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The characterisation of bacterial Nucleoside Diphosphate Kinase (NDK) proteins and their effects on haemopoietic stem cells

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Dear Reader,

This combined thesis is composed of 2 separate research projects, undertaken for the award of Masters of Research in Molecular and Cellular Biology, at the University of Birmingham.

Both projects focus on bacterial Nucleoside Diphosphate Kinase (NDK) proteins; and have many similarities. I have therefore consciously combined both projects so that they read as one with ease.

Part A of this thesis focuses on the effects of bacterial NDK proteins on haemopoietic stem cells, derived from bone marrow and leukaemic samples. This project was supervised by Dr. Farhat Khanim at the School of Biosciences, College of Life and Environmental Sciences, University of Birmingham.

Part B of this thesis focuses on the characterisation of bacterial NDK proteins and determines whether point mutations or deletions within the conserved protein domains of NDK could affect the function of the protein. This project supervised by Dr. Mark Webber at the School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham.

Yours faithfully

Monika Köpf

ABSTRACT

Introduction

Nucleoside Diphosphate Kinases (NDK) are a family of small conserved proteins present in all cells which are involved in the synthesis of nucleoside triphosphates (NTP) and nucleoside diphosphates (NDP) which maintain nucleotide pools in the cell. NDK play an important role in the cells metabolic processes, for example maintaining dNTP for nucleic acid synthesis, CTPs for lipid synthesis, UTPs for polysaccharide synthesis and GTP for protein elongation. Most NDK proteins are known to form hexameric quaternary structure; however a few prokaryotic NDKs are tetrameric. However regardless of their quaternary structure all NDK proteins share a common dimer unit and the structures surrounding the active histidine site is identical in all NDK.

The NDK protein found in humans named, non-metastatic 23 (NM23) was studied recently by Lily et al. A survival mechanistic role of the human NM23-H1 protein on haemopoietic stem cells was identified. It was shown that the NM23-H1 protein increased the survival and proliferation of stem cells from bone marrow and umbilical cord bloods and also acute myeloid leukaemia (AML).

Hypothesis

This study tested the hypothesis that bacterial NDK proteins can promote the survival of haemopoietic stem cells. Infection has been identified as a risk factor for progression to AML in patients with pre-leukaemic conditions.

Methods

The over expression of bacterial NDK from four bacterial species; *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, were cultured for 5 days with CD34-ve and CD34+ cells from umbilical cord bloods and leukaemic samples. FACS analysis showed the cell viability of CD34-ve and CD34+ve stem cells. The expression of NDK at mid-logarithmic and at stationary phase were analysed, and NDK knockout mutants and site directed mutants were produced from conserved domains of the NDK, to characterise important domains which could play a role in the NDK survival mechanism on haemopoietic stem cells.

Results

Preliminary results have suggested that bacterial NDK proteins may follow a similar pattern to increase the survival of haemopoietic stem cells, as the human NM23-H1 protein, however further work is needed to confirm such findings. The NDK mutants have shown to have a disrupted biology which leads to an altered expression in NDK. The Mutant 3, a disruption in the KPN loop, has shown a disruption in the structure of NDK.

Conclusion

The results suggest that bacterial NDK proteins could play a role in promoting the survival of leukaemic stem cells. This would impact patient care and treatment as well as requiring ongoing medical research into bacterial NDK proteins; and their interactions with leukaemic stem cells. The increased risk of neutropenic infections in patients undergoing chemotherapy could have a greater link to leukaemia than first

thought and NDK proteins may have a role in bacterial pathogenicity by disrupting haemopoiesis.

ACKNOWLEDGEMENTS

The writing of this thesis has been an academic challenge, and I would not have been able to complete my MRes in Molecular and Cellular Biology, without the support, patience and guidance of the following people;

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Professor Chris Bunce, who accepted me into his research group, and allowing me to contribute to such a fascinating field of research.

Dr Mark Webber who undertook responsibility as my 2nd project supervisor despite his many other academic commitments. His knowledge, support and commitment, inspired and motivated me.

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At last but not at all least, my family, my friends, especially my 2 best friends, Kirsty Hobbs and Debs Lord and my boyfriend Joe Scott for ultimate support, guidance, advice and encouragement throughout the last year.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
NDK	Nucleoside Diphosphate Kinase
NM23	non metastatic 23
NTP	Nucleoside Triphosphate
NDP	Nucleoside Diphosphate
dNTP	deoxy nucleoside triphosphates
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ATP	Adenosine-5'-triphosphate
LYS	Lysine
TYR	Tyrosine
ARG	Arginine
THR	Threonine
SER	Serine
ASN	Asparagine
GLU	Glutamine
ITS+	Insulin-transferrin-sodium
CB	Cord Blood
HSC	Haemopoietic Stem cell
LSC	Leukaemic Stem cell
AGM	aorta-gonads-mesonephros
K.pneu	<i>Klebsiella pneumoniae</i>
S.aur	<i>Staphylococcus aureus</i>
S. pneu	<i>Streptococcus pneumoniae</i>
HLA	Human leukocyte antigens
ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia

1. INTRODUCTION

1.1 NDK Superfamily

Nucleoside Diphosphate Kinases (NDKs) are a superfamily of proteins which are involved in the synthesis of nucleoside triphosphates (NTP), where a gamma-phosphate is transferred from one NTP to NDP to produce new NTPs and NDPs. i.e. $GTP+ADP \rightleftharpoons GDP+ATP$, this maintains a balanced nucleotide pool within the cell (Sikarwar, et al., 2013) (Schaertl, et al., 1999).

Extensive research has shown that the nucleoside diphosphate kinase proteins are highly conserved within eukaryotes, prokaryotes and archaea, all of which contain nucleoside diphosphate kinase (NDK) activity (Bilitou, et al., 2009). These proteins were first discovered by (Berg & Joklik, 1953) in yeast, and by (Krebs & Hems, 1953) in breast muscle of pigeon.

For the purpose of this thesis, the focus will be on Human non-metastatic clone 23 (NM23), and bacterial NDK. Human NM23, named because of its ability to function as a tumour suppressor in a number of solid tumours such as breast and ovarian cancers (Almgren, et al., 2004) play an identical role to bacterial NDK in maintaining nucleotide pools within the cell (Chakrabarty, 1998).

1.2 NDK Activity

NDK plays a role in all cells, their primary role is to maintain NTP and (deoxy derivatives) dNTP pools, which are used in the synthesis of RNA and DNA.

(Chakrabarty, 1998) NDK provide nucleoside triphosphates for several metabolic processes, for example – dNTPs for nucleic acid synthesis, cytidine triphosphates (CTP) for the synthesis of lipids, uridine triphosphates (UTP) for polysaccharide synthesis, and guanosine triphosphates (GTP) for the elongation of proteins, signal transduction as well as polymerisation of microtubules (Tonoli, et al., 2009).

1.2.1 Role in Bacteria

Bacterial NDK proteins are multifunctional housekeeping enzyme and use the reversible transfer of a gamma phosphate from an NTP to NDP for the essential generation of specific NTPs or dNTPs. The dNTPs are then used as precursor for the synthesis of RNA and DNA. (Ray & Matthews, 1992) *Escherichia coli* NDK protein can interact with several proteins that are involved in DNA synthesis. Research has shown that the NDK from *Escherichia coli* interacts with the uracil DNA glycosylase, increasing *Ung* catalytic activity. It also interacts with T4 bacteriophage encoded proteins during infection (Nordman & Wright, 2011).

Bacterial NDK also play an important role in bacterial growth and cell division. The nucleoside triphosphate, GTP is particularly important in cellular macromolecule production and signalling transductions within bacteria (Chakrabarty, 1998).

Sequencing of the NDK gene of *Mycobacterium tuberculosis* has shown that the NDK protein has a secondary function to nucleoside diphosphate kinase activity. It has shown to cleave single stranded DNA in the human c-myc promoter, (Kumar, et al., 2005) and it also acts as a GTPase activating protein (Chopra, et al., 2004).

The NDK protein had always been considered an important enzyme for cell growth and division in prokaryotic organisms, however it was found that enzymes such as Pk, adenylate kinase and polyphosphate kinase all contain nucleoside diphosphate kinase activity, thus making NDK non vital in bacterial such as *E. coli* and *P. aeruginosa* (Lu, et al., 1996) (Sundin, et al., 1996) (Munoz-Dorado, et al., 1990). However if any of these enzymes are restricted then cellular growth and division become dependent on the nucleoside diphosphate kinase activity of NDK.

