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Declaration of Authorship

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Abstract

Mitochondria have an essential role in the preservation of life. Life persists as a result of structure and energy, and the mitochondria are key components of energy production. Mitochondrial dysfunction is considered a characteristic cause of aging, as well as an involved factor in almost all chronic diseases. This makes it important to perform studies on the mitochondria to better understand mutational mechanisms that occur and the consequences they bring, and in the end develop appropriate treatments to prevent such outcomes. When performing such research, a common path is to procure isolate metabolically active mitochondria. When doing so, purity of sample is key to uncover the "true" nature of mitochondrial activity in controlled conditions. In this thesis, the objective was to develop a method of obtaining such a purified sample of intact mitochondria. Mitochondrial extracts have been performed on beef heart and liver, as well as on the human myeloblastic suspension cell line HL-60. Although progression was made, by the end of the practice a pure mitochondrial extraction was yet to be accomplished. Extractions made on beef heart seemed to be most promising, with clear concentrated protein complex bands observed from Native-PAGE. However, the samples were contaminated and before any methodical alterations could be made the availability of beef heart became scarce. Mitochondrial extraction from beef liver seemed most troublesome. After performing an additional purification with a percoll gradient, the sample did not seem to provide any protein complexes in the subsequent Native-PAGE that was performed. The mitochondrial extraction from HL-60 cells also proved challenging. However, progress seemed to be made in the effort to solve to issue of resuspending the pellet after lysing the cells with digitonin.

Abbreviations

ATP – Adenosine Triphosphate

ETC – Electron transport chain

OXPHOS – Oxidative Phosphorylation

NADH – Nicotinamide Adenine Dinucleotide

$FADH_2$ – Flavin Adenine Dinucleotide

DNA – Deoxyribonucleic acid

RNA – Ribonucleic acid

PBS – Phosphate-buffered saline

BSA – Bovine Serum Albumin

BCA – Bicinchoninic acid

TCA – Trichloroacetic acid

APS – Ammonium Persulfate

CCCP – Carbonyl Cyanide m-Chlorophenylhydrazone

RIPA – Radioimmunoprecipitation assay

SDS – Sodium Dodecyl Sulfate

PAGE – Polyacrylamide Gel Electrophoresis

DR – Deep Red

EDTA – Ethylenediaminetetraacetic acid

TMRM – Tetramethylrhodamine, methyl ester

TEMED – Tetramethylethylenediamine

β-DDM – Lauryl-β-D-maltoside

LDS – Lithium dodecyl sulfate

DTT – Dithiothreitol

TMK – Tris/Magnesium/Potassium containing solution

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1. Introduction

1.1 Mitochondria and its role in maintaining life

Mitochondria, which are present in virtually all eukaryotic cells as cytoplasmic organelles, have been thought to have arisen approximately 1.5 billion years ago in a symbiotic association between an oxidative bacterium and a glycolytic proto-eukaryotic cell [1]. Since this merger, the organelle has adapted to its intracellular environment, shrinking in size presumably by deleting unessential genes and transferring other genetic material into the nuclear genome. In turn, these nuclear genes are transcribed and transferred as complete modified proteins back to the mitochondria [2]. As a result of the reduces mitochondrial genome, their replication has steadily increased, leading to the existence of hundreds of mitochondria within each cell [3].

The mitochondrial role in preserving mammalian life has become essential. Life persists as a result of structure and energy, and the mitochondria are key components of energy production [4]. The principle function of mitochondria is to generate a proton gradient on either side of the inner mitochondrial membrane by way of an electron transport chain (ETC). In turn, the protons will pass through a "rotor" based protein mechanism called ATPase, resulting in the production of energetically charged adenosine triphosphate (ATP). This process is referred to as oxidative phosphorylation (OXPHOS). The ETC is comprised of four primary protein complexes numbered I-IV, which work together with mobile accessory electron carriers to transport electrons down an electronegative current. NADH and $FADH_2$, produced in the citric acid cycle, are the initial electron donors, and they donate their electrons to complex I and complex II respectively. The accepted electrons from both complexes are then passed to an intermediate carrier called Ubiquinone, which subsequently delivers them to complex III. From here, the electrons are given to cytochrome c, which finally donates them further to oxygen atoms carried by two heme groups in complex IV. Both heme groups (a, and a_3) have a tight grip on their respective oxygen atom using their iron and copper ions, until the oxygen is fully reduced producing water. Protons are the biproducts from many of these electron-transferring reactions, releasing them into the intermembrane space of the mitochondria, which in turn creates the proton gradient and fuels the OXPHOS system. An overview of the aforementioned ETC and OXPHOS processes with all their primary components are shown in figure 1.1 [5]. The end-product is ATP, which is the primary energy source for mammalian life.

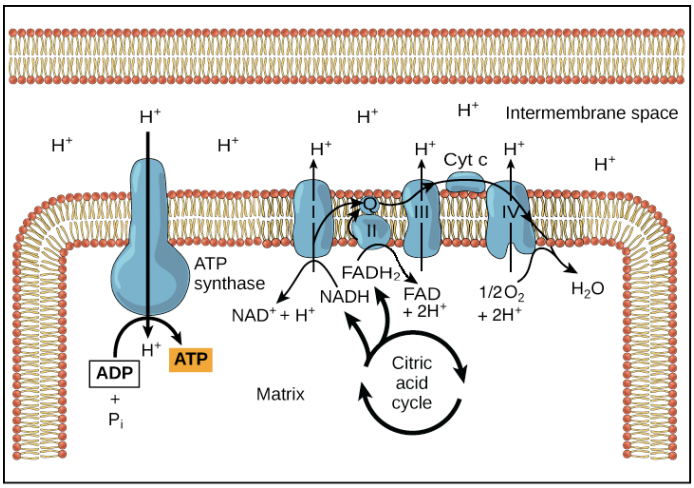

Figure 1.1: Overview of the electron transport chain and oxidative phosphorylation [5].

1.2 Mitochondrial genome and dysfunction

The mammalian mitochondrial DNA (mtDNA) molecule is comprised of over 16500 base pairs and are present as two to ten copies within each mitochondrion [6]. This means that on average, there are several thousand mtDNA copies inside each cell [7]. There are 13 polypeptide genes comprising the mtDNA genome, all of which encode basic protein subunits of the protein complexes of the ETC and OXPHOS system. Seven genes encode subunits ND1, 2, 3, 4, 4L, 5, and 6 in the 43 subunit complex I; one gene encodes the cytochrome b subunit in the 11 subunit complex III; three genes encode COX1, 2, and 3 in the 13 subunit complex IV, and two genes encode ATPase 6 and 8 in the 17 subunit complex V. The mtDNA also encodes the 12S and 16S rRNA genes and the 22 tRNA genes required for mitochondrial protein synthesis [3]. An overview of the genes in the mtDNA has been presented in figure 1.2. The remaining genes responsible for comprising the rest of the mitochondrial ETC and OXPHOS proteins, the DNA and RNA polymerases, the metabolic enzymes, the ribosomal proteins, and the mtDNA regulatory factors, such as mitochondrial transcription factor A, are all encoded from the nucleus [1].

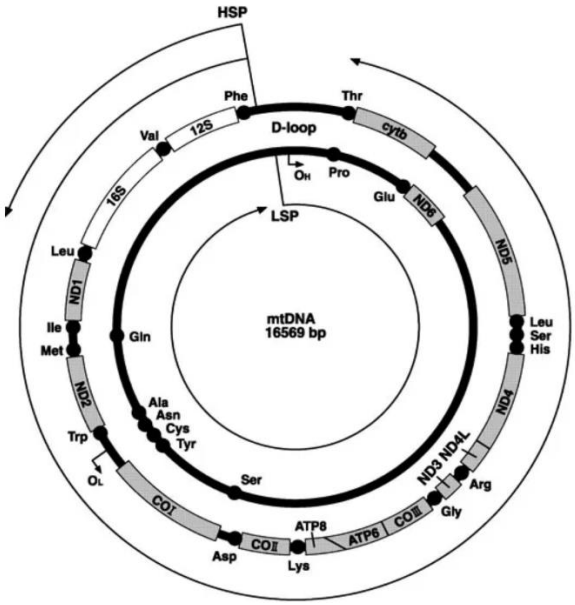

Figure 1.2: Distribution of genes in mammalian mtDNA. ND1, 2, 3, 4, 4L, 5, and 6 are subunits of complex I. Cyt b is a subunit of complex III. COI, II, and III are subunits of complex IV. ATPase6 and 8 are subunits of complex V. 12SrRNA and 16SrRNA are ribosomal RNA genes. 22 tRNA genes are shown as closed circles with three-letter abbreviations of amino acids. There are two forms each for Leu- and Ser-tRNAs. O_H and O_L indicate origin of replication of heavy (H) and light (L) strands. The outer circle represents the L-strand, while the inner represents the H-strand [6].

Mitochondrial dysfunction is characterized by the loss of efficiency of the electron transport chain, which in turn results in the decreased production of ATP. Such a dysfunction is considered a characteristic cause of aging, as well as an involved factor in almost all chronic diseases. Examples of such diseases include, neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, and Huntington's disease [8, 9]; cardiovascular diseases like atherosclerosis; diabetes and metabolic syndrome[10]; Autoimmune disease[11], and many more [12]. Age related mitochondrial dysfunction is thought to be due to a 17-fold higher mutation rate in mtDNA compared to nuclear DNA. These mutations are believed to occur near the inner mitochondrial membrane where oxidants are produced from the ETC. Also, in this area the mtDNA lack protective histones and DNA repair activity, which would play a role in why the mutation rate is so high [13]. Eventually the cell exists with both dysfunctional and wild-type mitochondria. The

mitochondria have a maternal inheritance, which means that the damaged and wild-type mitochondria will be passed randomly on the daughter cell. After many generations, the cell may become predominantly filled with dysfunctional mitochondria. During this process, the production rate of ATP has steadily been decreasing. Finally, the ATP levels will sink below the bioenergetic threshold for which the cell can no longer perform normally. This is when pathological symptoms may occur [3].

The manner of the occurring symptoms of mitochondrial dysfunction in patients are dependent on where in the body the mitochondrial dysfunction takes place, as well as which mutations that have occurred. For instance, parkinsonism may be caused by mutations in the 12S rRNA gene in mitochondria located in the basal ganglia [14]; or atherosclerosis, where mutations in several genes like ND1, 2, 5, and 6 in mitochondria in major vessels are suggested to play a role in its development [15]. However, given the stochastic and quantitative nature of mtDNA mutations, certain symptoms may occur from more than one mutation type. Also the same mutation may portray different symptoms in one patient than another[1]. Hence, the strive for understanding of the complexity of mitochondrial genetics and the effects that mutations may portray is of high importance. A way of doing so may be to isolate intact mitochondria, and study its behavior when modified with specific mutations.

1.3 The importance of purity and its challenges

In any biomedical or chemical context, purity of a sample is of the utmost importance to gather reliable data. Since purity is a key parameter of the "*true*" chemical nature of a substance, any residual contamination may result in false conclusions. Thus, a purity assessment is a logical prerequisite in obtaining accurate and reproducible data [16]. This is also the case when studying mitochondria. Many protocols for the extraction of intact mitochondria from various sources like tissue and cultured cells have been published [17-20]. However, these methods have been specifically calibrated to work efficiently on certain types of tissue, or for a specific species. Consequently, if any of these procedures were to be performed on biological matter other than that which was specified, the resulting purity and functionality of the mitochondrial extract could not be expected to be as high. This is understandably so, because different tissues or species possess various phenolic compounds or metabolic profiles, which if not considered could easily damage the integrity of mitochondria [21]. Also, eukaryotic cells exist in a lot of different sizes, making one universal method of mitochondrial extraction difficult.

1.4 Methodical theory

1.4.1 Polyacrylamide-gel electrophoresis (PAGE)

In this thesis, the primary tool for determining the purity of obtained mitochondrial extracts was through polyacrylamide-gel electrophoresis (PAGE). This is a technique for the separation of proteins and is performed by introducing an electromotive force which reacts with the charged ions on the protein, causing them to move through an acrylamide gradient gel. The proteins velocity and hence the distance of which the proteins travel are directly proportional to the ionic charged state and the electrical field strength that is exerted, and are inversely proportional to size or mass of the protein [22]. As other biomolecules like DNA and lipids also possess ionically charged ions, they too will pass through the polyacrylamide gel, and after staining the gel with Coomassie brilliant blue, they may be detected if present in the sample. Proteins may be separated either in their native or denatured form. Native-PAGE, for which unimpaired protein complexes are separated, was the focused method of protein separation in this project. However, if a purified mitochondrial extract was obtained, it would have been subject to a second-dimension sodium dodecyl sulfate-PAGE (SDS-PAGE). SDS is a string detergent which binds to the polypeptide backbone of a protein in a constant manner. As a result, the proteins unfold denaturing the protein complex into its individual subunits. By passing these proteins through an acrylamide gradient gel, the proteins are separated almost solely on the length of their polypeptide chain. Because of this, the subunit composition of the mitochondrial protein complexes could be determined, which would be very insightful when studying the behavior of mitochondrial functionality.

1.4.2 Solubilization with lauryl-β-d-maltoside (β-DDM)

The mitochondrial protein complexes I-V were solubilized using the mild detergent lauryl-β-d-maltoside (β-DDM), before being subject to Native-PAGE. At a concentration of over 0.17 mM (0.0087% w/v) β-DDM associates to form a multimolecular complex called a micelle. This micelle is comprised of a hydrophobic core and a hydrophilic surface. Subsequently, when introduced to the mitochondria, β-DDM destabilizes, and breaks apart the lipid bilayer of the mitochondrial membrane, detaching the protein complexes from its grip and into its micellular body. The protein is kept stable by keeping it hydrophobic segment within the micellular core, and its hydrophilic segment places outside on the surface.

1.4.3 BCA assay

Once the proteins were isolated, they could be quantified. For this part the BCA procedure was used. This method is based on peptide bonds in proteins reducing Cu^{2+} ions from copper(II)sulfate and forming Cu^{+} ions. The amount of Cu^{+} produced is directly proportional to the amount of protein in the sample. The resulting Cu^{+} ions are purple colored and absorb light at the wavelength of 562 nm. Therefore, by producing a standard protein concentration curve with samples of known concentrations, the concentration of an unknown protein sample may be deduced by comparison. The standard protein used in this case was Bovine Serum Albumin (BSA).

1.4.4 Spectrophotometry

Spectrophotometry is performed to measure how much a chemical substance absorbs light. All chemical compounds absorb, reflects or transmits light at a certain wavelength. Proteins for instance, have an absorbance peak wavelength of ~280 nm, while DNA/RNA have theirs at ~260 nm[23]. Therefore, by scanning a mitochondrial extract, it would be possible to get an idea of what is present in the sample.

1.4.5 TMRM, Mitotracker DR and TOMM20

During the project, HL-60 cells and some intermediate sample extracts were subject to staining using the two dyes TMRM and Mitotracker DR, as well as the antibody TOMM20. TMRM and Mitotracker DR are both mitochondrial specific dyes which passively diffuse across the membrane of metabolically active mitochondria and accumulate electrophoretically into the matrix [24]. However, if a cell that is undergoing apoptosis or is treated with CCCP, the mitochondrial membrane becomes depolarized and the signal is lost Because these dyes are dependent on functional mitochondria to become oxidized and fluorescent, they provide an extra confirmation that the mitochondria are undamaged. The anti-TOMM20 antibody on the other hand binds to the translocase of the outer mitochondrial membrane (TOMM) and therefore does not rely on metabolically active mitochondria to work [25].

1.4 Objectives

Mitochondrial dysfunction is considered a characteristic of aging, and essentially a participant co-factors in the development of almost all chronic diseases. Therefore, by gaining insight on mitochondrial behavior with specific mutations, it could become possible to gain the necessary understanding for which treatment to be made. A way of doing so, is by performing research on isolated mitochondria, but to get accurate results, the mitochondrial extract must be pure. The objectives for this thesis was:

- To develop a method for extracting a purified sample of intact mitochondria from beef heart and liver
- To develop a method for extracting a purified sample of intact mitochondria from HL-60 cultured cells.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

An assortment of chemicals was used throughout the duration of this thesis. An overview of all these chemicals, as well as their company of origin and their stock composition has been supplied in table 2.1.

Table 2.1: Overview of all the chemicals used in this project, including producer company and stock composition.

