	2
NP_035319.1	1	2	0	2	0	3	2	1	4	4	1	0	0	0	1	0	1	1	2
NP_001070997.1	2	0	0	0	2	0	4	2	2	3	2	0	0	3	3	0	3	0	3
NP_062708.1	0	0	0	1	0	0	2	0	0	1	0	1	0	0	0	0	0	0	0
NP_035845.1	0	0	0	0	0	1	1	1	2	3	0	0	0	3	1	0	0	0	1
NP_033934.2	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	3
NP_034608.2	2	1	1	2	4	0	1	2	0	1	0	0	1	1	2	0	2	1	1
NP_034551.2	0	1	0	3	0	2	1	1	1	1	1	0	0	1	0	0	0	0	1
NP_722490.1	0	0	0	0	0	1	1	0	1	0	2	2	0	0	0	0	2	0	0
NP_033482.1	0	0	1	2	1	0	2	2	1	0	2	0	0	0	1	0	2	0	1
NP_001288303.1	0	0	1	1	1	2	1	1	3	2	1	0	0	1	2	0	1	0	4
NP_064373.1	1	1	2	1	0	1	1	2	2	2	1	0	0	2	2	0	2	1	2
NP_035193.1	1	1	1	1	1	2	0	1	3	1	1	0	0	1	1	0	0	0	2
NP_001129534.1	0	1	0	0	0	0	1	1	2	2	0	0	0	1	2	0	0	0	2
NP_079672.1	0	0	2	0	0	0	2	1	0	0	3	0	0	0	0	0	1	0	1
NP_071720.1	0	0	3	5	1	2	0	2	1	2	0	0	0	0	0	0	0	0	0
NP_031819.1	1	0	0	0	0	1	1	0	3	0	0	0	0	2	0	0	0	0	2
NP_034712.2	0	0	0	1	0	0	0	1	1	1	0	0	0	2	1	0	2	0	2
NP_080405.1	0	4	0	2	2	3	3	1	0	1	1	0	0	0	1	0	1	1	1
NP_062615.1	4	2	2	2	2	2	2	4	0	0	2	0	0	0	0	0	3	1	1
NP_075891.1	0	1	1	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	1
NP_001139429.1	1	0	3	3	1	2	2	1	2	2	2	1	0	1	0	0	2	0	2
NP_034818.1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	1	0	2
NP_659095.1	0	0	1	1	0	1	1	0	2	0	0	0	0	0	2	0	0	0	2