1.2.2 Pathogenicity and NDK

Pathogenesis of bacteria is a complicated process, which is dependent on high energy levels. The bacterium is required to successfully infect and proliferate within the host, as well as produce virulence factors. To be able to successfully carry out these processes, the bacterium requires the maintenance of a good energy source. NDK proteins play an important role in maintaining this vital source (Chakrabarty, 1998). The bacterium can also obtain Adenosine triphosphate (ATP) from its host at the site of inflammation, which leads to cell death. (Hussain, et al., 2011) It has been suggested that bacterial NDK can help the cell utilise this eATP to favour their survival (Chopra, et al., 2003).

NDKs have been shown to have a secretory function (Zaborina, et al., 1999) which plays an important role in the survival of pathogenic organisms within the host (Hussain, et al., 2011). This secretory function has also been suggested to play a role in apoptosis of macrophages (Zaborina, et al., 1999).

1.2.3 Role in Humans

NM23, like the bacterial NDK is a housekeeping enzyme that plays a role in the transactivation on c-myc, and aids in the regulation of endocytosis. It also plays a role as a tumour suppressor; this is discussed in more detail in chapter 1.4 (Lee, et al., 2009). NDK also plays a role in the differentiation of cells and protein synthesis (Chakrabarty, 1998). However the cellular mechanism which regulates NM23 is not clearly defined (Lee, et al., 2009).

1.3 Protein Structure

Bacterial NDK proteins are known to form homohexameric or homotetrameric structures (Arai, et al., 2012). Most NDK proteins are known to form a hexameric quaternary structure; but a few prokaryotic NDKs are tetrameric such as *E. coli* NDK.

Regardless of their quaternary structure all NDK proteins share a common dimer unit and the structures surrounding the active histidine site are identical in all NDKs. Apart from c-terminal residues found in the hexamer and not the tetrameric structure the nucleoside binding sites are fully conserved (Janin, et al., 2000). Research suggests that this assembly of subunits in a tetrameric NDK differ from hexameric NDK and also alters the structure of the protein (Giartasia, et al., 1996). Hexameric NDK constitutes 3 dimers; whereas the tetramer constitutes only 2 dimers, this occurs by interactions at differing surface regions in the protein (Williams, et al., 1993).

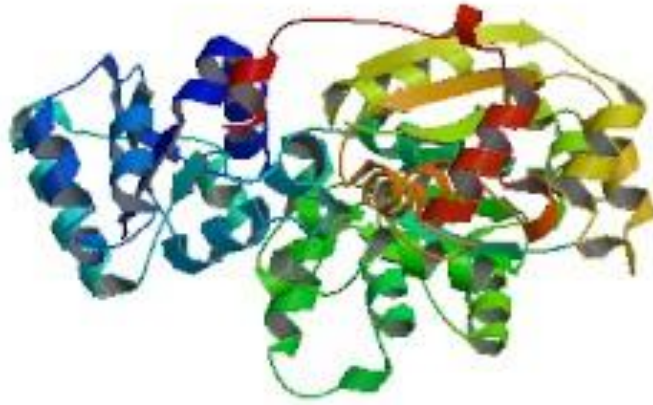


Figure 1 Ribbon Diagram of *S. aureus* (Srivastava, et al., 2011)

This image shows the hexameric structure of the bacteria *S. aureus* consisting of 3 dimers, all in 3 separate colours.

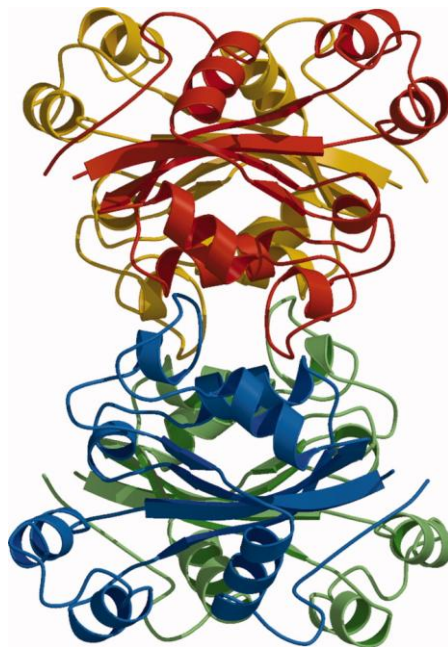


Figure 2 Ribbon Diagram of *E. coli* (L.Moynié, et al., 2007)

This image shows the tetrameric structure of *E. coli* consisting of 2 dimers.

The NDK kinase sequence is 60% homologous amongst eukaryotes and 45% homologous between eukaryotes and prokaryotes representing a very high degree of conservation (Georgescauld, et al., 2013).

Human NM23 has at least 10 isotypes, H1-H10 (Lilly, et al., 2011), -H1 to -H4 are considered to play a major role in cell development. An 88% similarity between the amino acid sequences of NM23-H1 and NM23-H2 was also found (Lacombe, et al., 2000). The high similarity between NM23 H1 and H2, allow the two isotypes to fuse together forming a hybrid (Milon, et al., 1997). This hybrid could be useful in the tumour suppressor role of NM23, because in many cancers the levels of NM23-H1 and NM23-H2 together are found to be elevated (Konishi, et al., 1993) (Godfried, et al., 2002).

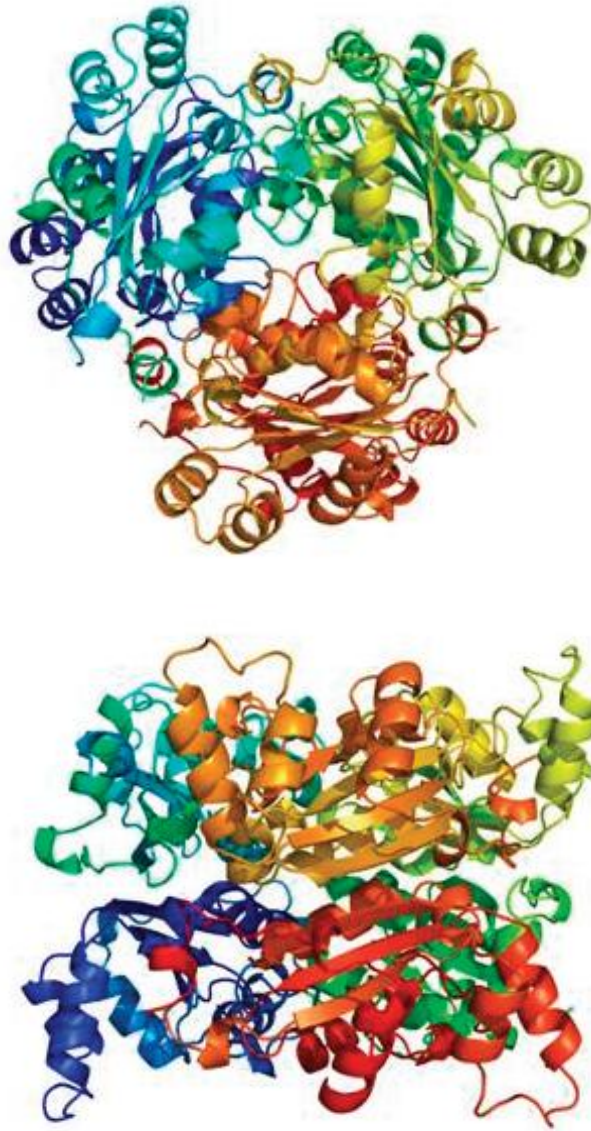


Figure 3 Ribbon Diagram of the hexameric structure of NM23 (Han, et al., 2010)

Both images show a hexameric structure of NM23, in different rotations. Both images show the 3 monomers, which make up the hexameric structure, these are all coloured differently.

1.3.1 Conserved Protein Domains of NDK

The NDK protein consists of several conserved protein domains. These domains play roles in the structure and functions of the NDK protein (Georgescauld, et al., 2013) (Tepper, et al., 1994). Current research has suggested that the c-terminal tail found in hexameric NDK structure plays a crucial role in the stability in its hexameric structure. It is also important in full enzymatic activity of the NDK protein. Breaking the salt bridges between Arg80 and Asp 93 shows a decrease in the thermal stability of the NDK (Georgescauld, et al., 2013).

Mutations in several conserved domains of *M. tuberculosis* have shown changes in the function and structural stability of the protein. A mutation in amino residues, Lys¹⁶ Arg¹⁰⁹ Asn¹¹⁹ have shown to interrupt sub-unit interactions thus decreasing the stability of the protein. The following amino acid residues Lys¹⁶, Tyr⁵⁶, Arg⁹², Thr⁹⁸, Arg¹⁰⁹ Asn¹¹⁹, Ser¹²⁴, Glu¹³³ have shown to decrease NDK activity and binding, and have been found to be conserved within all NDK proteins (Georgescauld, et al., 2013). The Kpn loop, named after the mutation 'killer of prune' in *Drosophila*, is thought to play a role in the stability of the quaternary structure of NDK (Tepper, et al., 1994).