Chemical	Company	Stock
Sucrose	MERCK	342.29 g/mol
Tris	VWR Chemicals	121.14 g/mol
HCl	MERCK	1L = 1.19 kg
Succinic Acid	SIGMA	118.09 g/mol
EDTA	AMRESCO	372.24 g/mol
Percoll	GE Healthcare Europe GMBH NUF	100%
TCA	Frederiksen Scientific AS	100%
Acetone	VWR International AS	100%
RIPA buffer	Thermo Fisher Scientific	1x
Glycine	MERCK	75.06 g/mol
SDS	MERCK	288.38 g/mol
Acrylamide	Bio-Rad	30%
TEMED	SERVA	100%
APS	GE Healthcare Europe GMBH NUF	10%
6-Aminohexanoic acid	MERCK	131.17 g/mol
BSA	Bio-Rad	2 mg/ml
BSA	SIGMA Life Technologies	66430.3 g/mol
Coomassie® Brilliant Blue G 250	SERVA	833.05 g/mol
Tricine	AMRESCO	179.2 g/mol
PBS	Life Technologies Europe BV	2005.5 g tablet
RPMI 1640	Life Technologies AS	1x
Protease Inhibitor	Sigma Aldrich Norway AS	100x
TMRM	Life Technologies Europe BV	100 µM
Mitotracker Deep Red FM	Life Technologies Europe BV	1mM
Hoechst	Life Technologies Europe BV	1 mg/ml
Cell-Tak	Corning B.V. Life Science	100%
Sodium Carbonate	VWR International AS	0.1 M, pH 8.0
TOMM20	Abcam	0.5 mg/ml
Methanol	Sigma Aldrich Norway AS	100%
FBS	Biowest SAS	10x
Penicillin	Life Technologies AS	100x
Tween 20	Sigma Aldrich Norway AS	100%
Ponceau S	Sigma Aldrich Norway AS	672.63 g/mol
NaCl	MERCK	58.44 g/mol
Glycerol	Chiron AS	92.09 g/mol
Bis Tris	Sigma Aldrich Norway AS	209.24 g/mol
Aluminum sulfate-(14-18)-hydrate	Thermo Fisher Scientific	666.42 g/mol
Ortho-phosphoric acid	VWR International AS	85% v/v
Ethanol	Thermo Fisher Scientific	95% v/v
β-DDM	Sigma Aldrich Norway AS	510.62 g/mol
Digitonin	MERCK	1229.3 g/mol
CCCP	Sigma Aldrich Norway AS	2.5 mM
6-Aminohexanoic acid	Sigma Aldrich Norway AS	131.17 g/mol
Bromophenol blue	Avantor Performance Materials Poland S.A.	669.96 g/mol
DTT	VWR International AS	1 M
LDS	Sigma Aldrich Norway AS	10%
$MgCl_2$	VWR International AS	95.21 g/mol
KCl	Sigma Aldrich Norway AS	74.55 g/mol

2.1.2 Cell Line

The human myeloblastic suspension cell line, HL-60, was used in this project.

2.1.3 Buffers & solutions

This section provides a summary of all the buffers and solutions that were prepared and used during this thesis. Each table represents the composition and concentrations of the various chemicals within each buffer/solution used in a specific procedure.

Table 2.2 represents the solutions used in the mitochondrial preparation from beef heart and livers. Each chemical was prepared in advance with a higher concentration which made it possible to prepare large quantities at one time. The chemicals were later diluted together into their corresponding concentrations at the day the procedure took place. ddH_2O was the solvent used in all the solutions.

Table 2.2: Solutions used in the mitochondrial preparation from beef hearts and livers

Chemicals	Concentrations in procedure	Prepared volumes	Prepared concentrations	Amount of stock chemicals
Mince Solution				
Sucrose	250 mM	500 ml	1250 mM	213.93 g
Tris-Cl (pH 7.8)	10 mM	250 ml	500 mM	15.14 g
Sucrose Solution				
Sucrose	250 mM	500 ml	1250 mM	213.93 g
Tris-Cl (pH 7.8)	10 mM	250 ml	500 mM	15.14 g (Tris)
Tris-Succinate (pH 7.8)	1 mM	250 ml	50 mM	1.5143 g (Tris)
EDTA	0,2 mM	250 ml	10 mM	0.9306 g
pH adjustment solution				
Tris (unneutralized)	2000 mM	30 ml	2000 mM	7.2684 g

Table 2.3 shows the dilution that was made to prepare the 30% percoll solution in ddH_2O. The sucrose solution mentioned in the protocol in methods section 2.2.2 was the same solution described in Table 2.

Table 2.3: Solution used in the mitochondrial purification with a percoll gradient.

Chemicals	Prepared concentration	Prepared volume	Stock concentration	Dilution volume
Percoll	30%	16 ml	100%.	4.8 ml

A summation of the composition of all the solutions and buffers used in the mitochondrial preparation from HL-60 cells are provided in table 2.4. Digitonin was made fresh the day of each procedure, while the rest could be prepared and store for later use. β-DDM was stored at -20°C, while the rest were kept at 4°C.

Table 2.4: Composition of all solutions used in the mitochondrial preparation from HL-60 cultured cells

Chemicals	Concentrations in procedure	Prepared volume	Quantity of stock chemicals used	Solvent
RPMI 1640 cell culture medium				
L-Glutamine	1%	500 ml	Already present in RPMI 1640 medium	RPMI 1640 medium
FBS	10% v/v		50 ml	
Penicillin	1% v/v		5 ml	
PBS				
Na_2HPO_4	10 mM	200 ml	Dissolved a 2005.5 mg readymade tablet	Ultrapure H_2O
KCl	2.68 mM			
NaCl	140mM			
Digitonin solution				
Digitonin	3.3 mM	1 ml	4 mg	PBS
Protease Inhibitor	1x		10 µl	
EDTA solution				
EDTA	500 mM	0.5 ml	93 mg	ddH_2O
Original mitochondrial buffer				
6-aminohexanoic acid	1.75 M	1 ml	230 mg	ddH_2O
Bis Tris	75 mM		15.7 mg	
EDTA	2 mM		4 µl of EDTA solution	
β-DDM solution				
β-DDM	10% w/v	1 ml	100 mg	ddH_2O
Protease Inhibitor	1x		10 µl	
Alternative mitochondrial buffer				
6-aminohexanoic acid	0.75 M	5 ml	490 mg	ddH_2O
Bis-Tris	50 mM		52.3 mg	

A 10x TMK stock solution was available ready made in the lab. Table 2.5 presents the composition of that solution. The TMK was diluted to a 1x concentration with ddH_2O before using it in the solubilization procedure.

Table 2.5: Composition of the TMK solution used in the solubilization procedure.

Chemicals	Prepared concentration	Prepared volume
Tris, pH 8.5	100mM	1 ml
$MgCl_2$	100mM	
KCl	200mM	

The contents of all the buffers used during SDS- and Native-PAGE are shown in table 2.6. The buffers were all prepared in higher concentrations and aliquots were diluted the day of the respective procedure. Ultrapure H_2O was the solvent used for all the buffers, and they were stored at 4°C.

Table 2.6: Buffers used for the SDS- and Native-PAGE procedures.

Chemicals	Concentrations in procedure	Prepared volume	Prepared concentrations	Amount of stock chemicals
SDS-PAGE running buffer (pH 8.4)				
Tris	25 mM	100 ml	250 mM	3.03 g
Glycine	192 mM		1920 mM	14.41 g
SDS-PAGE sample buffer (pH 6.8)				
Tris	62.5 mM	5 ml	250 mM	1.25 ml 1M Tris-Cl
Glycerol	10% w/v		40% w/v	2 g
SDS	2% w/v		8% w/v	0.4 g
Bromophenol blue	0.001% w/v		0.004% w/v	0.02 g
DTT	3.125 mM		12.5 mM	80 µl 1M DTT into 920 µl prepared sample buffer just before use
Native-PAGE anode running buffer (pH 6.8)				
Bis Tris	50 mM	1000 ml	1000 mM	209.2 g
Tricine	50 mM		1000 mM	179.2 g
Native-PAGE cathode running buffer (pH 6.8)				
Bis Tris	50 mM	250 ml	A 500 ml 1x dilution was made from the Native-PAGE anode running buffer, followed by adding 200 µl 10% LDS	
Tricine	50mM			
LDS	73.5 µM			
Native-PAGE sample buffer (pH 7.2)				
BisTris	50 mM	10 ml	200 mM	0.418 g
HCl	pH adjusting		pH adjusting	- - - - -
NaCl	50 mM		200 mM	0.117 g
Glycerol	10 % w/v		40 % w/v	4 g
Ponceau S	0.001 % w/v		0.004 % w/v	0.4 mg

The composition of the Coomassie staining solution has been provided in table 2.7. Aluminum sulfate-(14-18)-hydrate was first dissolved in 600 ml ddH_2O, followed by adding ethanol and homogenizing. Coomassie was then added and dissolve, and lastly the ortho phosphoric acid. ddH_2O was then added to a final volume of 1L.

Table 2.7: Chemical composition of the Coomassie staining solution used in SDS-, Native- and BlueNative-PAGE.

System	% in Coomassie solution	Amount needed for 1 L of Coomassie solution
CBB-G250	0.02 % (w/v)	0.2 g
Aluminum sulfate-(14-18)-hydrate	5 % (w/v)	50 g
Ethanol (95 %)	10 % (v/v)	105.4 mL
Ortho Phosphoric acid (85 %)	2 % (v/v)	23.5 mL

2.1.4 Gel casting

Polyacrylamide gels were prepared. For every preparation, three gels were made and store at 4°C for later use. The composition of the two gel types have been presented in tables 2.8 and 2.9.

Table 2.8: Recipe for the preparation of SDS-PAGE gels.

12% Separation gel	3 gels	4% stacking gel	3 gels
ddH_2O	5.922 ml	ddH_2O	2.978 ml
1.5 M Tris (pH8.8)	4.5 ml	1.5 M Tris (pH6.8)	1.25 ml
10% SDS	180 µl	10% SDS	50 µl
30 % Acrylamide	7.2 ml	30 % Acrylamide	667 µl
TEMED	18 µl	TEMED	5 µl
10% APS	180 µl	10% APS	50 µl
Total volume	18 ml	Total volume	5 ml

Table 2.9: Recipe for the preparation of Native-PAGE gels.

7.5% Separation gel	3 gels	4% stacking gel	3 gels
ddH_2O	10.2 ml	ddH_2O	1821 µl
1.5 M Tris (pH8.8)	5.25 ml	1.5 M Tris (pH6.8)	750 µl
30 % Acrylamide	5.25 ml	30 % Acrylamide	399 µl
TEMED	18 µl	TEMED	3 µl
10% APS	180 µl	10% APS	18 µl
Total volume	21 ml	Total volume	3 ml

2.2 Methods

2.2.1 Mitochondrial preparation from beef hearts and livers

The beef hearts were acquired fresh from Idsøe AS 1 day after slaughter, and the beef liver was purchased frozen from Meny AS. This protocol was inspired from Archie L. Smith's protocol from 1967 [18]. The entirety of the procedure was done at 4°C.

The beef heart/liver was trimmed for all fat and connective tissue with a meat knife. 300 g of meat was then sliced into approximately 1 cm cubes and placed in a 1 L plastic beaker. The sliced meat was further minced inside the beaker with a pair of scissors. 400 ml of 0.25 M sucrose and 0.01 M Tris-Cl (pH7.8) was then added. The pH of the mixture would drop to levels of 5.5 which may have been harmful to the mitochondria, so the pH was adjusted to 7.5 with 2 M unneutralized Tris while continuously stirring. The neutralized minced meat was then placed in a double layered cheesecloth and the solution was carefully squeezed out and discarded.

200 g of the neutralized mince was transferred into a new 1 L beaker and 400 ml 0.25 M Sucrose, 0.01 M Tris-Cl (pH7.8), 1 mM Tris-succinate, and 0.2 mM EDTA (hereby referred to as sucrose solution) was added. 3 ml 2 M unneatralized Tris was pipetted into the mixture, followed by blending the mixture with a hand-blender for 15 seconds. 1 ml more 2 M unneatralized Tris was then added before continuing to blend for 5 seconds. The pH was then adjusted to 7.8 using 2M unneatralized Tris.

The resulting homogenate was equally transferred into two 400 ml centrifuge containers which were compatible with the F10S-6x500y rotor for the Sorvall RC 6+ Centrifuge. A centrifugation was done at 1200 x g for 20 minutes to remove unruptured tissue and nuclei. The supernatant from both containers were then carefully decanted through a double layered cheesecloth into a 1 l beaker. pH was adjusted to 7.8 with 2 M unneutralized Tris, and the solution was then aliquoted into six 50 ml centrifuge tubes compatible with the HB-6 rotor for the Sorvall RC 6+ centrifuge. A new centrifugation was done at 26000 x g for 15 minutes. When the centrifugation was finished, the rest for the filtered supernatant was transferred into two more tubes and the centrifugation was done on them as well.

Three layers of pellet would appear after the centrifugation. The upper layer would be light brown and would contain damaged mitochondria. The middle layer would be dark brown and would contain intact mitochondria, and the bottom layer would be a tiny black brown button which would contain ruptured nuclei. 25 ml of supernatant from each tube was decanted, and the light brown pellet was carefully resuspended using a small paint brush. The rest of the supernatant was discarded, and the remaining pellet in one tube was resuspended in 10 ml sucrose solution. The resuspension was transferred into the next tube, and the pellet was resuspended. This was repeated until all pellets were resuspended in the same sucrose solution. The solution was then transferred into a tight-fitting glass-Teflon homogenizer (clearance 0.006 inch) and was homogenized by slowly passing the rod 2-3 times up and down with a slight rotation.

Sucrose solution was added to the homogenate to a total volume of 180 ml, followed by aliquoting the solution equally into four 50 ml centrifuge tubes. Centrifugation was then done at 26000 x g for 15 min. The supernatant was carefully discarded, and all the pellet were resuspended in 10 ml sucrose solution as described before. The resuspension was then homogenized and there was added more sucrose solution to a total volume of 60 ml. This solution was equally divided into two 50 ml centrifuge tube, and were centrifuged at 26000 x g for 15 min. The supernatant was discarded once more, and the remaining pellets were resuspended in 2 ml sucrose solution. The resuspension was homogenized and transferred in 200 µl aliquots into new 1.5 Eppendorf tubes. The samples that were not to be used immediately after were stored at -80°C.

2.2.2 Mitochondrial purification using a percoll gradient

To further purify the mitochondria after the preparation procedure in section 2.2.1, the product sample was sent through a percoll gradient with the help of an ultracentrifuge. This procedure was inspired from Wieckowski MR and crew's article from 2009 [26]. The entirety of this procedure was performed at 4°C.

The mitochondrial prepared sample was extracted from the -80°C freezer and thawed on ice. It was then filtered twice through a nylon sheath to get rid of any larger contaminants. 1 ml sucrose solution was also poured onto the nylon sheath after each filtration to ensure most mitochondria made it through. The flow through content was then centrifuged at 6300 x g for 10 min to pelletize the mitochondria. After discarding the supernatant, the pellet was resuspended in 5 ml sucrose solution. Next, 8 ml precooled 30% percoll solution was added to two 15 ml centrifuge tubes (tube code: 344061). 2 ml sample was then carefully pipetted into each tube, and approximately 3.5 ml sucrose solution on top. The sucrose solution was added while the tube was inside its corresponding rotor container and placed on a scale showing 4 decimal points to ensure that the final weight of the two components would be sufficiently equal. The components were then mounted onto the rotor, and the rotor was places inside the ultracentrifuge. Centrifugation was done at 95000 x g for 30 min. The band of functional mitochondria would have traveled furthest down the percoll gradient and was extracted by pipetting and discarding the above solution, followed by carefully transferring the mitochondrial band into a 1.5 ml Eppendorf tube. The content was pelleted through centrifugation at 6300 x g for 10 min. The supernatant was discarded, and the pellet was resuspended in 1 ml sucrose solution. The last washing step was performed twice more to remove any residual percoll from the sample.

2.2.3 Counting mitochondria

A coverslip was placed gently on a Brand™ Bürker Counting Chamber. 10 µl of sample was pipetted gently under the coverslip. The Bürker chamber was then studied under an Olympus CKX41 microscope and the mitochondria within the shaded region on the gridded area in figure 2.1 were counted. By deducing the average count from all five squares and multiplying by 10, the concentration of mitochondria/µl was deduced.

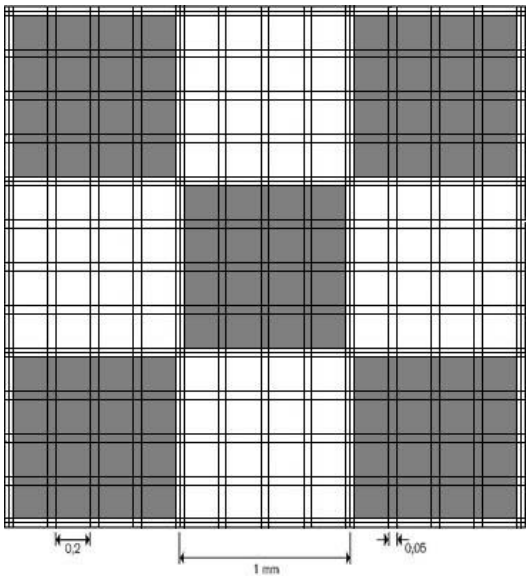

Figure 2.1: Picture indicating which region of the gridded area on a Brand™ Bürker Counting Chamber were used to deduce the concentration of mitochondria.

2.2.4 Protein Precipitation

To determine the protein concentration within the obtained mitochondria the proteins themselves had to be isolated. Protein precipitation was one of two methods used for this purpose. This protocol was based on isolating proteins through dissolution using a strong acid. Protein precipitation was also performed on pelleted chlorophyll as a positive control. This procedure was inspired from the 2018 lab course manual in protein biochemistry from the Unisversity of Stavanger[27].