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NP_033991.1	0	1	0	0	1	1	2	0	1	1	0	0	0	1	2	0	0	0	1
NP_700472.1	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0
NP_001272360.1	0	1	2	0	1	0	1	3	0	0	2	0	0	0	0	0	3	0	1
NP_061346.2	0	1	0	3	0	1	1	0	4	2	0	0	0	4	2	0	0	0	2
NP_033562.3	0	0	0	0	0	0	2	0	0	0	0	0	0	1	2	0	0	0	0
NP_001140179.1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP_001272418.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_083987.1	0	0	0	0	0	0	0	0	0	1	0	0	0	2	1	0	0	0	2
NP_033417.1	0	0	1	1	0	1	1	0	1	1	1	0	0	1	1	0	1	0	1
NP_001300823.1	0	0	0	0	0	1	0	0	2	4	0	0	0	1	0	0	0	0	3
NP_036098.1	2	1	4	1	1	0	2	2	0	0	2	0	0	0	1	0	1	1	1
NP_083640.1	0	1	1	2	2	2	1	1	1	1	1	0	0	0	1	0	0	0	0
NP_001288269.1	0	2	0	1	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0
NP_032257.3	1	1	2	2	0	0	2	2	1	2	2	0	0	1	0	0	2	0	2
NP_035279.2	0	0	0	2	0	1	1	0	1	3	0	0	0	2	1	0	0	0	1
NP_034249.1	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	1	0	0
NP_075747.3	3	1	3	3	0	1	2	2	0	1	1	0	0	0	0	0	1	1	0
NP_001025026.1	0	1	0	1	0	0	3	2	0	0	1	1	0	0	0	0	2	0	0
NP_031630.1	0	0	1	2	0	2	0	1	2	1	0	0	0	2	1	0	2	0	1
NP_001001491.1	0	1	0	0	2	1	0	2	0	0	1	1	0	0	0	0	0	1	0
NP_036099.1	0	0	1	2	1	0	3	1	1	1	4	0	0	0	0	0	1	0	0
NP_001153096.1	0	0	0	0	0	1	0	0	0	2	0	0	0	0	1	0	0	0	1
NP_001277698.1	0	1	3	0	1	0	2	3	0	0	0	1	0	0	0	0	1	0	0
NP_080305.2	0	0	0	0	0	1	0	0	1	2	2	0	0	2	2	0	0	0	3
NP_033850.1	0	0	0	1	0	3	0	0	2	3	2	0	0	1	0	0	0	0	2
NP_001033678.1	0	1	0	1	1	2	3	0	1	1	1	0	0	0	0	0	1	0	1
NP_059071.1	1	1	1	2	1	1	1	1	2	0	0	0	1	0	0	0	1	0	1
NP_033788.3	1	0	1	1	1	2	1	1	0	2	1	0	0	0	1	0	1	0	4
NP_001272352.1	0	1	0	2	0	0	0	2	0	1	0	2	0	1	1	0	0	0	0
NP_032405.3	0	0	2	2	0	0	2	1	0	0	1	0	0	0	0	0	1	0	0
NP_001157012.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_080270.2	0	0	4	0	3	1	4	4	0	0	1	0	0	0	0	0	5	0	0

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NP_031922.1	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	1	0	0
NP_001192242.1	0	0	0	2	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1
NP_001191804.1	0	3	0	1	0	0	2	1	1	2	3	0	0	2	1	0	0	2	1
NP_033399.1	0	0	0	0	0	2	0	0	1	3	0	0	0	1	2	0	0	0	2
NP_080283.3	0	0	1	1	0	0	0	1	1	3	0	0	0	1	0	0	2	0	1
NP_080645.2	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	2
NP_036006.1	0	0	2	0	1	0	1	2	1	0	1	0	0	0	0	0	2	0	1
NP_001159840.1	1	0	2	0	3	0	1	4	1	1	0	0	0	2	0	0	3	0	0
NP_001177382.1	0	1	1	1	1	0	2	1	0	0	0	0	0	0	0	0	3	0	0
NP_062422.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
NP_035909.2	0	1	1	0	0	2	2	1	1	2	0	2	0	0	1	0	0	0	2
NP_035159.3	0	1	0	0	0	2	1	0	0	0	1	0	0	0	0	0	0	0	0
NP_058540.1	1	1	1	1	1	1	4	3	1	1	1	0	0	0	0	0	2	3	1
NP_032169.1	0	0	0	1	0	1	0	0	0	1	0	0	0	1	1	0	0	0	1
NP_033927.2	0	1	0	2	0	1	1	0	2	1	0	0	0	2	1	0	1	0	1
NP_001240686.1	0	0	0	2	0	1	1	0	0	1	1	1	0	0	1	0	0	0	0
NP_033917.2	0	0	0	0	0	0	0	2	0	2	0	0	0	2	1	0	1	1	3
NP_080954.4	0	0	0	0	0	0	0	0	2	3	0	0	0	1	0	0	0	0	5
NP_001106670.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_542369.2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
NP_032643.2	1	3	3	4	1	1	4	3	1	1	2	0	0	0	0	1	2	1	1
NP_001104790.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_032876.1	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	0	1	0	0
NP_033828.2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
NP_032278.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
NP_031662.2	0	0	0	0	0	0	1	3	0	2	2	0	0	0	0	0	1	0	0
NP_001020432.1	0	1	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0
NP_080662.1	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
NP_061266.2	0	3	0	1	0	0	2	0	1	0	3	0	0	0	0	0	0	0	1
NP_001159881.1	0	0	2	1	1	0	1	2	0	0	1	1	0	0	0	0	1	0	0
NP_001291690.1	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1
NP_080828.1	0	0	0	0	0	2	0	0	3	2	0	0	0	2	1	0	0	0	2