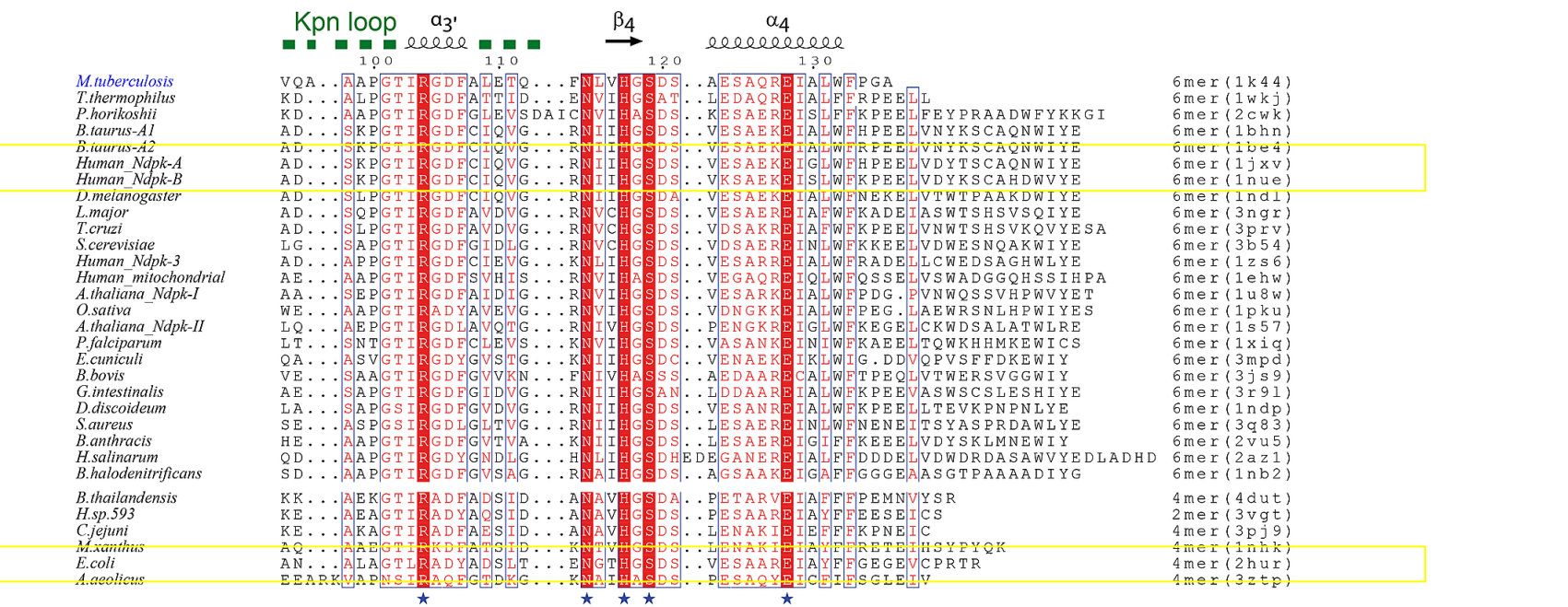
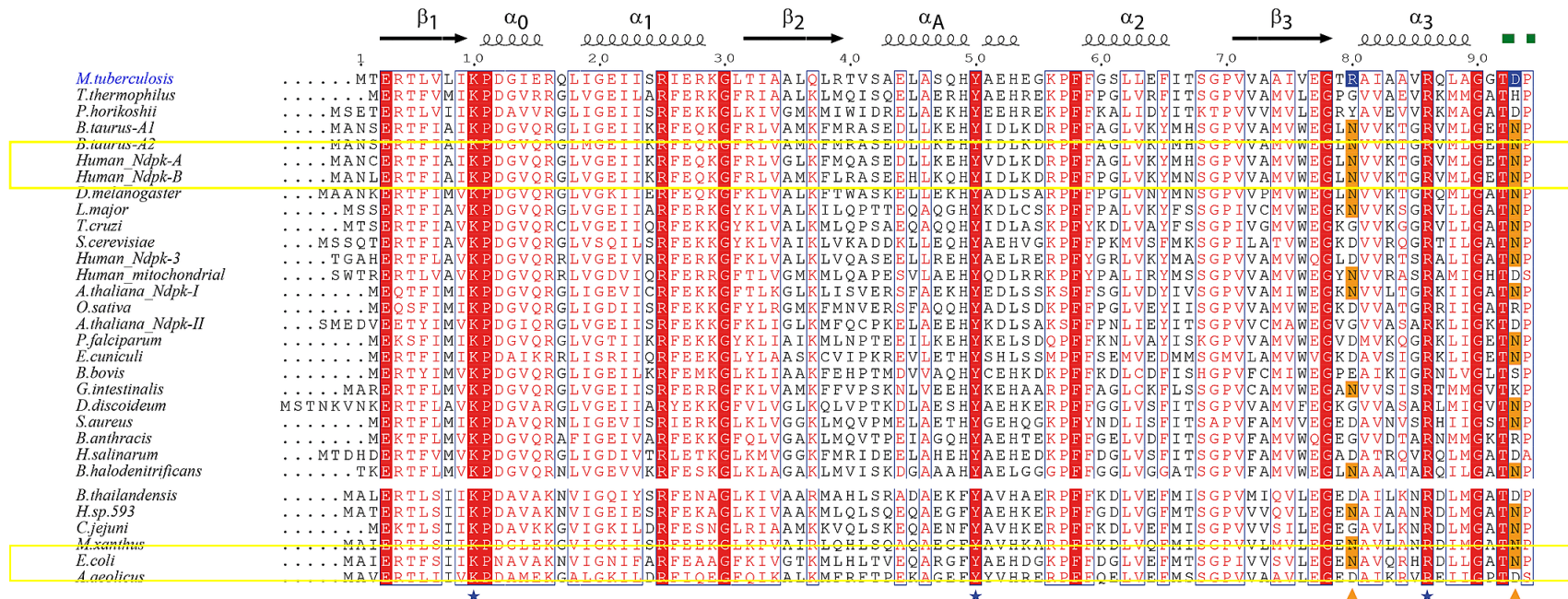


Figure 4 Sequence Alignments of NDK (Georgescauld, et al., 2013)

Figure 4 shows the sequence alignments of NDK of whose structures have been solved. Indicated in red are the conserved domains that span the whole NDK family. Active site residues are represented with a blue star. The important salt bridges are indicated by triangles on Arg80 and Asp93. Marked with a yellow box are human NM23 and *E. coli* NDK, to highlight the similarity between prokaryotic and eukaryotic NDK proteins.

1.4 NDKs association with Cancer

1.4.1 Tumour Suppressor Function

The NM23 gene and its role as metastatic suppressor was first noted in 1988 (Steeg, et al., 1988). In 1989 this gene was isolated from mouse melanoma cells by Rosengard (Rosengard, et al., 1989). A negative relationship between metastasis and gene expression was noted, and the gene named non metastatic clone 23. In 2000, this same pattern was noted in human breast carcinoma cell lines and human melanomas, suggesting NM23 plays a metastatic inhibitory role within these cancers (Hartsough & Steeg, 2000).

Research on the NM23 human NDK protein, is quite conflicting, (Postel, 2003). In certain cancers such as aggressive neuroblastomas or colorectal cancers, NM23-H1 and H2 RNA levels are in fact elevated. Because mutations in the NM23 gene are quite rare in cancers, it is thought that NM23 and its isotypes might become deregulated through expression at an RNA level rather than being activated through mutations. This would explain how an over or under expression of NM23 could be oncogenic.

The metastatic suppression quality of NM23-H1 has been studied in great depth by many researchers. In 1991, (Leone, et al., 1991) showed that metastatic k-1735 TK murine melanoma clones expressing NM23-H1 showed a decreased development in tumours. This metastatic suppression caused by NM23-H1 was also shown by many other researchers in; colorectal cancers, (Martinez, et al., 1995), in breast carcinomas (Howlett, et al., 1994) and in bladder cancers (Chow, et al., 2000).

In a study carried out by (Scambia, et al., 1996) a direct link between the associations of NM23-H1 and NM23-H2 levels were found, indicating that both proteins interact with each other to manage the metastatic suppression mechanism. In prostate carcinoma's levels of NM23-H1 and NM23-H2 were assessed; and all cases showed an increased level of both NM23-H1 and NM23-H2 expression (Konish, et al., 1993). In current research; work is being done to determine whether NM23-H1 can be over expressed in tumours to decrease its metastatic potential in patients (Junghee Lim, 2011) (Marshall, et al., 2010).