Two pelleted sample tubes of mitochondria/chlorophyll were extracted from the -80°C freezer and thawed on ice. Each pellet was resuspended in 100 µl of ddH_2O by carefully pipetting in the liquid and using the tip of the pipette to get loosen the pellets from the tube walls. The pellets were then smeared gently against the side of the tubes to spread the pellets into a greater surface area. Finally, the pellets were resuspended by slowly pipetting the solutions up and down.

The volumes of the resuspended solutions were determined using a pipette. Next, half of the resuspended content from one of the tubes was transferred into a new tube. Then half of the transferred solution was pipette further into yet another tube. Subsequently, ddH_2O was added to all tubes to obtain a final volume of 100 µl. There were now 4 tubes with resuspended solutions, having dilution factors: 1:1, 1:2, and two 1:4.

Furthermore, acetone and TCA was added to all samples so that the volumetric ratio became 1:1:8 (sample:TCA:Acetone). Acetone was added first, followed by TCA, inverting the tubes after each step. The samples were then left to precipitate at -20°C for 1 hour, after which they were centrifuged at 18000 x g for 15 min at 4°C. The supernatant was removed, leaving the protein pellet intact. The pellets were then washed with 100µl ice cold acetone. This was done by carefully overlaying the acetone and carefully pipetting the acetone up and down, until the pellets were completely homogenized. The samples were then centrifuged again at 18000 x g for 15 min at 4°C, and the supernatant was discarded. The pellets were washed and centrifuged twice more as described, and then placed under a hood to let all the acetone evaporate out of the tube. Finally, the resulting pellet was resuspended in 60µl ultrapure water.

2.2.5 Protein extraction using RIPA-Buffer

The second method for isolating proteins from the mitochondrial pellets was with the use of RIPA-buffer. The principle behind this procedure was to disrupt and break open lipid membranes and extract the proteins within into the solution. This procedure was inspired from Thermo Scientific [28].

One pelleted sample was extracted from -80°C and thawed on ice. 500 µl RIPA buffer was then added, and the pellet was resuspended by gently pipetting the solution up and down. The sample was incubated on ice for 15 min, dragging the tube back and forth through the ice every few minutes. This was followed by centrifuging the sample at 14000 x g for 15 min to pellet the lysed debris. Finally, the supernatant was transferred to a new Eppendorf tube for further analysis.

2.2.6 Bicinchoninic acid (BCA)

For this procedure, the BCA kit from Bio Rad was used.

A concentration series of BSA was prepared as shown in table x. Appropriate dilutions were then made from the protein isolated samples acquired from the protein precipitation/RIPA buffer procedure. Next, a total amount of samples, including the standards, were deduced. This was followed by making a working reagent (WR) with reagent A and reagent B from the BCA kit. 200 µl of WR was to be added to each sample, and the ratio between the two reagents was 50:1 (reagent A:reagent B). 25 µl of all samples were transferred into separate well in a 96 well microtiter plate. The WR was then added to into all occupied wells, and the plate was thoroughly shaken for 30 seconds before being covered with aluminum foil and incubated at 37°C for 30 min. The plate was then given time to cool down for 5 minutes before measuring the absorbance of all samples at 562 nm using the Spectramax® Paradigm® multi-mode microplate reader from Molecular Devices.

Table 2.8: Overview of the different standard (BSA) solution that were to be prepared for the BCA assay, showing concentration, final volume, amount of BSA stock solution, and amount of RIPA buffer for each dilution.

BSA solution	BSA µg/µl	Final volume µl	BSA Stock solution µl	RIPA buffer µl
A	2.0	300	300	0
B	1.5	500	375	125
C	1.0	650	325	325
D	0.75	350	175 of vial B	175
E	0.5	650	325 of vial C	325
F	0.25	650	325 of vial E	325
G	0.125	650	325 of vial F	325
H	0.025	500	100 of vial G	400
I (Blank)	0	400	0	400

2.2.7 Mitochondrial preparation from HL-60 cultured cells

HL-60 cells were cultured using standard aseptic techniques. The culture medium used was Roswell Park Memorial Institute (RPMI) 1640 with 1% L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin. The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. Mitochondrial isolation was performed when there were at least $3x10^7$ cells to work with. This protocol was inspired from Heidi A. Rossow and crew's article from 2018 [19].

On collection day, the HL-60 cells were counted using a Muse® count and viability assay kit, Muse® Cell Analyzer from MERCK. A volume corresponding to $3x10^7$-$5x10^8$ cells was then transferred into a 50 ml falcon tube using a pipette. The cells were spun down at 900 rpm for 5 min at 4°C, and the supernatant was aspirated. 20 ml ice cold Phosphate-buffered saline (PBS) was then added and the cellular pellet was resuspended. A new centrifugation was performed at 800 x g for 10 min at 4°C, subsequently discarding the supernatant. The last washing step was done once more, only before the centrifugation, a 500 µl extract was collected into a 1.5 ml Eppendorf tube to be used for protein quantification. The main cluster of cells were left pelleted with the supernatant until the protein amount was determined. For this, the proteins were isolated with RIPA buffer, and the proteins were quantified using the BCA protocol described in sections 2.2.5 and 2.2.6 respectively.

Once the protein quantification was completed the supernatant in the tube with the main cell cluster was discarded. The pellet was then resuspended in PBS with 1x protease inhibitor to a volume that created a 5mg/ml protein concentration. The resuspension was divided into 400 µl aliquots and transferred into 1.5 ml Eppendorf tubes. Some of these aliquots were stored at -20°C and could be used further in the following days. The mitochondria were then freed from the cells in the remaining tubes by adding 400 µl 3.3mM digitonin and incubated for 5 min on ice. This was followed by adding PBS with protease inhibitor to a final volume of 1.5 ml, and the tubes were centrifuged at 10000 x g for 10 min at 4°C to pelletize the mitochondria. The supernatant was discarded, and the pellet was resuspended in 200 µl mitochondrial buffer. Next, 10% lauryl maltoside (β-DDM) was added to each tube to a final concentration of 1%-3%. The mixture was then incubated from 15 min on ice, followed by a centrifugation at 20000 x g for 20 min at 4°C. The supernatants were collected into new 1.5 ml Eppendorf tubes.

2.2.8 Gel casting

SDS- and Native-PAGE gels were cast using the Bio Rad gel casting apparatus and were made with a 1.0 mm gel width. The method for this procedure has been given by Julie Nikolaisen from the University of Stavanger.

The glass chamber plate and cover plate were clamped together and mounted on a stand with a sponge sealing the bottom opening. ddH_2O was poured into the chamber to ensure no leakage from below. The water was then removed, and a comb was placed on the top opening. A mark was made on the outside glass about 0.5 cm below the bottom tip of the comb to show the transition point between the stacking and separating gels. The comb was subsequently removed, and the separating gel was the first to be added into the chamber. While preparing the gel mixture, TEMED and APS were the last substances to be added as these are the triggers for the polymerization of the acrylamide. After having added the separating gel to the marked point on the glass, isopropanol was pipetted on top to even out the gel. The separating gel was left to solidify for at least 15 min before the isopropanol was thoroughly removed and the stacking gel was added. Finally, the chamber was sealed by reinserting the comb. The gels were wrapped in soaked wiping paper and stored at 4°C.

2.2.9 Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

This method was inspired from the 2018 lab course manual in protein biochemistry from the Unisversity of Stavanger [29].

An SDS-gel was extracted from the refrigerator. Any remaining acrylamide was removed from the glass using filter paper, and the comb was removed. The gel was assembled onto the upper electrophoresis tank, facing the cover plate inwards into the chamber of the tank. The upper tank was then mounted to the bottom tank. 10x SDS running buffer was diluted down to 1x with ddH2O, and subsequently added to both the upper (cathode) and bottom (anode) tanks, making sure that the wells of the gel were filled with buffer. Finally, remaining polymers within the wells were carefully flushed out, avoiding damaging the gel spacers between each well.

22.5 µl of each protein sample was transferred into new 1.5 ml Eppendorf tubes. 7.5 µl 4x sample buffer (SB) was added to each tube, creating a 1x SB solution. The contents were then carefully mixed by pipetting up and down. Next, all samples were heated at 70°C for 2 min. This was followed by a centrifugation step at 16000 x g for 10 min at room temperature (RT) to sediment any contaminants. 20 µl of each sample, as well as 5 µl standard marker was loaded into individual wells. The safety lid was placed on the tank and the color-coded leads were properly attached to the voltmeter. The power supply was set to 150 V constant, with mA and W at max, and no time limitation. The electrophoresis was started and ran until the front had migrated to the bottom of the gel. The power was then stopped, and the gel was extracted from the tank. The glass plates were removed from the gel.

The exposed gel was placed on a tray filled with 100 ml ddH_2O. The tray was then placed on a shaker for 10 min, followed by discarding the water. This washing step was performed twice more. Next, 60 ml Coomassie staining solution was added to the tray, and the tray was left on the shaker for at least 1 hour (could also be left overnight). The Coomassie solution was then discarded into its own waste bottle, and the gel was washed thrice with 100 ml ddH_2O on the shaker for 10 min each time. Finally, the gel was scanned with an Odyssey scanner, using lasers with wavelengths 685 nm and 785 nm .

2.2.10 Solubilization

This method was inspired from the 2018 lab course manual in protein biochemistry from the Unisversity of Stavanger [30].

A tube of pelleted mitochondria was extracted from the -80°C freezer and thawed on ice. The pellet was resuspended in 30 µl 1x TMK and incubated on ice for 10 min. 4 µl 10% β-DDM, and 1 µl ddH_2O was subsequently added to the tube to make up a total volume of 40 µl. The sample was then centrifuged at 30000 x g for 30 min at 10°C, and the supernatant was transferred into a new Eppendorf tube. During this step it was important not to disrupt the pellet. Hence, a small volume of supernatant was left behind and discarded along with the pellet. 4x Native-SB was added to the supernatant to make a final 1x concentration in the sample solution. The tube was carefully snipped to mix the content evenly.

2.2.11 Native-PAGE

For the identification and isolation of native protein complexes within the mitochondria, Native-PAGE was performed. The entire procedure was executed at 4°C. The preparation of the Native-PAGE apparatus was done during the centrifugation step of the solubilization procedure. This method was inspired from the 2018 lab course manual in protein biochemistry from the Unisversity of Stavanger [31].

The prepared native-gel was extracted from the refrigerator. Any remaining acrylamide was removed from the glass surface with filter paper. The comb was carefully removed, and the gel was assembled to the upper tank module. A dummy plate was placed on the opposite side to seal the tank. Having then mounted the upper tank with the bottom tank, the running buffers were diluted, and poured into their respective tanks (cathode buffer in upper tank, and anode buffer in bottom tank). 20 µl for each sample; as well as 5 µl standard marker, was subsequently added to individual wells. The lid was mounted, and the color-coded leads were correctly plugged into the voltmeter. Settings were set at 150V constant (20V when run overnight), mA at max, and W at max, and the tank was kept in a styropor box cooled with cooling pads. The Native-PAGE ran for 3-4 hours (12-14 hours overnight), after which the gel was extracted from its glass entrapment and placed in a plastic tray filled with 100 µl ddH_2O. The gel was then washed, stained, and scanned as described in section 2.2.8.

2.2.12 Spectrophotometry

To gain better insight into what was present in the acquired mitochondrial prepared samples during this project, spectrophotometric scans were perform on a UV-2401 PC recording spectrophotometer. For this, an absorbance spectrum from wavelengths 190nm-700nm was observed. 300 µl sample was added to a micro quartz cuvette with a 1 mm slit width and a 10 mm light path. Baselines were chosen to be the solutes in the individual samples that were tested. The cuvette was washed with ddH_2O and acetone between each scan.

2.2.13 Immobilizing suspended HL-60 cells

In order to stain the suspended HL-60 cells they had to become immobilized. This was performed using Cell-Tak. Aseptic techniques were used while doing this procedure and all incubations were done at 37°C in a 5% CO_2 humidified atmosphere. This method, as well as methods sections 2.2.14 and 2.2.15 were provided by Tia Tidwell from the University of Stavanger.

280 µl 0.1 M sodium carbonate at pH 8.0 was pipetted into a 1.5 ml Eppendorf tube. Cell-Tak was then extracted from 4°C, and 20 µl was added to the tube. The solution was mixed gently by pipetting up and down, making sure not to create any bubbles while doing so. This was swiftly followed by pipetting 25 µl of the mixture into 10 wells of a 96 well plate. The plate was gently shaken to ensure the entire surfaces of the wells were covered with solution, and was then incubated 45 min. Next, the Cell-Tak solution was aspirated and the wells were rinsed twice with 100 µl prewarmed sterile water, discarding the water after each wash. The wells were subsequently filled with 100 µl cell culture containing 50000 HL-60 cells and incubated for 2 hours to permit the cells to settle and stick to the Cell-Tak coating on the well surface.

2.2.14 Staining mitochondria with TMRM and Mitotracker Deep Red (DR)

The HL-60 cultured cells as well as some intermediate samples from the mitochondrial preparation procedure were stained with TMRM and Mitotracker DR. In this procedure one sample was left untreated, and another was additionally treated with CCCP to act as negative and positive controls respectively. After the Cell-Tak immobilization procedure in section 2.2.13, the HL-60 cells were ready to be stained. To bring the biproduct samples from the mitochondrial extraction to the same stage, they were first pipetted in 100 µl aliquots into wells of a 96 well plate and subsequently centrifuged in a microplate centrifuge at 2000 x g for 10 min at 4°C.

The staining solution was then prepared by adding 1 µl 100 µM TMRM, 5 µl 1mM Mitotracker DR, and 1 µl 1mg/ml Hoechst into 1 ml of a relevant buffer solvent for each sample (RPMI 1640 for the cells, and mitochondrial buffer for the biproduct samples). The solutions in all wells were carefully aspirated, and 50 µl of staining solution was added to each well. The samples were then incubated at 37°C in a 5% CO_2 humidified atmosphere for 15 min. Finally, the samples were analyzed using a Leica TCS SP8 CSU confocal microscope. TMRM was scanned with a laser line at 552nm at 1% intensity, and a PMT detector from 557nm -631nm; Mititracker DR was scanned with a laser line at 638 nm at 1% intensity, and a HyD3 SMD detector from 648nm - 784nm; Hoechst was scanned with a laser line 405 nm at 4% intensity, and a HyD1 detector from 410nm – 531nm

2.2.15 Staining mitochondria with TOMM20

The samples, from the HL-60 mitochondrial extraction procedure, were also stained with TOMM20 antibodies. After aliquoting and centrifuging the samples as described in section 2.2.14, the supernatants were carefully aspirated. 100 µl methanol was then transferred to each well, and incubated at -20°C for 15 min, fixing the pelleted material from each sample to the surface of the wells. The methanol was subsequently removed, and the samples were gently washed three times with 100 µl PBS. The wells were then filled with 50 µl blocking buffer (BB) and left at room temperature (RT) for 2 hours. During this time, the TOMM20 antibody staining solution was prepared by making a 1:500 dilution of TOMM20 in BB. 50 µl staining solution then substituted the BB in each well, and the 96 well plate was covered with aluminum foil and left at 4°C overnight. Finally, the samples were analyzed using a Leica TCS SP8 CSU confocal microscope. TOM20 was scanned with a laser line at 488nm at 1% intensity, and a HyD3 SMD detector from 500nm - 720nm.

3. Results and Discussion

The aim for this thesis was to develop a method for extracting pure functional mitochondria. During this process a goal was also set to determine with certainty that mitochondria were present in the end-product. Beef heart was the first source for which mitochondrial extraction was attempted, as heart muscle were known to be rich with mitochondria and would therefore be a good source to start with.

3.1 Analysis of mitochondrial preparation sample from beef heart

A mitochondrial preparation was performed on a beef heart as described in methods section 2.2.1. One of the resulting aliquots was further diluted 1:100 in sucrose solution, followed by doing a mitochondrial count as described in methods section 2.2.3. An image taken right outside the gridded area of the Bürker chamber has been provided in figure 3.1. The small dark spots spread around the image were presumed to be mitochondria, while the larger light accumulated objects seemed to be contaminants of some sort. Having finished the mitochondrial count it was determined that there were 2.25×10^7 mitochondria/ml in the non-diluted sample solution.

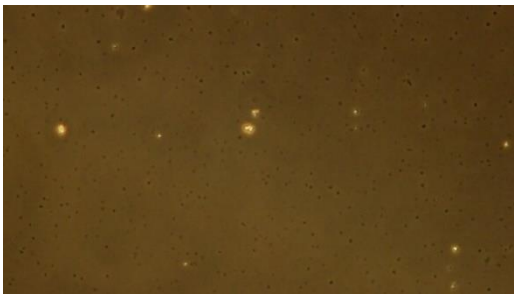

Figure 3.1: Image from the Olympus CKX41 microscope showing contents from the 1:100 diluted mitochondrial preparation sample from beef heart.

Having determined a crude concentration on what seemed to have been mitochondria in the prepared sample aliquots, the next step was an SDS- and Native-PAGE to further strengthen the claim that the contents in the sample in fact were mitochondria. The SDS- and Native-gels were prepared as described in methods section 2.2.8. First off was the SDS-PAGE, and three mitochondrial prepared samples were thawed and used for protein isolation. For this, both the protein precipitation and RIPA buffer procedures were performed as described in methods sections 2.2.4 and 2.2.5 respectively. By performing both procedures, it could be deduced which of them gave the best outcome regarding protein yield. Ready isolated chloroplasts were also subject to both protein isolation procedures to act as positive controls. The products from both protein isolations were further used in a BCA assay, as describe in methods section 2.2.6, to quantify the amount of protein in each respective sample. The resulting absorption values from the BCA assay were analyzed, and the initial protein amount from the mitochondrial prepared aliquot relative to each sample was calculated. These calculations have been provided in appendix section 5.1. Table 3.1 shows the final calculated quantities.