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NP_058022.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP_035007.1	0	0	0	1	0	2	1	1	0	0	1	2	0	0	0	0	1	0	2
NP_001074896.1	0	0	0	1	0	1	0	1	1	1	0	1	0	0	1	0	1	0	3
NP_001136422.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033966.1	0	0	0	0	0	1	1	0	0	2	0	0	0	0	0	0	0	0	1
NP_001165571.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP_082045.1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1
NP_071306.2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
NP_031534.2	0	0	0	0	0	0	3	1	0	0	2	0	0	0	0	0	0	0	0
NP_035934.1	0	1	0	3	0	3	0	1	0	0	0	0	0	0	0	0	0	0	0
NP_032733.1	1	0	1	0	0	1	0	1	1	0	1	1	0	0	0	0	2	0	1
NP_034947.1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
NP_080218.1	1	0	0	3	0	0	2	1	0	0	2	0	0	0	0	0	0	0	0
NP_068359.1	1	0	0	0	0	0	0	0	2	1	0	0	0	0	1	0	0	0	1
NP_001073853.1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1
NP_031447.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
YP_009359666.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2
NP_032124.1	0	0	0	1	0	0	0	0	1	2	0	0	0	1	1	0	0	0	1
NP_075631.2	0	0	0	0	0	1	0	0	1	2	0	0	0	1	2	0	0	0	3
NP_059068.1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
NP_034429.1	1	2	1	0	0	1	1	2	0	0	1	0	0	0	0	0	0	0	0
NP_742146.1	1	0	1	1	1	0	1	2	0	0	1	0	0	0	0	0	1	0	0
NP_061223.2	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	0
NP_058017.1	0	1	0	0	0	0	2	3	0	0	0	0	0	0	0	0	0	0	0
NP_035868.1	0	1	2	1	0	0	1	1	0	1	0	0	0	0	2	0	1	0	1
NP_780469.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001008702.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001239189.1	0	9	4	0	3	0	5	5	0	0	6	0	0	0	0	0	5	2	0
NP_598911.1	0	0	0	1	0	2	0	0	0	0	1	0	0	0	0	0	0	0	2
NP_542126.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1
NP_035305.1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_666231.1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1