1.4.2 NM23 and haematological malignancies

Whereas in solid tumours, expression of NM23-H1 acts as a metastatic suppressor; in haematological malignancies, this does not appear to be the case. In normal haemopoiesis, progenitor cells express a high level of NM23-H1 and –H2, however as these cells mature, the levels of NM23-H1 and –H2 decrease (Willems, et al., 1998). In fact the more recent research suggests that the NM23-H1 protein can act to promote the survival of haematological cancer cells. A study by (Lilly, et al., 2011) showed how NM23-H1 indirectly promoted the survival of acute myeloid blast cells. This was shown by the over expression of NM23-H1 in cell survival assays containing CD11b +ve cells. In other studies NM23-H1 levels seem to be increased in acute haematological malignancies, but not in chronic haematological malignancies (Yokoyama, et al., 1996) (Yokoyama, et al., 1998). While NM23 clearly has an important and diverse role in cancer, the mechanism by which this occurs is currently unclear.

1.5 Haemopoiesis

1.5.1 Haemopoietic System

During the first few weeks of development, the embryonic yolk sac is where the initial haemopoietic system resides. It consists of mainly nucleated erythroid cells, and feeds the embryonic tissues with oxygen. However as the embryo grows, a more multifaceted system takes over, which reside through adult life. This haemopoietic system consists of all blood cells, including erythrocytes, myeloid and lymphoid cells (Kennedy, et al., 1997). These cells originate from multipotent haemopoietic stem cells (HSCs) and are initially seen in the aorta-gonads-mesonephros (AGM) region, which is part of the embryonic mesoderm. These haemopoietic cells also termed haemangioblasts concentrate in the liver, spleen and bone marrow (Taylor, et al., 2010). After 6 weeks gestation the liver and spleen become the vital haemopoietic organs, until about 6/7 months into fetal life, where the bone marrow becomes the most important site. During childhood and adult life the bone marrow is the most important site in which haemopoietic stem cells are produced. Developing cells inhabit the region outside the bone marrow sinus, and once they have reached maturity are released into the sinus spaces and microcirculation and then into the general circulation (Hoffbrand, et al., 2006) (Lilly, et al., 2011).

1.5.2 Haemopoietic stem cells

Haemopoietic stem cells (HSCs) are multipotent stem cells, which differentiate into the mature blood cells; erythrocytes, megakaryocytes and all lymphoid (T cells, B cells and NK cells) and myeloid cells (monocytes, macrophages, neutrophils, basophils,

eosinophils, dendritic cells). They have the ability to self renew and undergo apoptosis if cells become damaged and are important in maintaining a constant haemopoietic system throughout life (Hoffbrand, et al., 2006).

1.5.3 Haemopoietic stem cell niche

The identification and characterisation of HSCs was initially carried out in mice. It was found that when HSCs were taken out of the bone marrow and its microenvironment, they lost their ability to self renew. This indicates that HSCs are dependent on the communication between themselves and other cells in their microenvironment or 'niche' to maintain the HSC system. This initial 'niche' theory was developed by (Schofield, 1978). The discovery of the stem cell niche in the 1970's promoted further research to identify and understand the different components that make up this niche. Currently there is a debate to whether 2 HSC niches actually exist; endosteal and vascular niche (Lilly, et al., 2011).

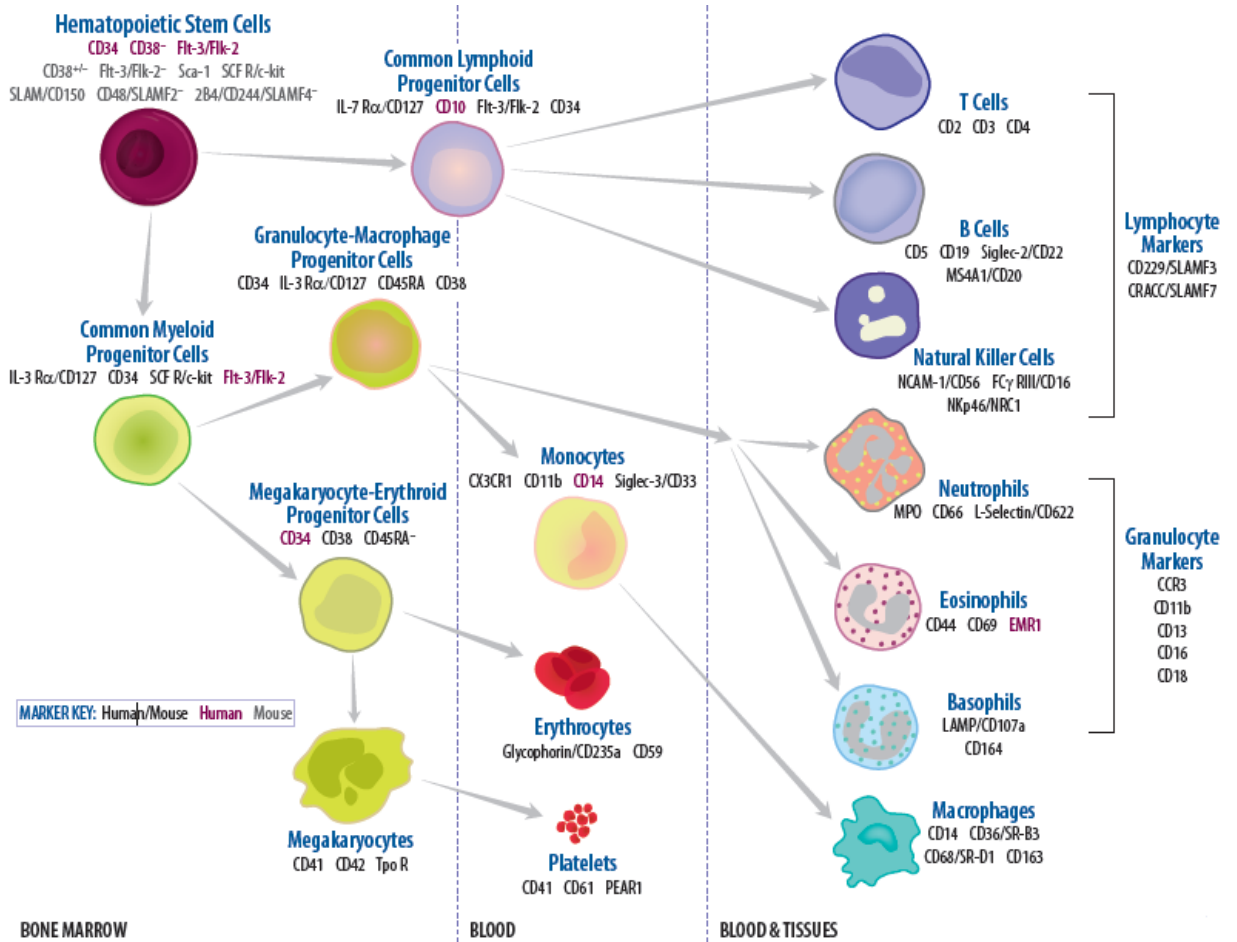


Figure 5 Haematopoietic Stem Cell and Lineage specific markers (R&D Tools for Cell Biology Research, n.d.)

Figure 5 shows the ladder of haemopoietic stem cell differentiation and the stem cell markers that can be used to identify between immune cells.

1.6 Leukaemia

Leukaemia is a cancer of blood and/or bone marrow, and is characteristic of an increased proliferation of immature white blood cells. Leukaemias are classified as acute and chronic. For the purpose of this research project, the concentration will be on acute leukaemias. Acute leukaemias are characterised by the rapid proliferation and accumulation of immature leucocytes in the bone marrow, which replace normal cells. Acute leukaemias are subdividing into lymphoid and myeloid, depending on which lineage of progenitor cells are affected. Acute lymphoid leukaemia (ALL) involved the lymphoid lineage and immature proliferation of lymphocytes. Acute myeloid leukaemia (AML) involves the myeloid lineage and immature proliferation of neutrophils, monocytes, eosinophils, basophils and megakaryocytes (BMJ, 1997).

1.6.1 Leukaemic stem cells

Morphologically the determination of ALL or AML are easy to characterise, as they are derived from different lineages. However the characterisation of ALL or AML blast cells is dependent on antigen expression (Dick, 2005). AML-stem cells express CD34 and CD38 along with a variety of myeloid antigen markers, including CD33, CD13, CD17 and CD123. (Horton & Huntly, 2012) ALL-stem cells express CD34, and antigen expression markers for T cells including CD7, CD4 CD5 and CD8 and antigen expression markers for B cells, CD19 and CD22 (Bachir, et al., 2009).