Table 3.1: Calculated amount of mitochondrial and chloroplast samples treated with protein precipitation and RIPA buffer from BCA readings.

Samples treated with protein precipitation	Initial amount of protein (mg)	Samples treated with RIPA buffer	Initial amount of protein (mg)
Undiluted mitochondria	0.0120	Undiluted mitochondria	5.6471
1:2 diluted mitochondria	0.0200	Undiluted chloroplasts	0.8840
1:4 diluted mitochondria	0.0086		
1:4 diluted mitochondria	0.0048		
Undiluted chloroplasts	0.0217		

By looking at the calculated amount of proteins in the various samples, it was evident that a large quantity of product was lost during the protein precipitation procedure. All samples started off with the same number of mitochondria as each aliquot from the mitochondrial preparation procedure was equally distributed into new tubes. Therefore, it would be expected that the protein amount should have ended up the same for all the mitochondrial samples. Thus, from this point on, the RIPA buffer would solely be used for protein extraction, as this method of extraction seemed to yield a far greater amount of proteins.

The protein extracted samples were thereafter subject to an SDS-PAGE as described in methods section 2.2.9. The purpose of this was to determine which amounts of protein should be added to a well to obtain the clearest result. As 20 µl solution (among which 15 µl were protein sample) was added to each well the corresponding amount of proteins used in each sample have been shown in table 3.2. The gel scans taken after the SDS-PAGE has been provided in figure 3.2.

Table 3.2: Protein amount of each sample added to their wells for the SDS-PAGE.

Samples treated with protein precipitation	Amount of protein in well (µg)	Samples treated with RIPA buffer	Amount of protein in well (µg)
Undiluted mitochondria	3.0	Undiluted mitochondria	169.4
1:2 diluted mitochondria	2.6	Undiluted chloroplasts	26.5
1:4 diluted mitochondria	0.5		
1:4 diluted mitochondria	0.3		
Undiluted chloroplasts	1.9		

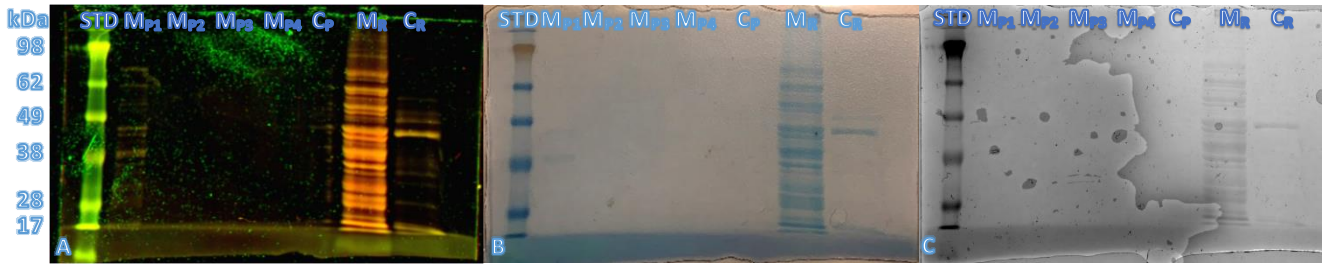

*Figure 3.2: SDS-PAGE gel showing bands of protein extracted from the product samples of the mitochondrial preparation from beef heart, using protein extractions from chloroplasts as positive controls. STD: Sea blue standard SDS marker, M_{P1}: undiluted mitochondrial prepared sample undergone protein precipitation, M_{P2},M_{P3},M_{P4}: respectively 1:2, 1:4, and 1:4 diluted mitochondrial prepared sample undergone protein precipitation, C_P: undiluted chloroplast sample undergone protein precipitation, M_R: undiluted mitochondrial prepared sample undergone protein extraction by RIPA buffer, C_R: undiluted chloroplast sample undergone protein extraction by RIPA buffer. **A)** Image from Odyssey scanner, **B)** White light image from camera, **C)** Image from Chemidoc scanner*

From the SDS-gel scans it could be seen that the protein precipitated samples had too low a concentration to obtain any clear readings. The samples treated with RIPA buffer however, were more promising. The mitochondrial prepared sample produced a variety of clear bands throughout the separation gel, although many bands were quite thick, suggesting the amount of proteins within the well was too high. From this, it was determined that an approximate amount of 80 µg proteins per well would be sufficient to obtain clear separation of bands in a gel.

The next step was to strengthen the claim of there in fact being mitochondria within the mitochondrial prepared samples. This would be done by performing a Native-PAGE. Two new aliquots from the mitochondrial preparation procedure were solubilized, as described in methods section 2.2.10, to extract native proteins from its contents. The two samples were split equally in two and each were treated with different concentrations of β-DDM (1.25%, 1.0%, 0.75%, and 0.5% (v/v)). A sample of chloroplasts was also solubilized, and treated with 1.0% β-DDM, to act as a positive control. The solubilized products were then used for a Native-PAGE as described in methods section 2.2.11, and the resulting gel images has been provided in figure 3.3.

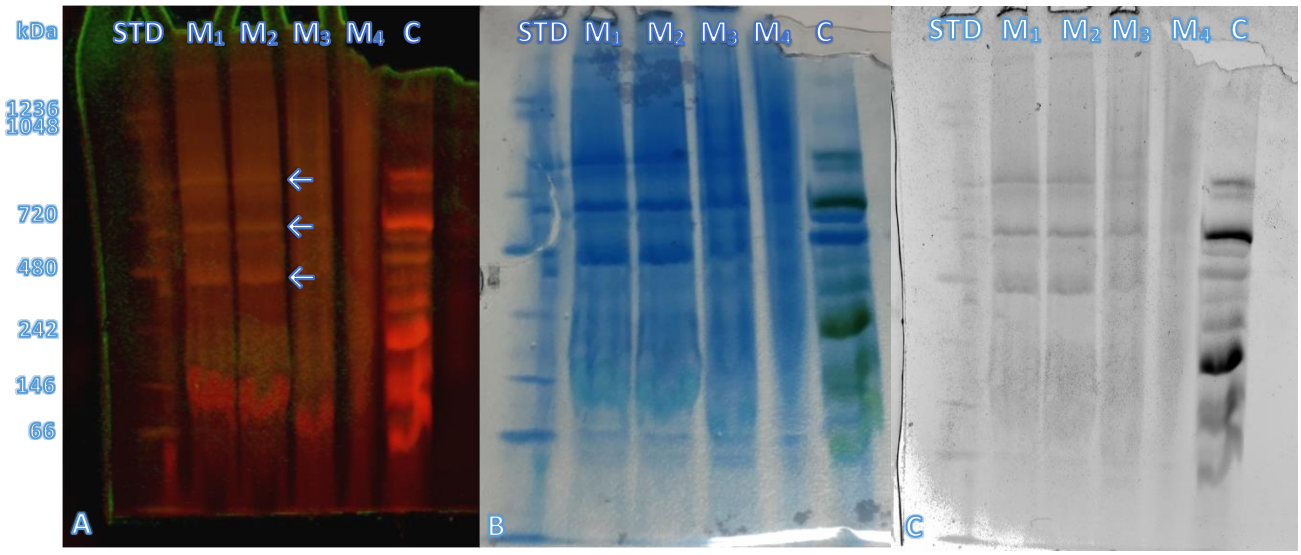

*Figure 3.3: Native-PAGE gel showing native protein bands extracted from the product samples of the mitochondrial preparation from beef heart, using native protein extractions from chloroplasts as positive control. STD: NativeMark, M_1, M_2, M_3, & M_4: Mitochondrial prepared sample from beef heart treated with 1.25%, 1.0%, 0.75%, & 0.5% β-DDM Respectively. **A)** Image from Odyssey scanner, **B)** White light image from camera, **C)** Image from Chemidoc scanner*

There were several bands present in all the mitochondrial samples. Lane M_2, containing the sample treated with 1% β-DDM, contained the most pronounced subfractionation of sample content. It was therefore chosen to be the best representation of what the mitochondrial sample contained. Three prominent bands stood out from among the rest (figure 3.3A). By comparing these band locations with that of the NativeMark (STD), their molecular weights were determined to range from ~400–900 kDa. If this was to represent mitochondrial protein complexes I (~980nm) , III (~600nm), and V (~750nm), it would seem that the complexes have lost a few subunits. If this was the case, judging by the fact that the protein bands present in the different lanes are aligned, it could mean that the mitochondria from the sample were damaged during the extraction process.

Although this Native-gel gave indications of the presence of mitochondria within the prepared sample, it could also be seen that the sample contained a fair amount of contamination, judging by the pronounced Coomassie stained regions throughout the entire lengths of the sample lanes. With a purified sample, the lane should contain little more that the protein separated bands as could be seen from the chloroplast control sample. In addition, the mitochondrial M_2 lane showed a band present at the threshold between the stacking and separating gels, indicating that there were larger substances in the sample being unable to pass into the pours of the 7.5% polyacrylamide separating gel. At this point, a decision could have been made to go forth with second dimension (2-D) SDS- or Native-PAGE to further sub fractionate the existing bands and investigate their monomeric composition. However, with the present contamination it would probably be difficult to acquire any reliable intel from the results. It therefore seemed more important to acquire a gel sample with less contamination, to make the results more reliable.

To address the issue of contamination, a new aliquot of mitochondrial prepared sample was extracted from -80°C. The pellet was resuspended in 100 µl sucrose solution and filtered through a 20-micron nylon sheath in an attempt to remove any larger contaminants. The flow-through product was then diluted 1:100, and a spectrophotometric scan of the sample was performed as described in methods section 2.2.12. This was done to gain insight into the content of the sample, both to detect signs of mitochondrial proteins, and any clear forms of contamination. The initial scan of the sample (not supplied) showed a high level of polysaccharide contamination, probably due to the sucrose solution the sample was suspended in. The solvent was changed to PBS and the sample was scanned once more, providing the absorption spectrum shown in figure 3.4.

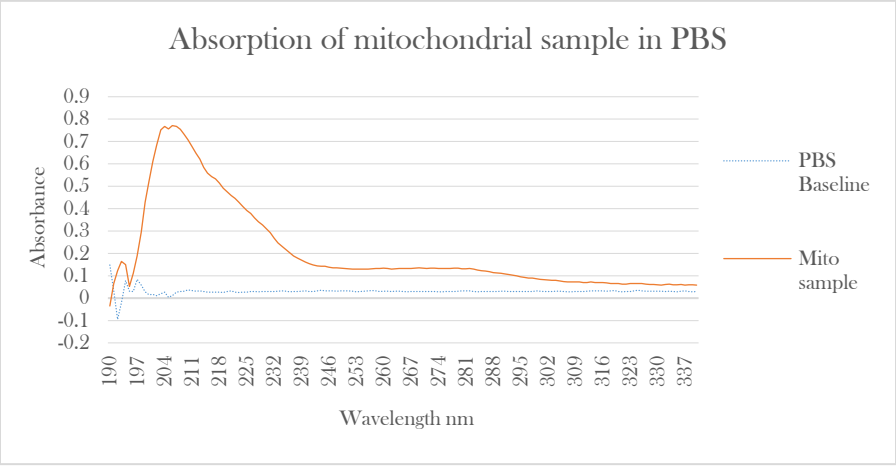

Figure 3.4: Spectrophotometric scan of a 1:100 diluted mitochondrial prepared sample from 190-340 nm, using the solvent PBS as a baseline.

Within the obtained absorbance spectrum there was firstly observed a large peak at ~205nm. This was presumably caused by the peptide bonds from the proteins within the sample. Other less obvious peaks were also noted at ~215nm, ~230nm, and ~280nm. This could indicate the presence of DNA within the sample.

After the spectrophotometric scan, the sample was retrieved and treated with RIPA buffer, as described in methods section 2.2.5, and was subsequently subject to a BCA assay, as described in methods section 2.2.6, to determine the protein concentration. During the BCA assay, the BSA standard from SIGMA™ was used in the interest of proceeding more economically. However, the resulting absorbance values of the BSA standards (appendix 5.1.2) were too poor to create a reliable standard curve. This may have been due to a poor preparation of the BSA dilution series, but it was also later informed that the BSA product was crude and impure. Thus, the BSA ready-made standard solution from BioRad was used henceforth.

3.2 Analysis of mitochondrial preparation sample from beef liver

The availability of beef heart had become scarce, resulting in exchanging the source of mitochondrial extraction to beef liver. A mitochondrial preparation was performed, as described in methods section 2.2.1, during which there were noticeable differences in behavior between the beef liver and the beef heart used previously. After the centrifugation step at 1200 x g while using the beef heart, there was a clear distinction between pellet and supernatant. However, at det same point for the beef liver, the supernatant was murky, and the pellet was still runny. Consequently, an additional filtration was performed, comprised of pouring the supernatant onto a new double layered cheese cloth, to accomplish a further isolation between the supernatant and the sludgy pellet. Although most of the pellet was hindered into the supernatant solution, the next centrifugation at 26000 x g showed that a fair amount passed through anyway. A comparison between processed beef heart and beef liver at this stage in the procedure has been presented in figure 3.5.

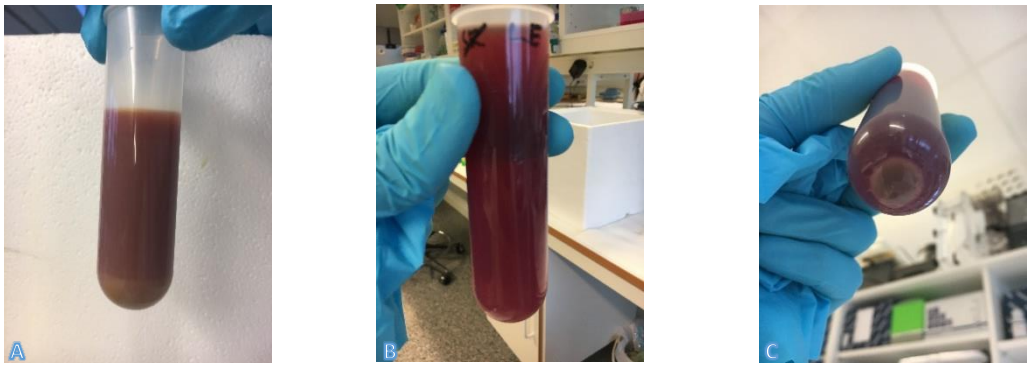

Figure 3.5: Images taken after the 26000 x g centrifugation from the mitochondrial preparation procedure from beef heart and beef liver. A) Result from beef liver. B) Result from beef heart. C) Shot taken from underneath tube with the beef heart sample.

By comparing the two samples at this stage of the procedure, it could be seen that the pellet from the beef liver (figure 3.5A) was considerably larger than that from the beef heart. Also, the supernatant from the beef liver seemed a lot murkier, suggesting that tube contained a significant amount of contamination. However, considering that the mitochondria in the sample were thought to represent the dark brown segment at the bottom of the pellet, the brighter pelleted matter was carefully resuspended into the supernatant, and discarded. The resulting pellet from the beef liver after the final centrifugation step at 26000 x g has been shown in figure 3.6.

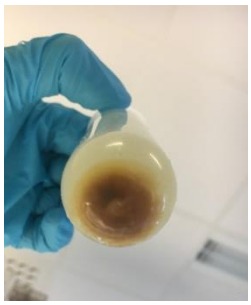

Figure 3.6: Final pellet after the last centrifugation step at 26000 x g in the mitochondrial preparation from beef liver.

Even though the end-product from this mitochondrial preparation looked more promising than that of the intermediate samples during the procedure, it could still be assumed that the product contained contamination. To purify the product further, the sample was sent through a 30% percoll gradient with the help of an ultracentrifuge as described in methods section 2.2.2. The resulting sedimentation has been provided in figure 3.7.

Figure 3.7: Mitochondrial prepared sample from beef liver sedimented through a 30% percoll gradient.

The bottom sedimented band, presumably containing mitochondria, was extracted, and washed in sucrose solution. It was later diluted into 1:1, 1:4, 1:8, and 1:16 aliquots of 300 µl and scanned in the spectrophotometer to check for any residual contaminants. The scan results have been provided in figure 3.8.

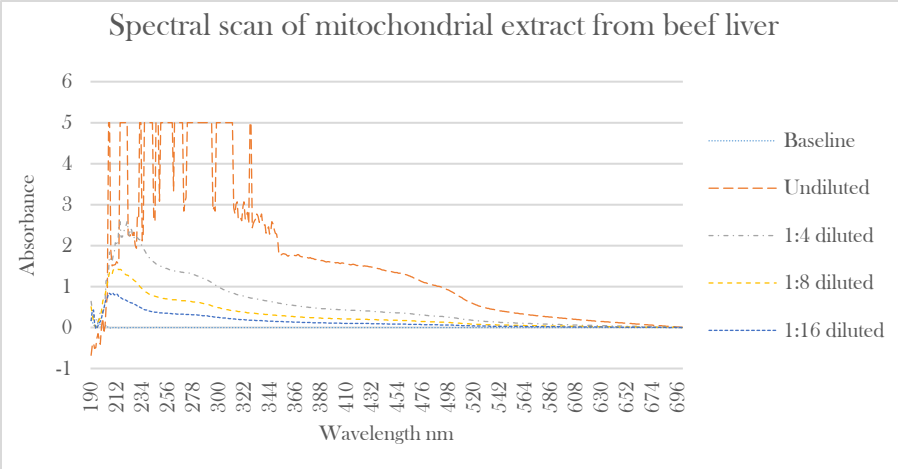

Figure 3.8: Spectral analysis of the mitochondrial prepared sample from beef liver purified in a 30% percoll gradient.