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NP_001191908.1	0	0	1	0	0	2	1	0	2	1	0	0	0	0	0	0	0	0	2
NP_032846.2	0	0	0	3	0	1	2	0	1	1	0	0	0	1	1	0	0	0	1
NP_032329.1	0	0	0	0	0	2	0	1	2	3	1	0	0	2	0	0	1	0	2
NP_033033.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2
NP_001239386.1	0	0	0	0	0	2	0	0	1	1	0	0	0	0	0	0	0	0	1
NP_035315.1	0	0	0	1	0	0	3	1	0	0	2	0	0	0	0	0	1	0	0
NP_033328.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_035908.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
NP_062745.1	0	0	0	1	0	1	0	0	0	2	0	0	0	0	0	0	0	0	1
NP_034370.2	0	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	2
NP_033138.1	0	0	0	0	0	0	1	0	0	2	0	0	0	1	1	0	0	0	1
NP_059500.2	1	2	1	0	0	1	0	2	0	0	0	0	0	0	0	0	2	0	0
NP_035427.2	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0
NP_077777.1	0	2	1	1	1	2	2	1	0	0	1	1	0	0	0	0	1	0	0
NP_001278037.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
NP_033347.1	1	2	2	0	1	0	1	1	0	0	1	1	0	0	0	0	1	0	0
NP_001258334.1	1	0	1	0	0	0	0	1	1	1	1	1	0	1	1	0	0	0	0
NP_032703.2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_663600.2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001157589.1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
NP_061359.2	0	0	1	0	0	0	1	1	0	1	0	0	0	1	1	0	1	0	0
NP_666312.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001300902.1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
NP_035286.1	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0
NP_081306.1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
NP_033118.1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
NP_035424.1	0	0	2	1	3	0	1	1	0	0	1	1	0	0	0	0	2	0	0
NP_001258467.1	0	1	0	0	0	0	1	0	0	0	0	2	0	0	0	0	0	0	0
NP_733467.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
NP_444338.2	0	0	0	0	1	1	2	0	1	3	2	0	0	0	0	0	0	0	1
NP_598487.1	1	1	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0
NP_034403.1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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NP_036160.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_001291457.1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_001276527.1	0	0	1	2	1	2	1	1	1	1	0	1	0	0	0	0	1	0	0
NP_032974.2	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP_062340.1	0	1	1	1	3	0	0	1	0	0	0	0	0	0	0	0	1	0	0
NP_001280550.1	0	0	0	1	0	2	0	0	0	0	0	0	0	2	0	0	0	0	1
NP_001106826.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001033581.1	0	0	0	0	0	2	0	0	1	1	0	0	0	0	0	0	0	0	1
NP_035869.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
NP_062754.1	0	0	0	0	0	0	0	0	1	2	0	0	0	2	0	0	0	0	3
NP_064337.1	2	1	2	1	2	0	2	2	0	0	1	1	0	0	0	1	1	0	0
NP_032801.2	0	0	0	2	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
NP_001074564.1	0	0	0	0	0	0	0	0	1	1	0	0	0	2	1	0	0	0	1
NP_001290352.1	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
NP_062750.2	0	0	0	1	0	0	0	0	0	1	0	0	0	2	0	0	0	0	0
NP_032970.2	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
NP_033882.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_084481.3	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	1	0	0
NP_766089.1	0	0	0	1	0	1	0	0	1	2	0	0	0	2	1	0	0	0	2
NP_080296.3	0	1	0	0	0	0	1	3	0	0	1	0	0	0	0	0	0	0	0
NP_001071635.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
NP_957678.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001139780.1	0	0	3	0	2	0	2	3	0	0	1	0	0	0	0	0	2	0	0
NP_056598.2	0	0	3	0	1	0	4	2	0	0	4	1	0	0	0	0	1	0	0
NP_036067.2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
NP_444409.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_079610.1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0
NP_001158133.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001070658.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
NP_035200.2	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0
NP_035430.1	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0
NP_001289006.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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NP_705727.1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
NP_034253.3	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
NP_032038.1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	1
NP_945175.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_035831.2	0	12	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
NP_075622.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_031448.2	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_001299578.1	0	0	0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
NP_001164011.1	0	0	3	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
NP_001157724.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
NP_035790.1	0	0	0	1	0	0	0	1	1	1	0	0	0	1	1	0	1	0	1
NP_032274.1	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
NP_663544.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033106.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
NP_077195.2	1	0	1	0	1	0	1	2	0	0	1	0	0	0	0	0	2	1	0
NP_001123480.1	0	0	1	0	1	0	1	1	0	0	1	0	0	0	0	0	1	0	0
NP_001299796.1	1	0	2	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
NP_001139592.1	0	1	0	1	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0
NP_033034.1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	1	0	1
NP_038691.1	0	0	0	0	0	2	0	0	1	1	0	0	0	1	0	0	1	0	1
NP_032543.2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_796300.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
NP_001029138.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
NP_035369.2	0	0	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	0	1
NP_573479.3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_075373.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_079863.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001257466.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_666262.2	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1
NP_033968.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_083379.2	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
NP_997406.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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NP_766523.2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_056550.1	2	3	2	1	1	0	2	2	0	0	2	0	0	0	0	0	0	1	0
NP_035629.2	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0
NP_032387.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_031536.2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001297611.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_032695.3	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_077135.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001035780.1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
NP_035995.1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
NP_001041526.1	0	1	0	0	0	1	0	1	0	0	2	0	0	0	0	0	0	0	0
NP_001278003.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001170840.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_036167.1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
NP_001132992.1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
NP_064377.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033802.2	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0	0	1
NP_035249.1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	2
NP_001152981.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_573461.2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_036082.1	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1
NP_031535.2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
NP_001139529.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001034790.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_598553.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033122.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033465.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001172111.1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0
NP_034566.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
NP_001171015.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
NP_001288082.1	0	0	2	0	0	0	1	2	0	0	1	0	0	0	0	0	2	0	0
NP_038615.2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