1.7 Neutropenic Infections

Leukaemia patients are prone to developing infections, due to the depletion in immune cells, due to the intensive chemotherapy treatment, and the lack of normal maturation of haemopoietic progenitor cells. In an article by (Chandran, et al., 2012) it is pointed out that even in today's clinical advances, infection remains a disastrous complication to leukaemic patients, often leading to death. Advances such as targeted therapies and the use of antibiotic therapies have shown a decline in neutropenic infections; however "infections in leukaemia remain a therapeutic challenge" (Chandran, et al., 2012).

The most common bacterial infections in leukaemia tend to be by gram negative bacteria, this include *Escherichia coli* and *Klebsiella pneumoniae*, however some gram positive bacteria are also high in causing infection in leukaemia patients such as streptococci and staphylococci bacteria. In this study, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* NDK proteins are investigated, due to its high infectious consequences to leukaemic patients (Sung, et al., 2007).

1.7.1 *Escherichia coli*

Escherichia coli (*E. coli*) are an Enterobacteriaceae that is part of the normal flora in the intestines of humans and animals. This Gram negative rod is one of the most diverse bacterial species with several pathogenic strains known to cause ill health. *E. coli* can grow in a range of conditions.

Wild type *E. coli* requires no additional growth factors, and can happily grow in media containing just glucose as a carbon source. It has the ability to convert glucose into all the vital macromolecules required to make up the cell and is very responsive to changes in environmental conditions; for example chemicals, pH or temperature. It has the ability to switch 'on' and 'off' certain functions, for example enzyme production for the synthesis of metabolites, this will only be produced if the metabolites are available in their habitat, thus using energy for processes of necessity at any given time (Madigan, et al., 2003) (Murray, et al., 2005).

E. coli is a major pathogen able to cause infection of the urinary and GI tract as well as being a leading cause of bacteremia.

1.7.2 *Staphylococcus aureus*

Staphylococci are Gram positive spherical bacteria, and part of the normal flora of skin and nasal cavity. There are over 20 species of *staphylococcus* bacteria, however only 2 are major human pathogens, *Staphylococcus aureus* and *Staphylococcus epidermidis*. *S.aureus* localise within the nasal passage, oral cavity, gastrointestinal tract (Franklin & Lowy, 1998) (Murray, et al., 2005) (Madigan, et al., 2003).

S.aureus can grow in a range of conditions and are a major cause of wound and bloodstream infections. *S. aureus* is a versatile and dangerous pathogen in humans and hospital acquired infections are still on the increase, as well as multi drug resistance *S. aureus*, posing an increased risk to immunodeficient patients (Patrick, et al., 2013).

1.7.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae are gram negative rod shaped bacteria along with *E. coli* they belong to the Enterobacteriaceae family. *Klebsiella pneumoniae* are part of the normal flora of the gastrointestinal tract, but are ubiquitous when it comes to their habitat association. In the laboratory *Klebsiella pneumoniae* grow happily in (Lysogeny) LB broth at 37°C (Murray, et al., 2005).

They are quite happy to thrive in sewage, drinking water, soil and vegetation. *Klebsiella pneumoniae* are one of the most common bacterium to cause *pneumoniae* infections. It has a mortality rate of around 50% in ICU patients due to their extracellular toxic capsule, which causes severe lung tissue damage. The majority of infections caused by *Klebsiella pneumoniae* occur in patients with a compromised immune system (Madigan, et al., 2003).

1.7.4 *Streptococcus pneumoniae*

Streptococcus pneumoniae are gram positive cocci which grow in chains. In common with all *streptococci* species they lack catalase and ferment glucose to lactic acid. *Streptococcus pneumoniae* are known to cause *pneumoniae* infections but it is also associated with bacterial meningitis. *Streptococcus pneumoniae* make up part of the normal flora in the upper respiratory tract. They grow best in 5% CO₂ cultures of *Streptococcus pneumoniae* require a source of catalase to neutralise hydrogen peroxide produced by the bacteria, where a blood source is efficient (Madigan, et al., 2003) (Murray, et al., 2005).

Streptococcus pneumoniae are prone to self destruction and contain an enzyme called autolysin. This enzyme has the ability to cause disintegration of the cell, this occurs when the bacteria hit stationary phase within their growth cycle and production of autolysin is induced making the growth of *Streptococcus pneumoniae* in the lab slightly more tedious (Madigan, et al., 2003).

2. PART A INTRODUCTION

Part A of this research project focuses on the effects of bacterial nucleoside diphosphate kinase (NDK) proteins on haemopoietic stem cells, derived from bone marrow and leukaemic samples.

2.1 Previous Studies

A study by Andrew J Lilly on the interactions between NM23-H1 and haemopoietic stem cells have shown that NM23 actually cause increased survival and proliferation of stem cells from bone marrow and umbilical cord bloods. In his PhD titled 'the significance of extracellular NM23-H1 protein in acute myeloid leukaemia and its role in regulating haemopoietic stem cell behaviour' he investigated the survival of umbilical cord blood stem cells, bone marrow stem cells treated with over expressed NM23-H1 protein. Importantly; he also investigated the affects of NM23-H1 protein on acute myeloblastic leukemic cells. A study by (Lilly, et al., 2011) shows how NM23-H1 can indirectly promote the survival and proliferation of acute myeloid Leukaemias, where it is stated that NM23-H1 has been found to act as an 'AML cell survival factor' The study incorporates investigations into the binding mechanisms of AML to NM23-H1, which was found to be through more mature CD11b+ve cells, and not directly to the CD34+ve stem cells.

2.2 Aims of the project

Considering the previous work on NM23-H1, and the similarity in structure and function of bacterial NDK bacterial proteins to human NM23-H1 proteins and the high infection rate of leukaemic patients; the survival of umbilical cord stem cells and leukaemic cells, in the presence of bacterial NDK proteins from *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* will be investigated. The over expression of bacterial NDK protein, and human NM23-H1 and elution buffer control in primary cell cultures of umbilical cord stem cells and leukaemic cells will be investigated to monitor and analyse the survival of stem cells.

I hypothesise that bacterial NDK proteins will promote the survival and proliferation of umbilical cord blood stem cells and acute leukaemic progenitor cell

3. PART A METHODS AND MATERIALS

3.1 The production of his-tagged recombinant NDK protein

3.1.1 Polymerase chain reaction

To amplify Bacterial NDK DNA for cloning, from *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. Each 1xPCR Mix (thermo scientific, (50uL) contains; 34uL dH₂O, 5uL (x10) Accuzyme Buffer, 1uL (50mM) MgCl₂, 5uL (10mM) dNTPs, 2uL NDK forward primer, 2uL NDK reverse primer, 2uL taq polymerase and 1uL NDK DNA.

Primer Sequences:

<i>E. coli</i> /K.pneu Forward	5' GTAGCATATGGCTATTGAACGTACTTTTTCCATC 3'
<i>E. coli</i> Reverse	5' GTAGGGATCCTTATTAACGGGTGCGCGGGCACAC 3'
K.pneu Reverse	5' GTAGGGATCCGATTAGCGAGTGC GCGGGCAAACC3'
S.aur Forward	5' GTAGCATATGGAACGTACATTTTTAATGATTAAAC 3'
S.aur reverse	5' GTAGGGATCCATTTTATTCATATAACCATGCATC 3'
<i>S. pneu</i> forward	5' GTAGCATATGGAACAAACATTCTTTATCATCAAA 3'
<i>S. pneu</i> reverse	5' GTAGGGATCCCTCTTAAAACCAAAGAGCAATTC 3

2 controls containing no NDK DNA were amplified. Control 1 containing forward and reverse primers for *E. coli* and *K. pneu* and Control 2 containing forward and reverse primers for *S. aureus* and *S. pneumoniae*.

PCR Conditions:

94°C	-->	5 minutes (denaturation)	
94 °C	-->	15 seconds (denaturation)	} x 40 Cycles.
60 °C	-->	15 seconds (primer annealing)	
72 °C	-->	45 seconds (primer extension)	
72 °C	-->	5minutes (final extension)	
8 °C	-->	hold, over night.	

To check the quality and quantity of amplified DNA product, after amplification 5uL of PCR NDK product was mixed with 2uL of 4x gel loading buffer (see appendix for buffer recipe) and run on a 1% agarose mini gel containing 5uL/100mL ethidium bromide (see appendix for gel recipe) and electrophoresed in running buffer (see appendix for buffer recipe) at 70V for 45 minutes. 5uL hyperladder™ IV (Bioline) added into first well, and 5ul sample added to consecutive wells. The product was observed under UV light.