The 1:4 diluted sample of the purified mitochondrial preparation was chosen to be subject to a Native-PAGE at different concentrations. 1, 2, 3, 5, and 10 µl sample was transferred into separate tubes, followed by adding TMK to a total volume of 35 µl The diluted samples were then solubilized as described in methods section 2.2.10, treating with 1% β-DDM as this had been observed earlier to produce the best protein extraction. The Native-PAGE was run at 150V at max mA and W for 3 hours, followed by staining the gel with Coomassie. The resulting gel has been presented in figure 3.9.

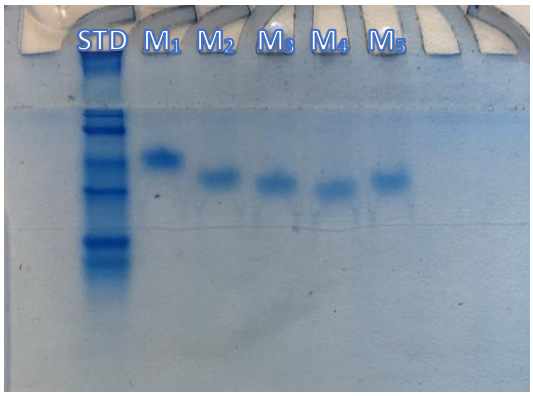

Figure 3.9: Coomassie stained Native gel containing diluted aliquots of solubilized 1:4 diluted mitochondrial prepared sample from beef liver. STD: NativeMark, M_1, M_2, M_3, M_4, & M_5: respectively 1, 2, 3, 5, 10 µl extracted volumes from of sample before solubilization

By looking at the gel it was clear that something was not right. Each sample lane contained one thick band. Judging from the fact that the substance in the gel seemed to be more concentrated in the sample containing less purified mitochondrial prepared product (M_1), and that there looked to be a trend where less initial product resulted in shorter travel through the gel, it was theorized that these bands could be accumulations of micellular β-DDM. If this was the case, it would make sense that the diluted samples did not have a high enough concentration, resulting in there being a large quantity of left over β-DDM in the loaded solutions. A decision was therefore made to perform a second Native-PAGE using the original undiluted sample of purified mitochondrial prepared sample. This time dilutions of 10, 20, 50 and 70 µl were made, pelletizing the sample before proceeding with the solubilization once more. The resulting stained gel has been provided in figure 3.10.

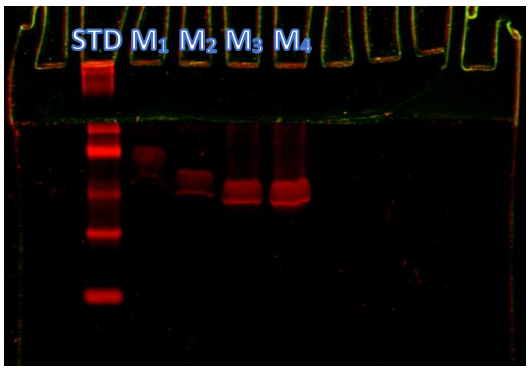

Figure 3.10: Coomassie stained Native gel containing diluted aliquots of solubilized undiluted mitochondrial prepared sample from beef liver. STD: NativeMark, M_1, M_2, M_3, & M_4: respectively 10, 20, 50, 70 µl extracted volumes of sample before solubilization

Unfortunately, the same curious result occurred with only one thick band appearing in all the sample lanes. At this point, it was very unlikely that the concentration of product within the last test was too low, seen as the initial mitochondrial sample prepared was not divided into aliquots before being purified, and that most of the mitochondrial extract was used in this Native-PAGE. It was equally unlikely that there was too much sample in the loaded solutions, as this would result in there being undigested matter accumulated at the bottom of the wells, not being able to enter the gel. Also, judging by the peaks at 190nm on the spectrophotometric scans in figure 3.8, there were indications of proteins being present the sample, leading the question of where these proteins ended up. Perhaps the β-DDM was ineffective in its role in digesting the sample content and extracting the proteins into the supernatant, causing the proteins to become trapped within the pellet which was unfortunately discarded during the solubilization process.

However, to test the theory of the β-DDM being ineffective, a new solubilization was performed on a pellet of pre-isolated chloroplasts. After having resuspended the pellet containing $6.7x10^7$ chloroplast in TMK, the sample was further diluted as shown in table 3.3. All six diluted samples of chloroplasts underwent solubilization with 1% β-DDM and were subject to a Native-PAGE, producing the gel results given in figure 3.11.

Table 3.3: Overview of the diluted samples of chloroplasts used for solubilization and Native-PAGE

Diluted sample name	Number of chloroplasts
C_1	$3.35x10^7$
C_2	$1.0x10^7$
C_3	$5.0x10^6$
C_4	$1.0x10^6$
C_5	$5.0x10^5$
C_6	$1.0x10^5$

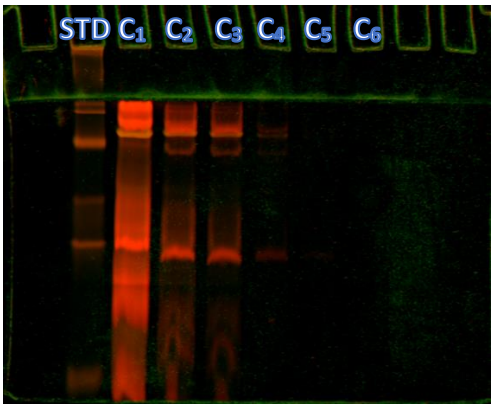

Figure 3.11: Native-PAGE of solubilized chloroplasts in various concentration treated with 1% β-DDM. STD: NativeMark, C_1, C_2, C_3, C_4, C_5, & C_6: respectively $3.35x10^7$, $1.0x10^7$, $5.0x10^6$, $1.0x10^6$, $5.0x10^5$, & $1.0x10^5$ chloroplasts in solubilization samples

By looking at the gel it could be deduced that the theory of β-DDM not performing protein extraction was false. Although not all the expected protein bands were present as did occur when the chloroplasts were used as control in figure 3.3. Because of this, a new Native-PAGE was performed in the interest of determining the optimal amount of β-DDM to acquire the best protein extraction. From figure 3.11 it was determined that a chloroplast number of $1.0x10^7$ was representative for a clear separation of protein bands.

Five chloroplast samples were solubilized with β-DDM concentrations of 2.0, 1.0, 0.5, 0.25, & 0.125%, and were subsequently run through a native gel. The resulting stained gel has been presented in figure 3.12.

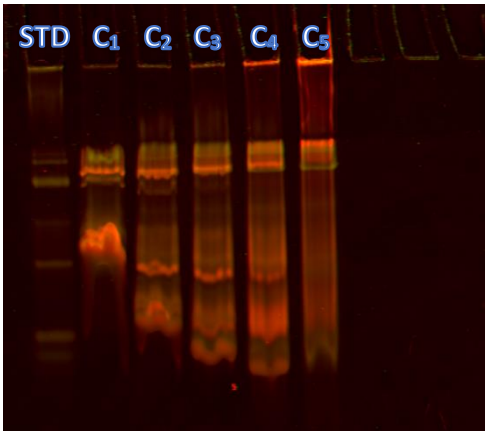

Figure 3.12: Native-PAGE of 1.0x10⁷ chloroplast samples solubilized with various concentrations of β-DDM. STD: NativeMark, C_1, C_2, C_3, C_4, & C_5: 1.0x10⁷ Chloroplast samples solubilized with 2.0, 1.0, 0.5, 0.25, & 0.125% β-DDM respectively.

There were a couple views to consider from the resulting gel to identify which concentration of β-DDM that gave to best extraction and separation of protein complexes. Firstly, it was the matter of accumulated substances still existing within the loading well (C_4 and C_5). This indicated that there was a shortage of β-DDM to solubilize the entire content of the sample, causing larger undigested matter to be completely stopped by the polyacrylamide gel. Next, it could be observed that too high quantities of β-DDM would cause there to be leftover micelles of detergent, which would accumulate and clog the pours in the gel, preventing smaller proteins from passing further (C_1 and C_2). The last consideration was the effectiveness of β-DDM to extract the largest variety of protein complexes. Having these criteria in mind, the lane which seemed to produce the best results was C_3 containing chloroplasts treated with 0.5% β-DDM. The lane presented minimal matter residing in the loading well, as well as it did not seem to be very affected by an excess quantity of detergent micelles clogging up the pours of the gel. At the same time, it presented an assortment of protein complexes.

At this point the choice was made to take what had been learned and move on to extracting mitochondria from HL-60 culture cells. Since this mitochondrial extraction process was more time consuming than initially expected, and it seemed like it would take a lot longer to obtained a pure mitochondrial extract from beef liver, it seemed more important gain new experience in extracting mitochondria from cultured cells.

3.3 Analysis of mitochondrial prepared samples from HL-60 cultured cells

A mitochondrial preparation procedure was attempted on the HL-60 cultured cells as described in methods section 2.2.7. The initial aim was to prepare a large amount of mitochondrial extract which was the sample obtained after the cells had been treated by digitonin in the procedure. Aliquots would then be frozen at -80°C to be used from later analysis.

At the day of cell extraction, the cell count was read to be $4.5x10^8$ cells in total, and so roughly $3.0x10^8$ cells were extracted to be subject to the mitochondrial preparation procedure. After the BCA assay, the protein quantity in the main cluster of cells was calculated to be 42.98 mg as shown in appendix section 5.1.3. The cellular pellet was therefore resuspended in 8.60 ml PBS with protease inhibitor to acquire a protein concentration of 5 mg/ml. Having subsequently made 450 µl aliquots of the resuspended cell solution and treated them all with 3.3 mM digitonin (from a 10 ml stock solution of 3.3 mM digitonin as there was so much material), a complication arose after removing the centrifuged supernatant and attempting to resuspend the pellets in mitochondrial buffer. The pellets had a soft rubbery consistency, being flexible but would not be homogenized in the mitochondrial buffer.

A theory to why the pellet would not be resuspended was that the cells had not been completely digested while treated with digitonin. Perhaps the concentration of digitonin was too low, leading to partly digested lipid membranes joining in a cluster to shield their hydrophobic segments from the hydrophilic solution. Another possibility could be that a 5-minute incubation with digitonin was too short, and that there was leftover digitonin in the centrifuged supernatant. Luckily, the supernatant had been kept on ice, so the latter theory could be tested. Two sample aliquots were centrifuged at 10000 x g for 10 min at 4°C and the mitochondrial buffer was aspirated from each tube. 1 ml of the previous supernatant was added back to both tubes and the pellets were detached from the tube walls. The samples were incubated on ice for 15 min, inverting the tube 3 times every 2 minutes. When the incubation was over, there did not seem to be any change in neither size nor consistency of the pellets, and after a new centrifugation and changing the solvent to mitochondrial buffer again, the pellet would still not be resuspended.

Judging by there not being any mention in the source article of this procedure about pellets neglecting to become resuspended, a new theory was made that perhaps a human error had occurred at some point. Because of this, the decision was made to perform the procedure once more. It was deduced from the previous procedural attempt that a far smaller quantity of cells was needed to obtain an appropriate number of sample aliquots. Therefore, a cell number of $6.24x10^7$ was harvested this time. The quantity of proteins within the cell sample was calculated from the BCA assay to be 15.81 mg, as shown in appendix section 5.1.4, and so the cellular pellet was resuspended in 3.16 ml PBS with protease inhibitor, producing seven 400 µl aliquot samples. One aliquot was further treated with digitonin as the protocol instructed, but the resulting pellet turned out the same, and would still not be resuspended in the mitochondrial buffer.

Despite this reoccurring complication, the procedure was resumed, and the sample was further treated with 1% β-DDM, in the hopes that this detergent would manage to break down the components of the pellet and extract the target native protein complexes. This was followed by performing a Native-PAGE on the resulting supernatant at different concentrations. The outcome of this Native-PAGE has been presented in figure 3.13.

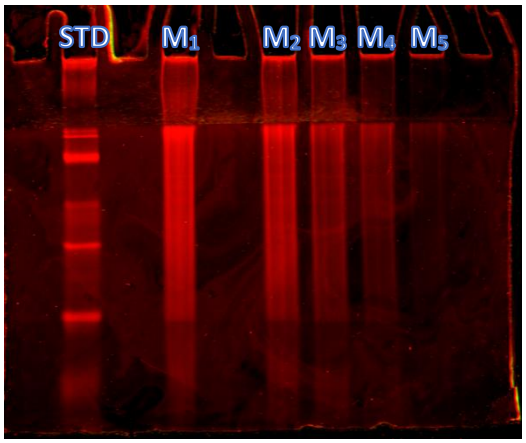

Figure 3.13: Native-PAGE showing lysed HL-60 cells treated with 1% β-DDM and loaded with various concentration of sample. STD: NativMark, M_1, M_2, M_3, M_4, & M_5: respectively 15, 10, 5, 2, & 1 µl sample in total 20 µl loading solution.

The sample lanes within the gel seemed to be containing a large quantity of contamination, which made it hard to identify any clear bands of protein. On the other hand, there did not seem to be any β-DDM left in the sample as there was no micellular accumulation clogging the acrylamide pours of the gel, as was the case with the chloroplasts shown in figure 3.12. Thus, a new treatment of 1% β-DDM was performed on the remainder 100 µl of the same sample aliquot. When preparing the loading sample solutions for a new Native-PAGE, calibrations were made to provide the same sample concentrations within each well. The resulting scan of the Native-PAGE has been shown in figure 3.14.

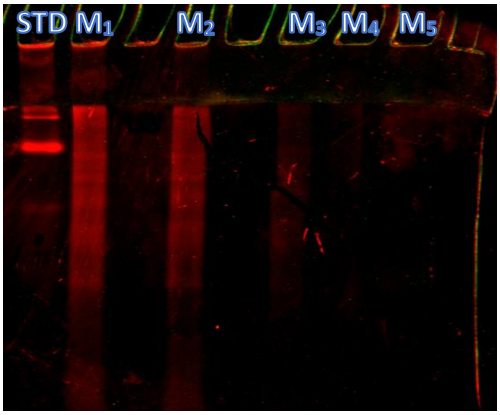

Figure 3.14: Native-PAGE showing lysed HL-60 cells treated twice with 1% β-DDM and loaded with various concentration of sample. STD: NativMark, M_1: 16.5 µl sample in total 22 µl loading solution, M_2, M_3, M_4, & M_5: respectively 11, 5.5, 2.2, & 1.1 µl sample in total 20 µl loading solution.

The sample lanes in this Native gel, although not free from contamination, looked significantly more promising than the previous run. A smaller amount of matter resided in the loading wells and stacking gel. Also, having adjusted the intensity of the absorbance signal, there could clearly be observed distinct bands of proteins. It would have been interesting to observe the effects of even more detergent to the supernatant sample but judging by the weak presence of the different protein bands, it was suspected that a lot of sample had been trapped by the unsuspended pellet. It therefore seemed more pressing to investigate why the mitochondrial prepared pellet would not become resuspended after the digitonin treatment.

As an attempt to gain insight into how digitonin affected the HL-60 cells, a microscopic analysis was performed both before and after the cells were treated with digitonin. Therefore, a third mitochondrial preparation procedure was performed (protein quantitation shown in appendix section 5.1.5) where, intact HL-60 cells resuspended in PBS with protease inhibitor, and the centrifuged supernatant from after the digitonin treatment, were isolated. The two samples were then studied under the Olympus CKX41 microscope at 40x magnification. Four representative images of both samples have been provided in figure 3.15 showing what was observed.