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NP_001280579.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_036097.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001300890.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_032871.3	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_001186117.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_080276.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001180574.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_598738.1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
NP_034572.1	0	6	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
NP_001288293.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033970.3	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
NP_031520.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_056622.1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1
NP_001076803.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001181975.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP_038913.1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	1
NP_001264042.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_035853.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033124.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_444444.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_598862.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_079976.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
NP_035780.1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_631888.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_035597.2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP_038675.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_035470.3	0	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0
NP_084514.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_067485.1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
NP_113553.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_038497.2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_032997.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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NP_032186.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001103709.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_035426.1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
NP_001275507.1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
NP_570959.1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
NP_035443.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1
NP_033139.2	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0
NP_057886.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
NP_058676.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001240680.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
NP_001297469.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001029037.1	0	0	0	0	0	0	0	2	0	0	0	0	0	2	2	0	0	0	0
NP_079824.2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_033971.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001153711.1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
NP_084011.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_032474.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001157308.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_598599.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001070733.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001001932.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_038714.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_079561.1	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
NP_001153189.1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0
NP_001157287.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
NP_001295379.1	0	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
NP_061352.2	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0
NP_001152823.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
NP_775539.1	0	0	0	0	0	0	0	1	2	1	0	0	0	0	0	0	1	0	0
NP_033937.2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
NP_035960.2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
NP_033967.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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NP_001159899.1	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
NP_620087.3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
NP_032973.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_032155.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_075860.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
NP_473398.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_057932.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001192259.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_080239.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
NP_035488.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_570934.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_001158149.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_997044.2	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
XXX_YP_177962.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001077424.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
NP_766635.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_081245.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_067499.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_058655.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_032968.2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_075898.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001017959.1	0	1	0	0	0	1	0	0	0	2	0	0	0	2	0	0	0	0	3
NP_001034481.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
NP_036096.1	0	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0
NP_058086.2	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_938045.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_038574.3,	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_080112.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033121.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001186194.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_077753.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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NP_035318.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_666338.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001139374.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001104301.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_035945.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_034390.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
NP_033908.2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1
NP_031570.2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001300865.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
NP_077754.3	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
NP_033842.3	0	0	0	3	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_038668.2	1	0	2	1	0	0	0	1	0	0	0	0	0	0	0	0	2	0	0
NP_598912.4	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_065625.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_035863.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_080170.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_058572.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_031646.2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_001242984.1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_031650.3	0	0	0	0	0	0	2	1	0	0	1	0	0	0	0	0	0	0	0
NP_033822.2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
NP_001020609.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_058066.2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP_001303251.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_032240.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_034507.2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_032040.1	0	0	2	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0
NP_033173.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001292748.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001272966.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_613069.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001103662.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

NP_001278126.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_077228.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_919444.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_080097.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
NP_001161980.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001278115.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001033789.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_033298.1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
NP_031664.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001278158.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_031412.2	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	1
NP_032433.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
NP_038653.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
NP_001073598.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
XXX_NP_001156610.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
NP_001153222.1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
NP_034455.1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
NP_032430.2	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
NP_001033320.1	0	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
NP_035800.2	0	0	0	0	0	0	2	1	1	0	2	0	0	0	0	0	0	0	0
NP_001077407.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_058587.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_659074.2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
NP_001095874.1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
NP_034369.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
NP_058054.2	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001182350.1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
NP_001157134.1	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_034607.3	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP_001171080.1	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0
NP_077198.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0

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NP_001076429.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S3: Contingency table from 11 experiments of 5 different treatments, 55 comparisons in total. The table was created to determine P-values using the Fisher Exact Test, highlighted in grey are observed significant p-values.