3.1.2 Agarose gel electrophoresis and DNA purification

Amplified NDK products run on 1% agarose gel containing 5uL/100mL ethidium bromide. (see appendix for gel and buffer recipe) 50uL of PCR NDK product was mixed with 12uL of 4x gel loading buffer (Bioline) and electrophoresed in running buffer at 100V for 45 minutes. 5uL of Hyperladder™ IV (Bioline) added into first well and 40uL of sample was added to each consecutive well. The product was observed under UV light.

After completed gel run, NDK bands (500bp) were extracted under UV light and weighed. NDK DNA bands were purified using QIA Quick PCR purification kit (Qiagen) according to manufacturer's instructions.

3.1.3 Cloning bacterial NDK into pET15b Plasmids

pET15b expression vector (Novagen, Merck Chemicals)

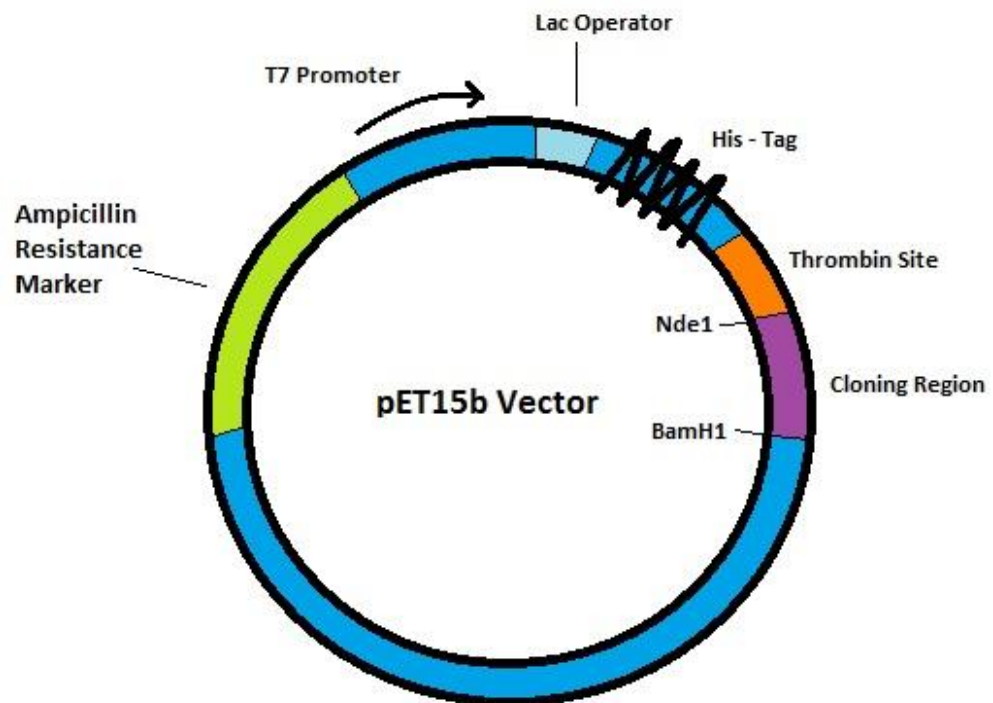


Figure 6 pET15b Expression Vector

Restriction Enzyme Digest:

Bacterial NDK PCR products and pET15b plasmid were digested with Nde1 and BamH1 restriction enzymes, in 10x R.E buffer (thermo scientific) overnight at 37°C. 1x

Restriction enzyme digest mix contains 31uL dH₂O, 5uL 10x RE buffer, 2uL Nde1 and 2uL BamH1-HF restriction enzyme, and 10uL NDK DNA.

To purify the digested pET15b vector, sample (50uL) was mixed with 12uL 4xGLB and run on a 1% agarose gel, in running buffer at 100V for 45 minutes. 5uL of hyperladder™ IV (Bioline) was added to the first wells and 40uL samples added to consecutive wells. Bands at 5Kbp were extracted and purified using QIA Quick Spin Gel Extraction Kit (Qiagen) according to manufacturer's instructions.

45uL of digested NDK PCR product was purified using QIA Quick PCR purification kit (Qiagen) according to manufacturer's instructions. Purified digested NDK are run on a 1% agarose gel, at 100V for 45 minutes.

Digested NDK DNA is sent for sequencing to check for mutations. 2uL NDK DNA mixed with 1uL 5uM Primers (forward and reverse primers specific to NDK) and 7uL dH₂O.

Sent to genomics for sequencing.

pET15b and NDK Ligation Reaction:

T4 DNA ligase is used to ligate the digested bacterial NDK protein coding region into the multiple cloning site of pET15b vector. 1x ligate mix contained; 2uL 10x ligase buffer (thermo scientific) 5uL Vector DNA, 10uL NDK DNA, 2uL T4 DNA Ligase and 1uL dH₂O. Ligation reaction left over night at room temperature.

Competent Bacterial Transformation:

Ligated NDK and pET15b vector are transformed into DH5 alpha competent *E. coli* cells, which have high transformation efficiency. Competent *E. coli* cells are thawed on wet ice. 3uL ligated NDK are added into 50uL competent *E. coli* cells, a control, 2uL vector only is added into competent *E. coli* cells, mixed well and incubated on ice for 30 minutes. Cells were heat shocked in a 42°C water bath for 45 seconds and then back on ice for 1 minute. 950uL of SOC media (Qiagen) is added and rocked at 37°C for 1 hour. Centrifuge at 15,000RPM for 5 minutes, discard 800uL supernatant and resuspend pellet in remaining fluid. 100uL transformation suspension are plated on ampicillin resistant agar plates under aseptic conditions and incubated over night at 37°C.

Colony PCR:

To screen colonies for NDK insert, 5 colonies were chosen from each agar plate. 10uL dH₂O was mixed with each colony. 2 controls, Vector only and Negative control for each bacterial NDK was mixed with 10uL dH₂O. Bacteria lysed at 95°C for 10 minutes. 1x PCR mix contains, 3.4uL dH₂O, 2uL 10x accuzyme buffer (0.6uL 50mM MgCl₂, 1uL 10mM dNTPs, 1uL Redtaq DNA polymerase, 1uL 10uM T7 polymerase primer (forward), 1uL 10uM NDK (Reverse) Primers and 10uL bacterial lysate.

PCR Conditions:

94°C	-->	5 minutes (denaturation)	}	x 40 Cycles.
94 °C	-->	20 seconds (denaturation)		
55 °C	-->	20 seconds (primer annealing)		
72 °C	-->	45 seconds (primer extension)		
72 °C	-->	5minutes (final extension)		
8 °C	-->	hold, over night.		

5uL GLB was added to PCR product, and run on a 1% agarose gel, run at 100V for 45minutes.

Miniprep DNA Purification, (Qiagen)

Colonies were selected from colony PCR, and an overnight culture was set up in 2mL L-broth containing 100uL/mL ampicillin per bacterial NDK, over night in a shaker at 37°C. Plasmid DNA was purified using a DNA miniprep purification kit, following manufacturer's instructions.

Mini prep plasmid DNA was sent for sequencing, 1x reaction mix contained; 7uL dH₂O, 2uL plasmid and 1uL either T7 promoter primer, or NDK Reverse primer.

Plasmid DNA is also checked by restriction enzyme digest. 1x digest mix contains; 15.5uL dH₂O, 5uL plasmid DNA, 2.5uL 10x Buffer 4, 1uL Nde1 and 1uL Bam-H1-HF. Incubated at 37°C for 90 minutes. 5uL digested samples were mixed with 5uL GLB, and 20uL dH₂O and 5uL hyperladder™ I (Bioline) added into first well, 15uL of digested and

undigested plasmid DNA added onto consecutive wells and run on a 1% agarose gel, run at 100V for 45 minutes.

3.1.4 Transformation into BL21 (DE3) protein expression cells

Using the sequence DNA plasmid digest results 1 colony of NDK from each bacterial species is selected. Each NDK colony is cultured overnight in 2mL Lysogeny-broth (LB) media plus 100uL/mL ampicillin in a shaker at 37°C.

A LB agar plate is streaked with each NDK, under aseptic conditions.

1.5mL overnight cultures are purified using mini prep DNA purification Kit, (Qiagen) under manufacturer's instructions.