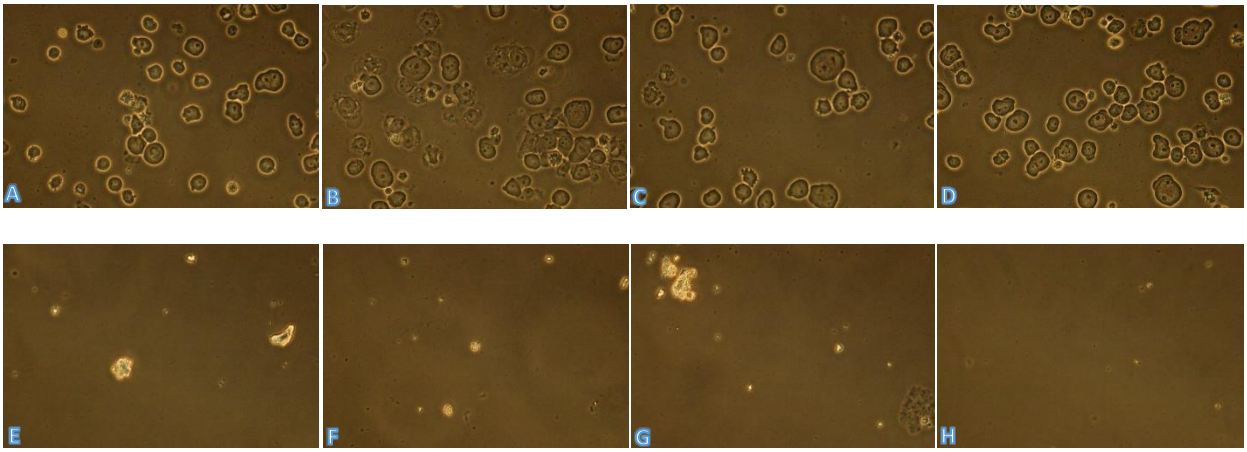

Figure 3.15: Microscopic observations of HL-60 cells before (A, B, C, D) and after (E, F, G, H) being treated with digitonin

Observations showed that the HL-60 looked to be mostly intact before the treatment of digitonin, apart from a few regions were some cells seemed to have undergone apoptosis (illustrated in figure 3.15B). The supernatant extracted after the digitonin treatment also showed the existence of substances the same size as the cells themselves. However, the intact HL-60 cells could be observed being dark and hollow on the inside, while the substances in the supernatant seemed to be compact. Seen as the lipid cell membrane of the HL-60 cells lighted up the same way as the compact substances in the supernatant, this gave an indication that the substance might be accumulated portions of lipid debris. If this was the case, the initial theory of insufficient quantity of digitonin being added seemed more plausible. The next step was to observe the supernatant once more, having treated an HL-60 cell sample twice with digitonin.

A new aliquot of intact HL-60 cells was first treated once with digitonin, followed by centrifuging and removing the supernatant. However, when PBS with protease inhibitor was added to the tube before treating the sample again with digitonin, the pellet became resuspended. This was a curious discovery, to find out that the pellet resuspended in PBS solution but not in the mitochondrial buffer. At this point it was noted that for all the HL-60 samples treated, no alterations had been made to the protocol to come to this stage of the procedure. The only difference was the solvent in which the pellet was attempted to be resuspended in. Thus, it could be assumed that the composition of the two solvents played an important part in what happened to the pellet. The ionic concentrations of the two solvents were compared, revealing that the ion content in the mitochondrial buffer far exceeded that of the PBS solution. Because of this the osmolarity of the pellet content was most likely in completely different stages when suspended in the two solvents. When the pellet was in solution with the mitochondrial buffer, the pellet was presumably hypertonic. Given the high osmotic pressure from the solvent, the mitochondria would exert water to compensate for the ionic imbalance on both sides of its outer membrane. This would cause the mitochondria to shrivel, creating a larger surface area for the mitochondrial to interact with each other and other components within the pellet. This could in return strengthen the entire pellet, making it difficult to

resuspend in the mitochondrial buffer. In comparison, PBS with protease inhibitors had an osmotic pressure which seemed similar that of mitochondria. This resulted in the mitochondria keeping its spherical form, presumably keeping any stronger interactions between themselves and other matter from developing, and therefore allowing the pellet to be resuspended.

With this new knowledge having come to light, the next priority was to prepare an alternative mitochondrial buffer comprised of a lower concentration of osmotic ions. Having found one that could prove promising, the plan was to perform a new Native-PAGE comparing the quality of samples suspended in the original mitochondrial buffer, the alternative mitochondrial buffer, and the PBS solution with protease inhibitor. Samples suspended in the two latter solvents would also be treated with 1, 2, and 3% β-DDM, while since 1% and 2% β-DDM had previously been tested with the original mitochondrial buffer, it would only be treated with 3% this time. Three new aliquots of HL-60 cells in PBS with protease inhibitor from the thirst harvest was extracted from -80°C and used for this purpose. After treating all the three samples with digitonin and centrifuging as before, the supernatant of each sample was exchanged with one of the aforementioned buffers. As expected, the pellet in the original mitochondrial buffer did not resuspend, but the pellets in the two other buffers did, providing confirmation of the theory on the osmotic pressure preventing resuspension. The two resuspended samples were aliquoted evenly into three tubes each, treating them with 1, 2, and 3% β-DDM. The unsuspended sample in the original mitochondrial buffer could not be diluted and was therefore threated with 3% β-DDM in its singular tube. Loading solutions were prepared for each sample and the Native-PAGE was performed giving the resulting gel presented in figure 3.16.

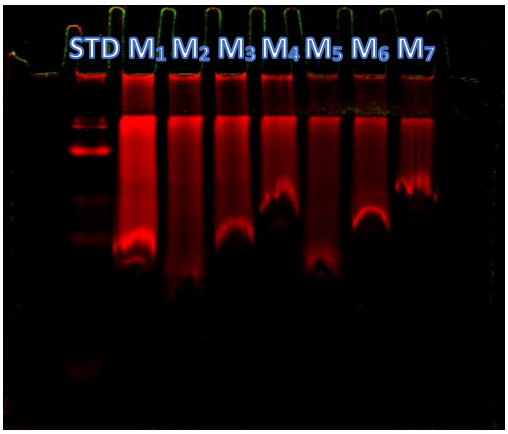

Figure 3.16: Native-PAGE showing digitonin digested HL-60 cells suspended in various buffers and treated with different concentrations of β-DDM. STD: NativeMark; M1: suspended in original mitochondrial buffer treated with 3% β-DDM; M2, M3, & M4: suspended in alternative mitochondrial buffer treated with 1, 2, & 3% β-DDM respectively; M5, M6, & M7: suspended in PBS solution with protease inhibitor treated with 1, 2, & 3% β-DDM respectively

By looking at the resulting gel, it could be seen that micellular accumulations of detergent had occurred in all samples treated with 2% and 3% β-DDM. The lane containing sample in original mitochondrial buffer had traveled further down the gel compared to the other two samples threated with 3% β-DDM. This was probably because of the larger quantity of digested HL-60 cells present in the original mitochondrial buffer tube while the β-DDM treatment was in progress, resulting in more detergent being used up, and less micelles being accumulated in the gel. An observation of note however, was that since the β-DDM had started to accumulate, there were presumably no more lipid debris for it to break down, meaning that the contamination fogging up the lanes in the gel, was something other than lipids. The remaining content of

each sample was therefore analyzed spectrophotometrically to see if anything other than the protein complexes would be picked up. The readings from the spectrophotometer has been shown in figure 3.17.

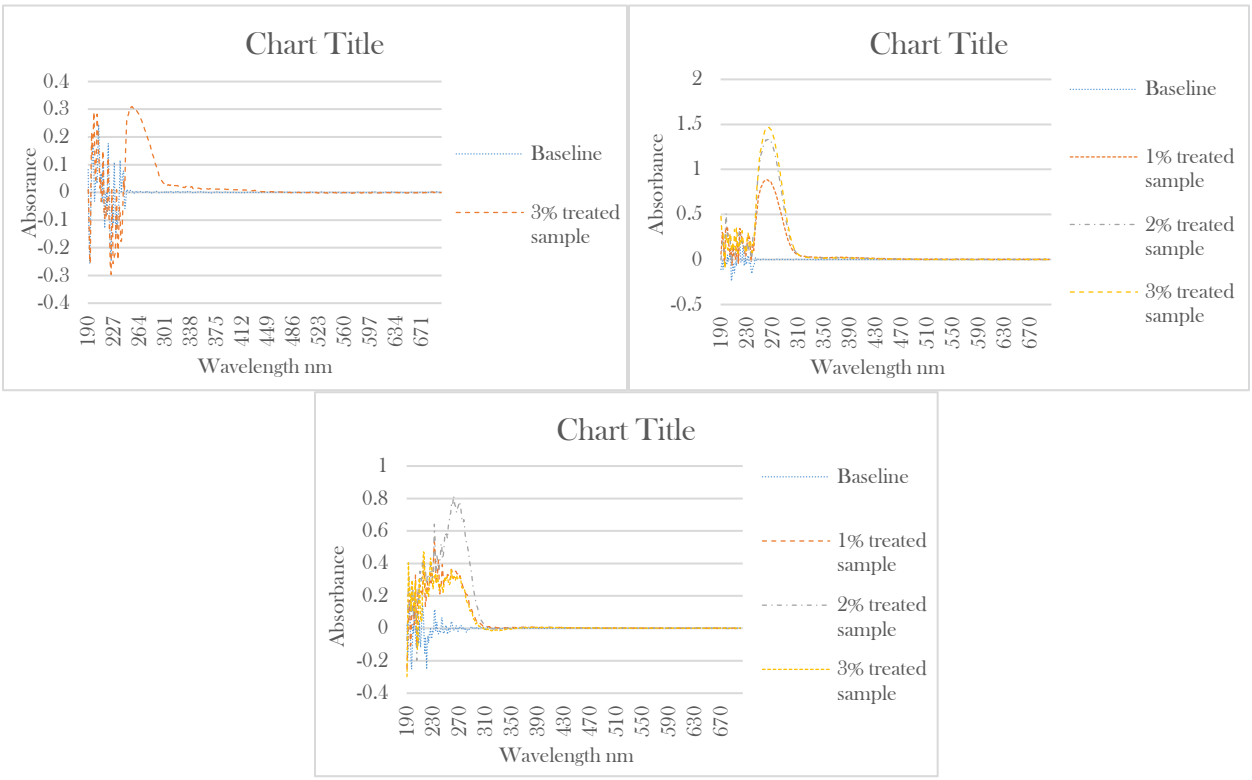

Figure 3.17: Spectral analysis of protein extracted samples from HL-60 cells contained in three different buffers and treated with different concentrations of β-DDM.

An absorbance peak at ~262 nm was observed in most of the scanned samples, especially in those suspended in the alternative mitochondrial buffer. This indicated that the samples could be contaminated with DNA. A choice could therefore have been made to perform a DNase procedure to break down any DNA contaminants and thereby perhaps obtain purified protein complexes. However, looking back at the last Native-PAGE results (figure 3.16) there were only faint bands of protein complexes present, implying that mitochondria could have been lost during the extraction step with digitonin. It therefore did not seem productive to perform a DNase procedure on a sample already lacking sufficient quantities of target material (mitochondrial protein complexes). Instead it seemed more important to investigate where the mitochondria ended up during the HL-60 cell lysing process using digitonin. This was done by staining both the pellet and the supernatant from the centrifugation step prior to the digitonin treatment with TMRM and Mitotracker DR. As these dyes are dependent on active mitochondrial respiration to enter the mitochondria and become attached, this would also provide insight in the functionality of the mitochondria in the sample. The TMRM and Mitotracker DR were first applied to intact HL-60 cells from cell culture to provide an initial view of the mitochondria within the cells. The HL-60 cells were immobilized and stained as described in methods sections 2.2.13 and 2.2.14 respectively. CCCP was also added to one of the sample wells to act as a positive control. Analysis was performed using a Leica TCS SP8 CSU confocal microscope as described in methods section 2.2.16. The resulting observations have been provided in figure 3.18.

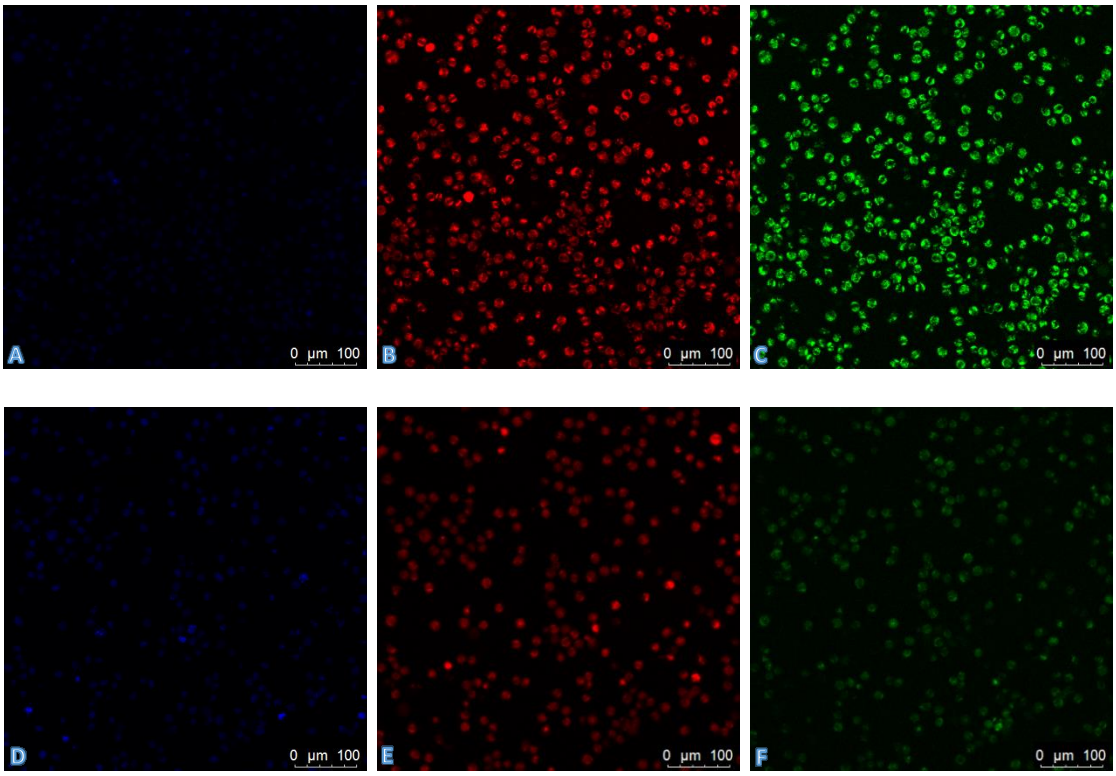

Figure 3.18: *Images of immobilized HL-60 cells stained by Hoechst, TMRM and Mitotracker using a Leica TCS SP8 CSU confocal microscope. A, B & C: respectively Hoechst, Mitotracker, & TMRM stained cells, D, E, & F: respectively Hoechst, Mitotracker, & TMRM stained cells with CCCP.*

The two dyes seemed to have stained the cells nicely, showing a rich content of active mitochondria. Also, the CCCP showed successful inhibition of the dyes. The HL-60 cells underwent a fourth culture harvest, and the mitochondrial preparation procedure was performed to the point of having made 5 mg/ml protein concentrated aliquots of cells in PBS with protease inhibitor (Appendix section 5.1.6). Three aliquots were then treated with digitonin, only this time they were first centrifuged at 1000 x g for 5 min. The supernatant was then transferred to a new tube and was subject to the original centrifugation at 10000 x g for 10 min. The intention behind this additional centrifugation step was based on the previous mitochondrial preparation procedure done on beef heart and liver. In that protocol an initial centrifugation was performed at low centrifugal force to isolate the mitochondria from larger debris. It would therefore be interesting to see if the same technique could be applied to purify the mitochondria from cell debris contamination. The supernatant from the last centrifugal step was again transferred to a new tube, and alternative mitochondrial buffer was added to the two obtained pellet containing tubes for resuspension. However, this time the pellets proved difficult to resuspend once more. The final pellet was successfully fractionated into smaller pieces, while the first pellet had the same rubbery consistency that occurred when using the original mitochondrial buffer. This was puzzling, as the same prepared stock of alternative mitochondrial buffer was used. Even so, it would still be interesting to see if the mitochondria were successfully isolated after the first centrifugation step, and therefore not present in the first pellet. All three samples were transferred to a 96-well plate and stained, as described in methods section 2.2.14, and analyzed under the Leica TCS SP8 CSU confocal microscope. The resulting observations have been shown in figure 3.19.