Set1	**BCG2**	**BCG1**	**BCG1**	**BCG1**	**BCG1**	**BCG1**	**BCG1**	**BCG1**	**BCG1**	**BCG1**
Set2	**BCG2**	**BCG3**	**Control1**	**Control2**	**Control3**	**H37Rv1**	**H37Rv2**	**R179**	**Smeg1**	**Smeg2**
PSMTotal1	**1237**	**1237**	**1237**	**1237**	**1237**	**1237**	**1237**	**1237**	**1237**	**1237**
PSMTotal2	**1164**	**1146**	**908**	**1225**	**1120**	**1742**	**1587**	**1259**	**825**	**1170**
NP_071855.2	0.989	0.988	0.989	0.209	1.000	0.137	0.039	0.395	0.192	0.999
NP_033773.1	1.000	1.000	0.285	1.000	1.000	0.095	0.046	0.315	0.894	1.000
NP_035732.2	1.000	0.991	0.026	0.693	0.963	0.616	0.613	0.095	0.603	1.000
NP_001139425.1	0.891	0.974	1.000	0.767	1.000	0.364	0.408	0.516	0.359	0.993

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Set1	**BCG2**	**BCG2**	**BCG2**	**BCG2**	**BCG2**	**BCG2**	**BCG2**	**BCG2**	**BCG2**	**BCG3**
Set2	**BCG3**	**Control1**	**Control2**	**Control3**	**H37Rv1**	**H37Rv2**	**R179**	**Smeg1**	**Smeg2**	**Control1**
PSMTotal1	**1164**	**1164**	**1164**	**1164**	**1164**	**1164**	**1164**	**1164**	**1164**	**1146**
PSMTotal2	**1146**	**908**	**1225**	**1120**	**1742**	**1587**	**1259**	**825**	**1170**	**908**
NP_071855.2	0.594	0.666	0.002	0.939	0.001	0.000	0.011	0.003	0.889	0.676
NP_033773.1	1.000	0.003	1.000	1.000	0.000	0.000	0.003	0.172	1.000	0.003
NP_035732.2	0.246	0.000	0.005	0.101	0.002	0.002	0.000	0.005	0.659	0.000
NP_001139425.1	0.851	1.000	0.422	1.000	0.094	0.117	0.189	0.118	0.939	1.000

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Set1	**BCG3**	**BCG3**	**BCG3**	**BCG3**	**BCG3**	**BCG3**	**BCG3**	**Control1**	**Control1**	**Control1**
Set2	**Control2**	**Control3**	**H37Rv1**	**H37Rv2**	**R179**	**Smeg1**	**Smeg2**	**Control2**	**Control3**	**H37Rv1**
PSMTotal1	**1146**	**1146**	**1146**	**1146**	**1146**	**1146**	**1146**	**908**	**908**	**908**
PSMTotal2	**1225**	**1120**	**1742**	**1587**	**1259**	**825**	**1170**	**1225**	**1120**	**1742**
NP_071855.2	0.003	0.942	0.001	0.000	0.012	0.004	0.894	0.003	0.914	0.001
NP_033773.1	1.000	1.000	0.000	0.000	0.003	0.175	1.000	1.000	1.000	0.386
NP_035732.2	0.053	0.378	0.025	0.026	0.000	0.048	0.916	0.995	1.000	0.995
NP_001139425.1	0.200	1.000	0.024	0.033	0.064	0.039	0.819	0.020	1.000	0.001