Purified pET15b-NDK construct is transformed into BL21 (DE3) expression *E. coli* cells (Novagen) according to manufacturer's instructions. BL21 (DE3) are high level expression *E. coli* bacterial cells, which induces the T7 polymerase gene using Isopropyl-1-thio- β -Dgalactopyranoside (IPTG) (Sigma-Aldrich). BL21 (DE3) cells are thawed on wet ice. 2uL of pET15b-NDK plasmid construct was added to 50uL of competent cells. These were incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 45 seconds, and then incubated on ice for a further 2 minutes. 950uL of L-broth added to each cell suspension. Shake at 200RPM for 1 hour at 37°C. Cell suspension is centrifuged at 15,000RPM for 5 minutes, 800uL supernatant is discarded and pellet resuspended in remaining fluid. 100uL transformation suspension is plated on ampicillin resistant agar plates under aseptic conditions, (see appendix for buffer recipe) and incubated overnight at 37°C.

3 colonies were chosen and a colony PCR was done to check inserted NDK gene. Colony PCR product was run on agarose gel.

Glycerol stocks were made by adding 200uL of 100% glycerol to 800uL of BL21 (DE3) cells, and these are stored at -20°C, until used.

3.1.5 Growth Kinetics – optimisation of IPTG induction for r-NDK expression

From the glycerol stock, 7.5mL L-Broth was inoculated from each pET15b-NDK BL21 (DE3) cells and incubated overnight at 37°C in a shaking incubator. The next day, the overnight cultures are taken down to microbiology, where growth kinetic analysis was done. 20uL of bacterial culture of each bacterial NDK was added to 10mL L-broth media. 195uL of diluted culture was added to a 96 well plate, 5uL of 40mM stock IPTG (Sigma-Aldrich) was added, at differing time intervals. See table below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	EC	SA	EC	SA	EC	SA	EC	SA	EC	SA	
B	B	EC	SA	EC	SA	EC	SA	EC	SA	EC	SA	
C	B	EC	SA	EC	SA	EC	SA	EC	SA	EC	SA	
D	B	EC	SA	EC	SA	EC	SA	EC	SA	EC	SA	
E		KP	SP	KP	SP	KP	SP	KP	SP	KP	SP	
F		KP	SP	KP	SP	KP	SP	KP	SP	KP	SP	
G		KP	SP	KP	SP	KP	SP	KP	SP	KP	SP	
H		KP	SP	KP	SP	KP	SP	KP	SP	KP	SP	

	IPTG added at:		
	No IPTG	EC	<i>E. coli</i>
	0 Hours	KP	<i>K.pneu</i>
	2 hours	SA	<i>S.aur</i>
	5 hours	SP	<i>S.pneu</i>
	8 hours		
Blank	No IPTG		

Table 1 1 IPTG Induction of bacterial NDK at differing time intervals

pET15b-NM23-H1 BL21 (DE3) cells and BL21 (DE3) cells were used as control/comparable samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	NM23	BL21	NM23	BL21	NM23	BL21	NM23	BL21	NM23	BL21	
B	B	NM23	BL21	NM23	BL21	NM23	BL21	NM23	BL21	NM23	BL21	
C	B	NM23	BL21	NM23	BL21	NM23	BL21	NM23	BL21	NM23	BL21	
D	B	NM23	BL21	NM23	BL21	NM23	BL21	NM23	BL21	NM23	BL21	

	IPTG added at:
	No IPTG
	0 Hours
	2 hours
	5 hours
	8 hours
Blank	No IPTG

Table 2 IPTG Induction of NM23-H1 and BL21 (DE3) only at differing time intervals

Following the optimisation of IPTG induction, 2mL Bacterial over night cultures were diluted the next day in 100mL L-broth Media (1 in 50 dilution). These were incubated for approximately 4 hours until OD_{600nm} had reached 0.4. (Optimum for IPTG induction). At time points of every hour, (up to 6 hours) and then the next day (18 hours) 1mL of culture was centrifuged at 15,000RPM for 1 minute, supernatant discarded and pellet resuspended in 100uL of 1xGLB.

Each time point sample for each pET15b-NDK construct was run on a 1.5% acrylamide gel. (See appendix for recipe) bacterial pellet in 1x GLB buffer is boiled for 5 minutes, to denature proteins. 5uL of Protein Marker is added into first well of each gel, and

50uL of bacterial sample at each time point is added to consecutive wells. Gels were run at 120V for 1hr 30minutes. Gels were stained in Coomassie blue stain (See appendix for recipe), for 2 hours and de-stained in 7% acetic acid and 5% methanol over night.

3.1.6 Expression and His-tag Purification of r-NDK proteins

pET15b-NDK BL21 (DE3) cells are cultured over night in 5mL of L-broth media with 100 mg/ml ampicillin at 37°C at 220 RPM. The next day 2uL of culture is diluted into 100mL (1 in 50 dilution) of L-broth media with 100 mg/ml ampicillin, and incubated 37°C, at 220 rpm for 4/5 hours, until OD_{600nm} reaches 0.4. 1mmol IPTG is added, and incubated overnight (18 hours) at 37°C at 220 rpm.

The next day, cultures are spun down at 15,000RPM for 10 minutes, supernatant is discarded, and wet pellet is weighed. Bacterial pellet was resuspended in 5mL/1g weight pellet of BugBuster™ (Novagen). 1uL/1mL benzonase is added to suspension, and is incubated at 50rpm for 1 hour to lyse bacterial cells and release NDK protein.

His-tagged purification kit (Novagen) is used to purify the His-tagged NDK protein from the lysed suspension, following manufacturer's instructions. 2mL His bind resin is added to chromatography column, once storage buffer has moved through the column, 3mL dH₂O is added to the column, followed by 5mL charge buffer, and then 3mL binding buffer. Once binding buffer has moved through the column, lysed bacterial suspension is added to the column. Once the bacterial suspension has moved through the column, 10mL binding buffer is added, and then column washed in 6mL

wash buffer. His-tagged protein is then eluted in 1mL fractions in elution buffer. *Buffers are made up under manufacturer's instructions.*

30uL of each fraction is mixed with 10uL 4xGLB, samples are boiled for 5 minutes, and run on a 1.5% acrylamide gel. 5uL protein marker (Bioline) is loaded into first well, and 20uL of each fraction is loaded into consecutive wells. Gels are run at 120V for 1 hour 30 minute. Gels are stained in Coomassie blue stain for 2 hours and destained in 7% acetic acid and 5% methanol overnight. Protein fractions are observed for quantity and purity of protein present. The concentration of protein in each fraction was determined. Protein concentration was further determined using western blot analysis.

3.1.7 Protein Determination

Fractions with most protein are pooled together. 2uL of 0, 0.625, 1.25, 2.5,5 and 10ug protein standards are added to a 96 well plate. 2uL of NDK protein are added to a 96 well plate. 20uL of Reagent S (Thermo scientific) is added per 1mL of Reagent A (Thermo scientific). 25uL of Reagent A+S is added to each well. 200uL of Reagent B (Thermo scientific) is added to each well and incubated at room temperature for 15 minutes. Plate is read on a plate reader, measuring OD_{645nm} . Protein concentration is determined in excel. Standard curve is plotted and equation taken from trend line, to determine concentration of protein. Proteins are then diluted to 200ug/mL and stored in the cold room until used.

3.2 NDK Protein Analysis

3.2.1 Western blot analysis

15% gels are prepared, consisting of an 8mL resolving layer and a 2mL stacking layer, made to 1.5mm thickness. The 1.5mm plate and small glass plate are held together in a casting frame and stand. The resolving layer (see appendix for recipe) is poured into the plate, and 600uL of dH_2O is added on top to ensure a straight surface. Once the gel has set (~10minutes) the stacking layer is added, (see appendix for recipe) and 1.5mm comb inserted carefully to ensure no air bubbles. Protein samples are boiled for 5 minutes to denature protein bonds and unfolded proteins and are run on the gel at 120V.

After gel has run, protein gels are transferred onto a PVDF membrane, using a semi-dry transfer tank. PVDF membrane is rehydrated with methanol for 3 seconds, and then washed in dH_2O , before transferred into transfer buffer (see appendix for recipe). 12x 3mm filter paper is also soaked in transfer buffer. 6 pieces of filter papers are layered onto the transfer tank, then the PVDF membrane is added, on top of which the protein gel is layered, and lastly another 6 pieces of filter paper. This is transferred for 1 hour at 25V.

After the transfer the membrane is blocked in 5% milk for 1 hour on the rocker at 20RPM. Membranes are then washed in TBS-T (see appendix for recipe) x3 for 5 minutes. Membranes are then probed with primary antibody; (Santa cruz) NM23-H1

antibody is added 1:1,000 dilution into 5% milk solution and incubated overnight in the cold room on the rocker at 20RPM. Membranes are washed x3 in TBS-T and probed with secondary anti-rabbit, diluted 1:1,000 in 5% milk. Membranes were incubated for 2 hours on rocker at 20RPM. Membranes were washed x3 in TBS-T and then 1.5mL

Supersignal West Pico Chemiluminescent substrate (Pearce), Stable peroxide solution and Luminol/Enhancer solution were added in equal parts were added onto the membranes, for 5 minutes. Excess solution was drained and membranes were wrapped in cling film. Membranes were placed into a film cassette and photographic film exposed to the membrane and developed in the dark room.