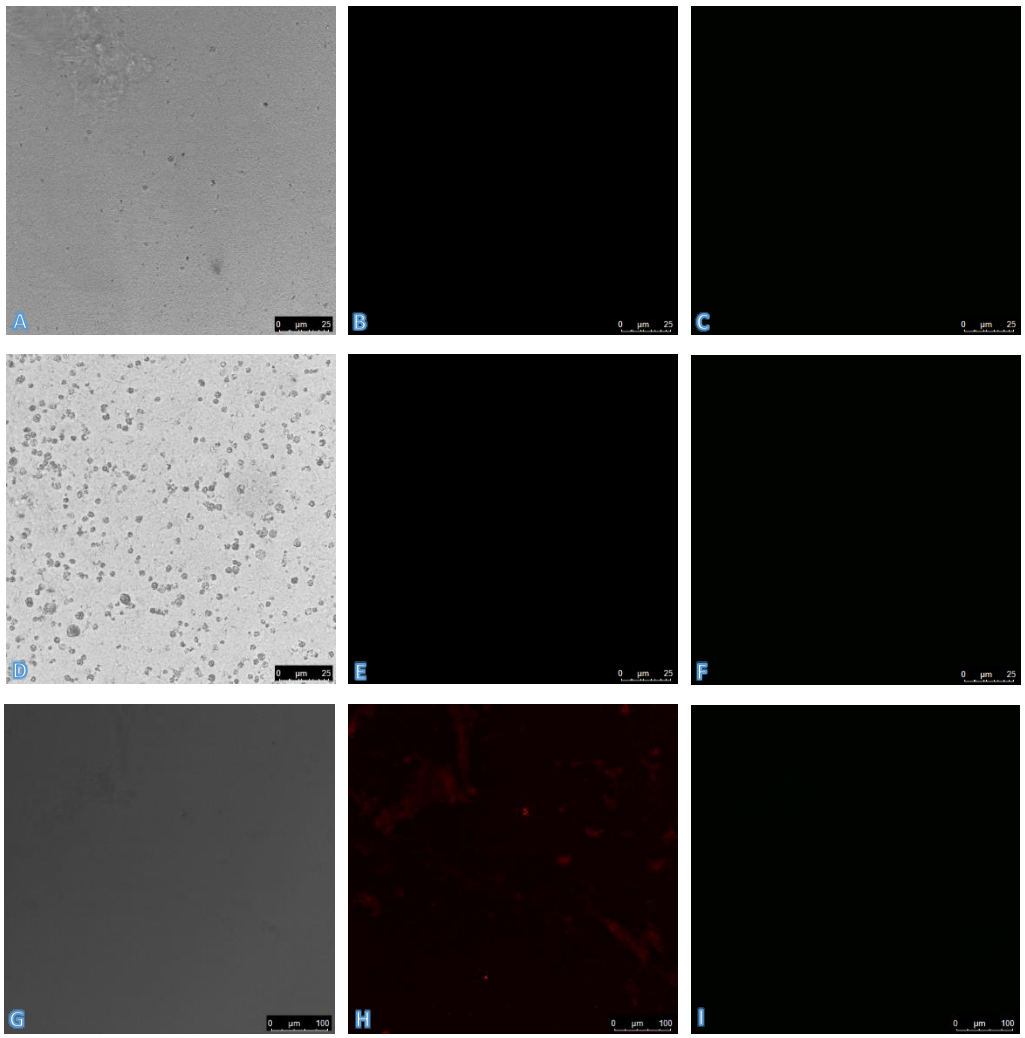

Figure 3.19: Intermediate samples of mitochondrial extraction from HL-60 cells using digitonin, stained with TMRM and Mitotracker DR. A, B, & C: Final pellet suspension showing bright field view, Mitothracker DR stain, and TMRM stain respectively. D, E, & F: Debris pellet suspension from first centrifugation showing bright field view, Mitothracker DR stain, and TMRM stain respectively. G, H, & I: Final supernatant showing bright field view, Mitothracker DR stain, and TMRM stain respectively.

The TMRM and Mitotracker DR dyes did not seem to have attached themselves to many mitochondria. This may indicate that the mitochondria were no longer functional after the digitonin treatment. Nevertheless, the location of mitochondria within these samples were not obtained, so it was decided to stain the same samples again with TOMM20 antibodies as described in methods section 2.2.15. Antibodies are more expensive that dyes, which is why the dyes were used first. However, the antibodies were not dependent on active respiration in the mitochondria to become attached, so the location of the mitochondria was much more likely to be determined. The resulting microscopic observations have been provided in figure 3.20.

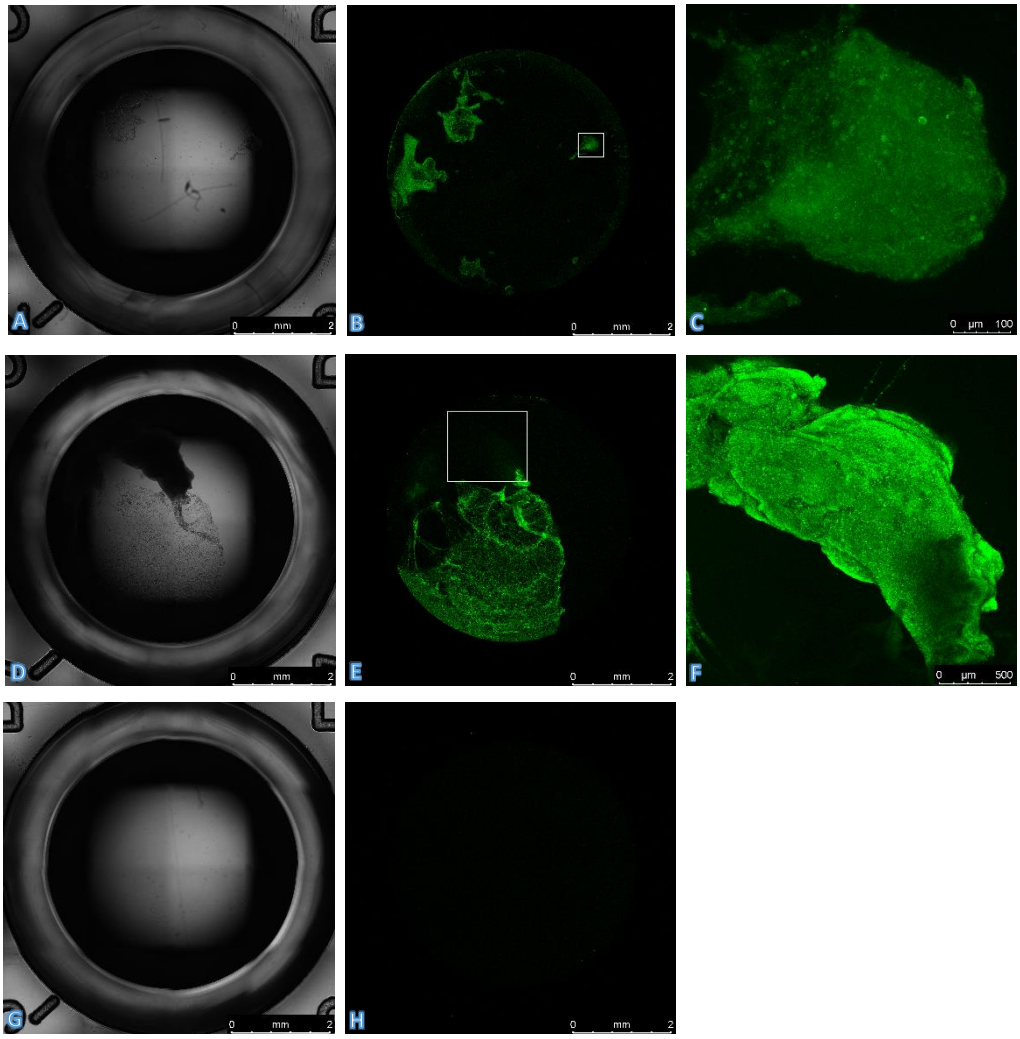

Figure 3.20: TOMM20 stained intermediate samples of mitochondrial extraction from HL-60 cells using digitonin. A, B, & C: Final pellet suspension respectively showing bright field view, TOMM20 stain, and a stack shot in the Z-plane from the white boxed area of the TOMM20 stained image. D, E, & F: Debris pellet suspension from first centrifugation respectively showing bright field view, TOMM20 stain, and a stack shot in the Z-plane from the white boxed area of the TOMM20 stained image. G, & H: Final supernatant respectively showing bright field view, and TOMM20 stain.

Although mitochondria were observed to be present in the final pellet suspension, most still existed within the pellet from the first centrifugation. In both cases the mitochondria appeared to be bundled together within each respective pellet unit. In the stack image (figure 3.20C) from the final suspension sample there could be seen lots of small areas giving off higher fluorescent intensities. This could illustrate bundles of closely packed mitochondria, which could indicate that the portions of pellet with lower intensity were segments of contaminants connecting the mitochondrial patches together. The supernatant on the other hand, did not appear to contain any significant quantities of mitochondria (figure 3.20H), suggesting that a centrifugation of 10000 x g for 10 min was enough to pelletize the mitochondria. Also, this showed that the pellets used further for protein extraction with β-DDM in the previous procedures contained almost all the mitochondria from their respective cells. From all these observations, the same objective persisted in trying

to figure out a way to isolate the mitochondria from all the contamination joining them in the pellet after the digitonin treatment. Perhaps if the lysed HL-60 cells were to be filtered with a 20-micron nylon sheath before undergoing centrifugation, the larger contaminants would be held back, and the resulting pellet would be easier to handle. This was tested on an aliquot of intact HL-60 cells from a fifth cell harvest (appendix section 5.1.7. The resulting pellet was smaller and was successfully solubilized in alternative mitochondrial buffer. The suspension was subsequently treated with 1% β-DDM followed by adding Native-SB and running four equally concentrated aliquots through a Native-PAGE. The resulting gel was stained and scanned on the Odyssey scanner, providing the image shown in figure 3.21.

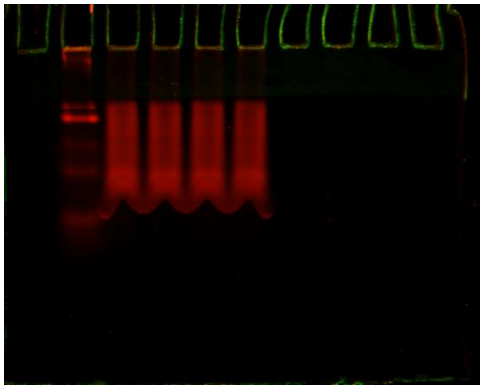

Figure 3.21: Native-PAGE of lysed HL-60 cells that had been filtered before centrifugation and treated with 1% β-DDM

By looking at the gel it seemed like a new accumulation of β-DDM had occurred once more, preventing smaller matter from proceeding further. As mention, this would indicate the lipids had been completely broken down, meaning that the contamination persisting in the lanes most likely was DNA. Also, although there seemed to be faint protein bands in the sample lanes, the concentration of there proteins were very low, suggesting that a lot of the mitochondria may have been stuck to the larger contaminant during the filtration process and therefore been lost.

3.4 Future Prospects

Unfortunately, there was no more time to continue with any practical work at this point. Although advances had been made in the process of figuring out how to obtain a pure mitochondrial extract, a successful result was yet to be made. Revising the observations made from the mitochondrial extraction from beef heart, it seemed as though the sample was rich with protein complexes, seen as there existed thick, concentrated protein bands after the Native-PAGE (figure 3.3). From later experience, the contamination in the mitochondrial sample lanes could presumably be lipid debris, since there were no clear signs of a build-up of β-DDM. Thus, if the sample had perhaps been treated with a higher concentration of detergent, there may have been less contamination within the sample. There was also a possibility for DNA contamination indicated by the spectrophotometric scan in figure 3.4. The deal with this, there could have been perform a DNase procedure.

The results obtained from the mitochondrial extraction from beef liver was promising at first. Even though the minced liver behaved differently than that of the beef heart during the mitochondrial extraction, the positioning of the mitochondrial band, after performing the purification procedure with a percoll gradient, was exactly were it was expected to be according to description from the article from which the procedure was inspired from [26]. Also, the subsequent spectrophotometric scan (figure 3.8) showed no

clear signs of DNA contamination. However, the spectrophotometric analysis did present a steadily declining absorbance in the range 300nm – 700nm. Had the sample been pure with intact mitochondria, there would presumably not be picked up any absorbance in this range. It was after the first Native-PAGE (figure3.9) where it became clear that something may have gone wrong when there did not seem to be any solubilized protein in the sampled lanes. Even though this could have been plausible as there was an initial volume of 300 µl of the 1:4 diluted sample and only 1-10 µl were used in the various wells, a second Native-PAGE attempt was performed with a much higher concentration of mitochondrial extract was run which produced the same results. The theory then, was that no protein complexes had been solubilized from the mitochondrial sample, and the only substance in the resulting gel was micellular β-DDM. However, the efficiency of the β-DDM was proven, by the subsequent analysis of pre-isolated chloroplasts (figure 3.11 &3.12), to be fine as clear protein bands from all sample lanes were produced. To compare the mitochondrial extraction from beef liver with that of the beef heart, the difference in the method was the extra performance of purification with a percoll gradient. Perhaps there was some residual percoll in the samples which may have affected the subsequent solubilization. If this challenge was to be explored further, a solution could be to perform an alternative purification procedure on the crude mitochondrial extract, like for instance filtration, followed by a new series of differential centrifugation to remove any larger contaminants.

The extraction of mitochondria from cultured HL-60 cells was the final objective that was made in this thesis. Here challenges arose almost immediately when the centrifuged pellet, after the cells had been lysed by digitonin, did not solubilize in the initial mitochondrial buffer. After deducing that this was not simply a performance error of the procedure, an attempt to solubilize protein complexes from the pellet was made. Although a 2% β-DDM treatment did manage to extract what seemed like mitochondria protein complex bands, the contamination was still too high and the yield of proteins too low. It was then observed that osmotic pressure may have had a role in the pellet not resuspending, when the pellet became resuspended in PBS, and subsequently an alternative mitochondrial buffer. However, after performing a new digitonin treatment using alternative buffer again for resuspension, the pellet once again did not allow itself to be resuspended. Perhaps there has been performed an error during the BCA assay when trying to determine the protein concentration of the harvested cells. If that was the case, the cells could have been suspended in a protein concentration larger than 5 mg/ml. This could result in there being too many cells in the solution when treated with digitonin, resulting with a larger amount of cell debris in the pellet, which may have accumulated with strong intramolecular forces and cause the pellet to not become resuspended. If there was time for further progression into performing a successful mitochondrial extraction from HL-60 cells, an idea would be to investigate the osmolarity of mitochondria further and procure a mitochondrial buffer well suited to support the functionality of the mitochondria. The reason for this was because, even though the alternative mitochondrial buffer did manage once to suspend the centrifuged pellet after the digitonin treatment, the protein yield from the solubilization was very poor. Also, from the resulted staining of the mitochondrial extract using TMRM and Mitotracker DR, there did not seem to be and metabolic activity from the mitochondria. This could suggest that the mitochondria in the extracted sample were damaged, which could also mean that the protein complexes were becoming denatured, explaining the low protein yield from the Native-PAGE results. An additional approach could also be to increase the concentration of digitonin after an appropriate mitochondrial buffer was made. Perhaps this would lower the quantity of cell debris accumulating in the pellet, and the mitochondria would be easier to access.

If a successful extraction of pure protein complexes had been obtained, the resulting Native-gel would have been subject to a second dimension SDS-PAGE to further sub fractionate the protein subunits within. With this done, it would be possible to determine with near certainty that the proteins in the sample in fact came from mitochondria. Later, a new objective could have been made to introduce protein complex inhibitors to the mitochondrial sample like rotenone and piericidin A, which regulate the functionality of complex I [32]. There could then have been perform a respiratory analysis of the mitochondria to see how this inhibition caused the metabolic activity to differ from wildtype mitochondria. Another approach could be to extract mtDNA and perform a study on their sequential changes after certain mutations occur. There were many doors that could be opened once a purified mitochondrial extract had been obtained. The only problem was getting there.

4. List of Referenses

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5. Appendix

5.1 Analysis of BCA assay

This section provides all the raw data obtain from the different BCA assays that were performed during this thesis, as well as the calculations done to determine the protein concentration of samples.

5.1.1 First mitochondrial extract aliquot from beef heart

After scanning all samples in the microplate reader, the absorbance values have been provided in table 5.1.

Table 5.1: Absorption values from the BCA assay analyzing protein extractions from both protein precipitation and RIPA buffer of mitochondrial sample from beef heart.

Samples in ddH_2O	Absorbance	Samples in RIPA buffer	Absorbance
BSA 2.0 mg/ml	4.157	BSA 2.0 mg/ml	4.152
BSA 1.5 mg/ml	3.475	BSA 1.5 mg/ml	3.518
BSA 1.0 mg/ml	2.735	BSA 1.0 mg/ml	2.561
BSA 0.75 mg/ml	2.233	BSA 0.75 mg/ml	2.137
BSA 0.5 mg/ml	1.635	BSA 0.5 mg/ml	1.588
BSA 0.25 mg/ml	1.022	BSA 0.25 mg/ml	0.984
BSA 0.125 mg/ml	0.632	BSA 0.125 mg/ml	0.658
BSA 0.025 mg/ml	0.282	BSA 0.025 mg/ml	0.308
BSA 0 mg/ml	0.141	BSA 0 mg/ml	0.207
Undiluted mitochondrial sample	0.242	Undiluted mitochondrial sample	1.255
1:2 diluted mitochondrial sample	0.225	Undiluted chloroplast sample	0.196
1:4 diluted mitochondrial sample 1	0.159		
1:4 diluted mitochondrial sample 2	0.151		
Undiluted chloroplast sample	0.206		

The absorbance value of the blank BSA standards were subtracted from all the other samples un their respective solvents, followed by producing a standard curve of all the BSA standards presented in figure 5.1.