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Set1	**Control1**	**Control1**	**Control1**	**Control1**	**Control2**	**Control2**	**Control2**	**Control2**	**Control2**	**Control2**
Set2	**H37Rv2**	**R179**	**Smeg1**	**Smeg2**	**Control3**	**H37Rv1**	**H37Rv2**	**R179**	**Smeg1**	**Smeg2**
PSMTotal1	**908**	**908**	**908**	**908**	**1225**	**1225**	**1225**	**1225**	**1225**	**1225**
PSMTotal2	**1587**	**1259**	**825**	**1170**	**1120**	**1742**	**1587**	**1259**	**825**	**1170**
NP_071855.2	0.000	0.013	0.004	0.854	1.000	0.474	0.225	0.756	0.496	1.000
NP_033773.1	0.256	0.661	0.973	1.000	1.000	0.000	0.000	0.002	0.162	1.000
NP_035732.2	0.994	0.816	0.984	1.000	0.929	0.479	0.478	0.052	0.489	0.999
NP_001139425.1	0.002	0.004	0.003	0.317	1.000	0.199	0.235	0.335	0.218	0.977

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Set1	**Control3**	**Control3**	**Control3**	**Control3**	**Control3**	**H37Rv1**	**H37Rv1**	**H37Rv1**	**H37Rv1**	**H37Rv2**
Set2	**H37Rv1**	**H37Rv2**	**R179**	**Smeg1**	**Smeg2**	**H37Rv2**	**R179**	**Smeg1**	**Smeg2**	**R179**
PSMTotal1	**1120**	**1120**	**1120**	**1120**	**1120**	**1742**	**1742**	**1742**	**1742**	**1587**
PSMTotal2	**1742**	**1587**	**1259**	**825**	**1170**	**1587**	**1259**	**825**	**1170**	**1259**
NP_071855.2	0.000	0.000	0.000	0.000	0.524	0.260	0.822	0.556	1.000	0.945
NP_033773.1	0.000	0.000	0.003	0.180	1.000	0.390	0.831	0.994	1.000	0.912
NP_035732.2	0.087	0.090	0.003	0.128	0.973	0.556	0.056	0.554	1.000	0.062
NP_001139425.1	0.000	0.001	0.002	0.001	0.261	0.614	0.710	0.527	0.999	0.675

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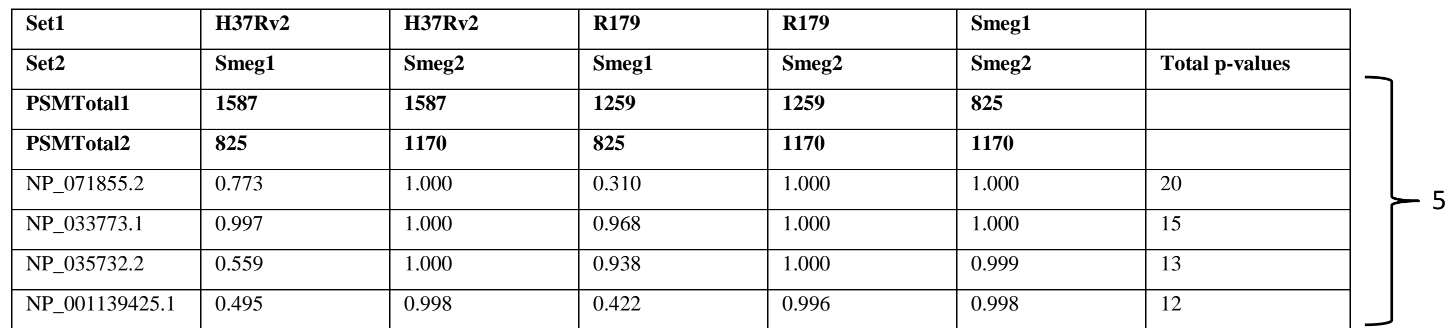

Set1	**H37Rv2**	**H37Rv2**	**R179**	**R179**	**Smeg1**	
Set2	**Smeg1**	**Smeg2**	**Smeg1**	**Smeg2**	**Smeg2**	**Total p-values**
PSMTotal1	**1587**	**1587**	**1259**	**1259**	**825**	
PSMTotal2	**825**	**1170**	**825**	**1170**	**1170**	
NP_071855.2	0.773	1.000	0.310	1.000	1.000	20
NP_033773.1	0.997	1.000	0.968	1.000	1.000	15
NP_035732.2	0.559	1.000	0.938	1.000	0.999	13
NP_001139425.1	0.495	0.998	0.422	0.996	0.998	12

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