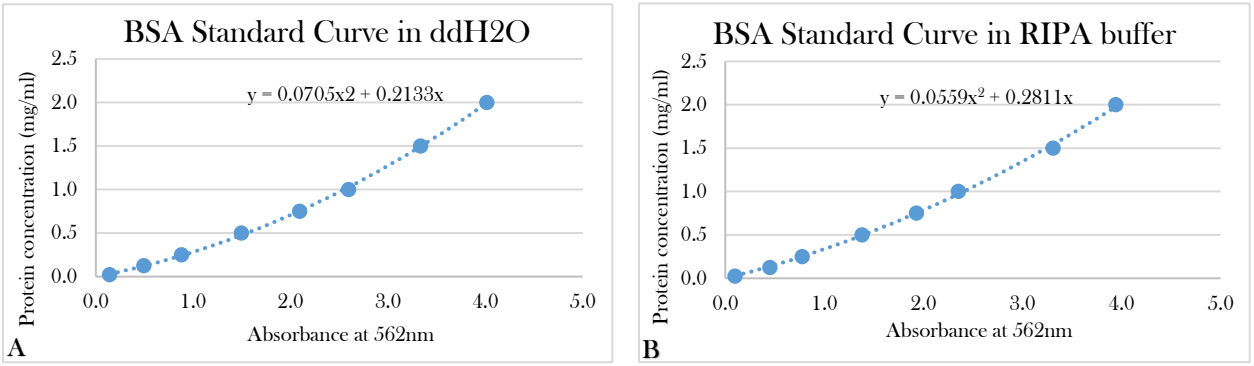

Figure 5.1: Graphs showing protein concentration (mg/ml) vs absorbance (562 nm) of the BSA standard solutions in ddH_2O (A) and RIPA buffer (B). The equations from the trendlines of the respective standard curves have also been provided.

Using the equation of the trendlines for each respective standard curve, the concentrations of the unknown mitochondrial/chloroplast samples were calculated. They were then multiplied by their dilution factor to determine the concentration of the protein isolated sample, and finally multiplied again by the volume (ml) of the isolated sample to deduce the amount (mg) of protein. The resulting calculation have been presented in table 5.2.

Table 5.2: Calculated concentrations of protein in the different mitochondrial samples acquired from beef heart.

Samples	Concentration in microplate (mg/ml)	Concentration in protein isolated sample (mg/ml)	Initial amount of protein (mg)
Samples in ddH_2O			
Undiluted mitochondrial sample	0.022	0.200	0.012
1:2 diluted mitochondrial sample	0.019	0.333	0.020
1:4 diluted mitochondrial sample 1	0.004	0.143	0.009
1:4 diluted mitochondrial sample 2	0.002	0.080	0.005
Undiluted chloroplast sample	0.040	0.362	0.022
Samples in RIPA buffer			
Undiluted mitochondrial sample	1.255	11.29	5.647
Undiluted chloroplast sample	0.196	1.768	0.884

5.1.2 Second mitochondrial extract aliquot from beef heart

After scanning all samples in the microplate reader, the absorbance values have been provided in table 5.3.

Table 5.3: Absorption values from the BCA assay analyzing protein extractions using RIPA buffer of the second mitochondrial extract aliquot from beef heart.

Samples in ddH_2O	Absorbance
BSA 2.0 mg/ml	0.163
BSA 1.5 mg/ml	0.150
BSA 1.0 mg/ml	0.140
BSA 0.75 mg/ml	0.150
BSA 0.5 mg/ml	0.145
BSA 0.25 mg/ml	0.138
BSA 0.125 mg/ml	0.132
BSA 0.025 mg/ml	0.135
BSA 0 mg/ml	0.126
Mitochondrial sample	1.396

5.1.3 Protein determination of the first HL-60 cell harvest

After scanning all samples in the microplate reader, the absorbance values have been provided in table 5.4.

Table 5.4: Absorption values from the BCA assay analyzing protein extractions using RIPA buffer of the first HL-60 cell harvest.

Samples in ddH_2O	Absorbance
BSA 2.0 mg/ml	0.727
BSA 1.5 mg/ml	0.600
BSA 1.0 mg/ml	0.425
BSA 0.75 mg/ml	0.345
BSA 0.5 mg/ml	0.246
BSA 0.25 mg/ml	0.181
BSA 0.125 mg/ml	0.135
BSA 0.025 mg/ml	0.100
BSA 0 mg/ml	0.090
HL-60 protein sample	0.570

The absorbance value of the blank BSA standards were subtracted from all the other samples, followed by producing a standard curve of all the BSA standards presented in figure 5.2.

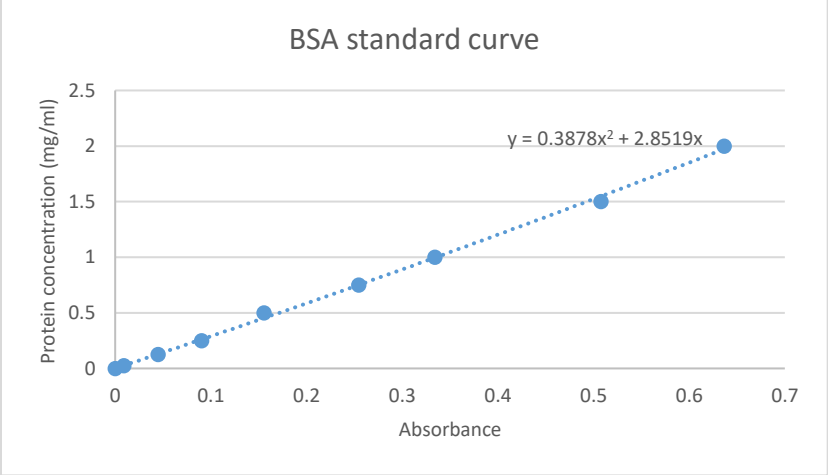

Figure 5.2: Graph showing protein concentration (mg/ml) vs absorbance (562 nm) of the BSA standard solutions in RIPA buffer used to calculate the protein concentration of the first HL-60 cell harvest. The equation from the trendlines of the standard curves have also been provided.

Using the equation of the trendlines for the BSA standard curve, the protein concentration of the HL-60 sample was calculated to be 1.457 mg/ml. As the volume of the sample aliquot was the same as the volume of RIPA buffer added for protein extraction, the concentration was multiplied by the volume the main cluster of cells were in (29.5 ml) to determine the total quantity of protein that was 42.98 mg.

5.1.4 Protein determination of the second HL-60 cell harvest

After scanning all samples in the microplate reader, the absorbance values have been provided in table 5.5.

Table 5.5: Absorption values from the BCA assay analyzing protein extractions using RIPA buffer of the second HL-60 cell harvest.

Samples in ddH_2O	Absorbance
BSA 2.0 mg/ml	1.836
BSA 1.5 mg/ml	1.572
BSA 1.0 mg/ml	1.059
BSA 0.75 mg/ml	0.882
BSA 0.5 mg/ml	0.637
BSA 0.25 mg/ml	0.397
BSA 0.125 mg/ml	0.253
BSA 0.025 mg/ml	0.146
BSA 0 mg/ml	0.097
HL-60 protein sample	0.937

The absorbance value of the blank BSA standards were subtracted from all the other samples, followed by producing a standard curve of all the BSA standards presented in figure 5.3.

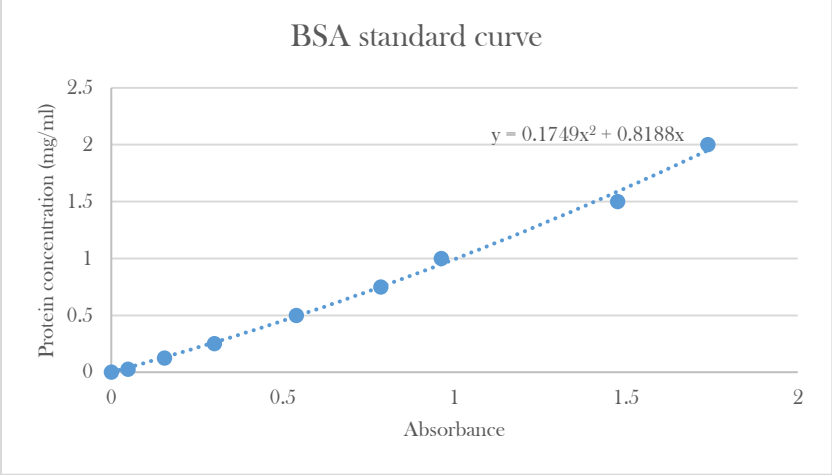

Figure 5.3: Graph showing protein concentration (mg/ml) vs absorbance (562 nm) of the BSA standard solutions in RIPA buffer used to calculate the protein concentration of the second HL-60 cell harvest. The equation from the trendlines of the standard curves have also been provided.

Using the equation of the trendlines for the BSA standard curve, the protein concentration of the HL-60 sample was calculated to be 0.811 mg/ml. As the volume of the sample aliquot was the same as the volume of RIPA buffer added for protein extraction, the concentration was multiplied by the volume the main cluster of cells were in (19.5 ml) to determine the total quantity of protein that was 15.81 mg.

5.1.5 Protein determination of the third HL-60 cell harvest

5.43x10^7 cells were harvested from cell culture. After scanning all samples in the microplate reader, the absorbance values have been provided in table 5.6.

Table 5.6: Absorption values from the BCA assay analyzing protein extractions using RIPA buffer of the third HL-60 cell harvest.

Samples in ddH_2O	Absorbance
BSA 2.0 mg/ml	1.6808
BSA 1.5 mg/ml	1.4618
BSA 1.0 mg/ml	1.065
BSA 0.75 mg/ml	0.8549
BSA 0.5 mg/ml	0.629
BSA 0.25 mg/ml	0.399
BSA 0.125 mg/ml	0.2597
BSA 0.025 mg/ml	0.1427
BSA 0 mg/ml	0.099
HL-60 protein sample	0.7115

The absorbance value of the blank BSA standards were subtracted from all the other samples, followed by producing a standard curve of all the BSA standards presented in figure 5.4.

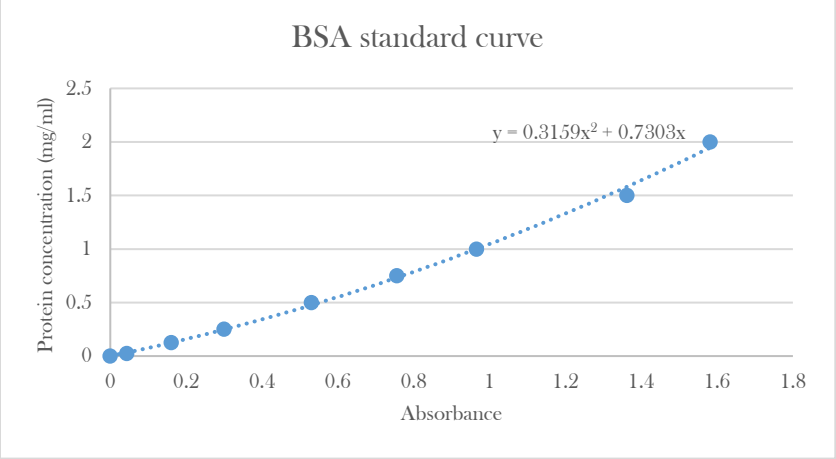

Figure 5.4: Graph showing protein concentration (mg/ml) vs absorbance (562 nm) of the BSA standard solutions in RIPA buffer used to calculate the protein concentration of the third HL-60 cell harvest. The equation from the trendlines of the standard curves have also been provided.

Using the equation of the trendlines for the BSA standard curve, the protein concentration of the HL-60 sample was calculated to be 0.566 mg/ml. As the volume of the sample aliquot was the same as the volume of RIPA buffer added for protein extraction, the concentration was multiplied by the volume the main cluster of cells were in (19.5 ml) to determine the total quantity of protein that was 11.04 mg. Therefore, the cellular pellet was resuspended in 2.208 ml PBS with protease inhibitor and divided into 400 µl aliquots.

5.1.6 Protein determination of the fourth HL-60 cell harvest

$7.19x10^7$ cells were harvested from cell culture. After scanning all samples in the microplate reader, the absorbance values have been provided in table 5.7.

Table 5.7: Absorption values from the BCA assay analyzing protein extractions using RIPA buffer of the fourth HL-60 cell harvest.

Samples in ddH_2O	Absorbance
BSA 2.0 mg/ml	1.6723
BSA 1.5 mg/ml	1.1807
BSA 1.0 mg/ml	0.7834
BSA 0.75 mg/ml	0.6146
BSA 0.5 mg/ml	0.5664
BSA 0.25 mg/ml	0.344
BSA 0.125 mg/ml	0.205
BSA 0.025 mg/ml	0.1382
BSA 0 mg/ml	0.0914
HL-60 protein sample	0.6237

The absorbance value of the blank BSA standards were subtracted from all the other samples, followed by producing a standard curve of all the BSA standards presented in figure 5.5.

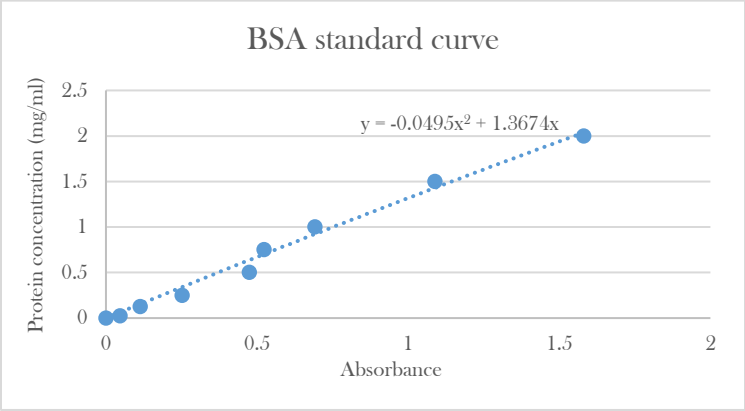

Figure 5.5: Graph showing protein concentration (mg/ml) vs absorbance (562 nm) of the BSA standard solutions in RIPA buffer used to calculate the protein concentration of the fourth HL-60 cell harvest. The equation from the trendlines of the standard curves have also been provided.

Using the equation of the trendlines for the BSA standard curve, the protein concentration of the HL-60 sample was calculated to be 0.714 mg/ml. As the volume of the sample aliquot was the same as the volume of RIPA buffer added for protein extraction, the concentration was multiplied by the volume the main cluster of cells were in (19.5 ml) to determine the total quantity of protein that was 13.92 mg. Therefore, the cellular pellet was resuspended in 2.785 ml PBS with protease inhibitor and divided into 400 µl aliquots.

5.1.7 Protein determination of the fifth HL-60 cell harvest

$3.28x10^7$ cells were harvested from cell culture. After scanning all samples in the microplate reader, the absorbance values have been provided in table 5.8.

Table 5.8: Absorption values from the BCA assay analyzing protein extractions using RIPA buffer of the fifth HL-60 cell harvest.

Samples in ddH_2O	Absorbance
BSA 2.0 mg/ml	2.0108
BSA 1.5 mg/ml	1.7092
BSA 1.0 mg/ml	1.2138
BSA 0.75 mg/ml	1.0128
BSA 0.5 mg/ml	0.7587
BSA 0.25 mg/ml	0.4672
BSA 0.125 mg/ml	0.2976
BSA 0.025 mg/ml	0.164
BSA 0 mg/ml	0.1072
HL-60 protein sample	0.6235

The absorbance value of the blank BSA standards were subtracted from all the other samples, followed by producing a standard curve of all the BSA standards presented in figure 5.6.

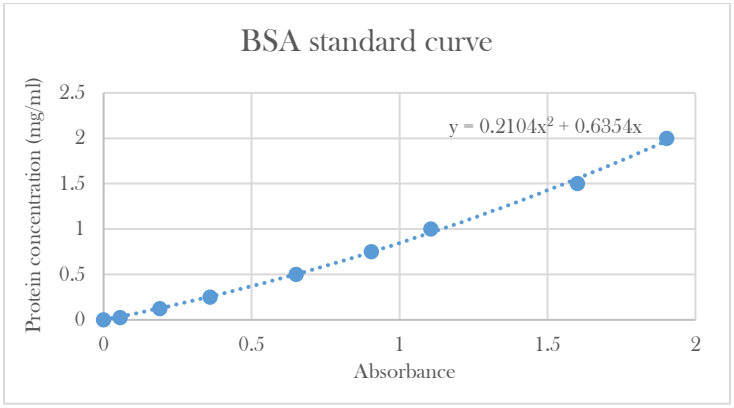

Figure 5.6: Graph showing protein concentration (mg/ml) vs absorbance (562 nm) of the BSA standard solutions in RIPA buffer used to calculate the protein concentration of the fifth HL-60 cell harvest. The equation from the trendlines of the standard curves have also been provided.

Using the equation of the trendlines for the BSA standard curve, the protein concentration of the HL-60 sample was calculated to be 0.384 mg/ml. As the volume of the sample aliquot was the same as the volume of RIPA buffer added for protein extraction, the concentration was multiplied by the volume the main cluster of cells were in (19.5 ml) to determine the total quantity of protein that was 7.488 mg. Therefore, the cellular pellet was resuspended in 1.498 ml PBS with protease inhibitor and divided into 400 µl aliquots.

5.2 Standard markers for SDS- and Native-PAGE

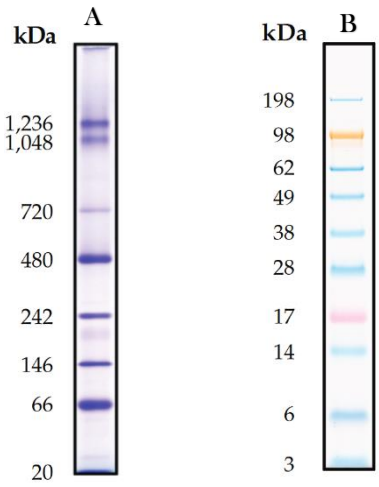

Figure 5.7: *Standard markers.* ***A)*** *NativeMark used for Native-PAGE,* ***B)*** *SeaBlue marker used for SDS-PAGE [33, 34]*