3.2.2 Coomassie blue staining

This technique can be used to determine purity of protein present on a gel. SDS page protein gel is stained in Coomassie blue dye (see appendix for recipe) for approximately 5 hours, until gels are sufficiently stained blue. They are then de-stained in 7% acetic acid and 5% methanol over night. Gels are then viewed under UV light.

3.2.3 NDK cross reactivity with NM23 Antibody

12ul of each NDK protein is mixed with 4uL 4xGLB, and run onto 3 1.5% acrylamide gel; 4uL of protein is added into wells. Each gel consists of NM23, *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* protein and run at 120V for 1 hour and 30 minutes. Following the western blot protocol, 3 different NM23-H1 antibodies are used to determine the cross reactivity with NDK protein.

3.3 Primary sample purification and cell culture

Umbilical cord blood (CB) samples were provided by the Tissue Bank, University of Birmingham and Leukaemic samples are provided by School of Dental and Medical sciences, University of Birmingham. The umbilical cord blood samples and leukaemic sample are handled under the approval by the ethical committee.

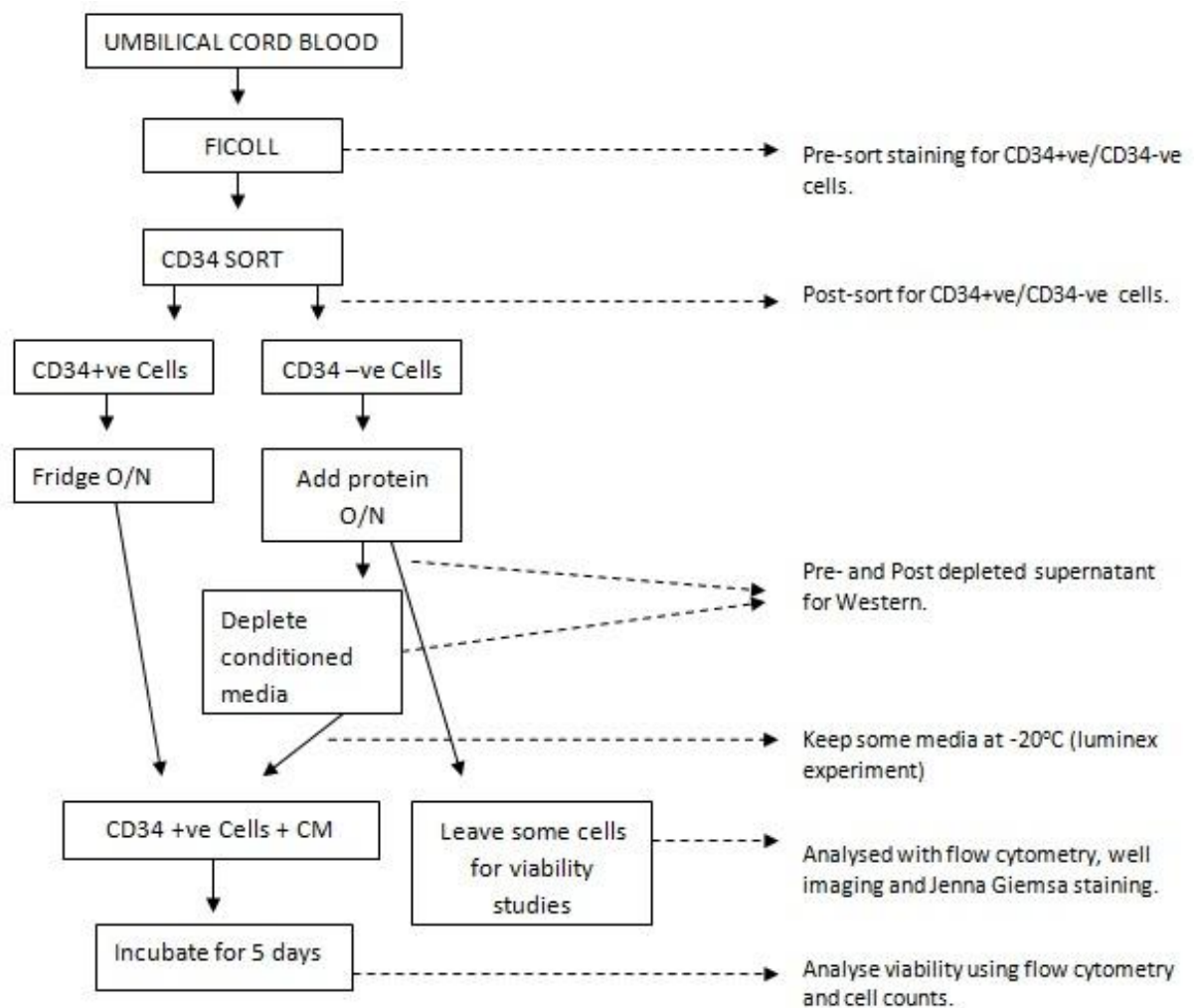


Figure 7 Schematic Diagram of Primary Sample Preparation and CD34-ve/CD34+ve viability studies.

3.3.1 Ficoll-paque™ Purification

Umbilical cord blood samples were diluted 1 in 2, into RPMI 1640 media (Sigma Aldrich), plus 5mL of Penicillin Streptomycin. 12mL Ficoll-paque™ PLUS (G.E.Healthcare, UK) is added into 50mL tubes and 30mL of umbilical cord blood is gently layered on top. Samples are centrifuged at 1340RPM for 40 minutes with no break. Supernatant is pipetted off, and mononuclear layer is retrieved carefully and added into fresh 50mL tubes.

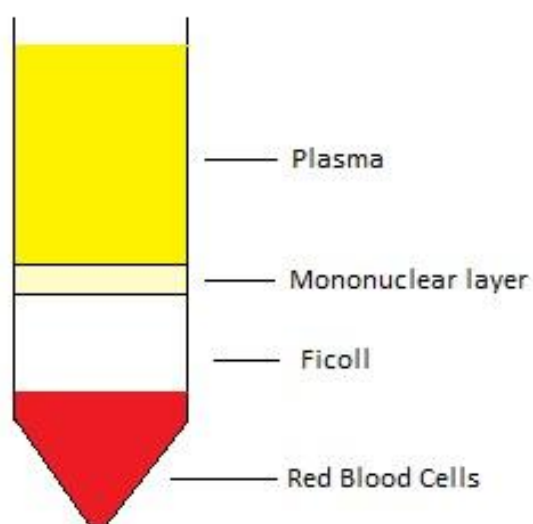


Figure 8 Ficoll-paque™ Purification

Mononuclear cells are collected carefully from the separated Ficoll tube. Mononuclear cells are washed 1 in 3 with RPMI media, (Sigma Aldrich) and centrifuged at 374g for 10 minutes. Supernatant is discarded and resuspended in 30mL of RPMI Media (Sigma Aldrich) and centrifuged at 249g for a further 10 minutes. This is repeated. The

supernatant discarded and cells are resuspended in 10mL RPMI Media (Sigma Aldrich) with Insulin-transferrin-sodium (ITS+). Leukocytes are counted using haemocytometer.

3.3.2 CD34+ve cell sorting

The mononuclear cells are sorted for CD34+ve using CD34 Microbead kit (Miltenyi Biotec UK) following manufacturer's instructions. Cells are centrifuged in RPMI Media (Sigma Aldrich) at 300g for 10 minutes, and resuspend in 400uL/10⁸ cells in cold (4oC) Magnetic Activated Cell (MACs) buffer. (see appendix for recipe). 100uL/10⁸ cells of FcR block is added, and 100uL/10⁸ cells CD34 Hapten-antibody are added to cell suspension in MACs buffer. Mix well and incubate at 4°C for 15 minutes. Cells are washed in 5mL/x10⁸ cells MACs buffer, centrifuged at 300g for 10 minutes. MACs buffer is discarded and cells are resuspended in 400uL/10⁸ cells of MACs buffer. 100uL per 10⁸ cells of anti-hapten microbeads are added to cell suspension, and centrifuged at 300g for 10 minutes. Cells are washed in 2mL/10⁸ cells, MACS buffer and centrifuged at 300 g for 10 minutes and then resuspended in 500uL/10⁸ MACs buffer. Following manufacturer's instructions, cells are sorted using MACs columns. Dependant on cell number MS or LS columns are used.

Column	Max. No. of labelled cells.	Max. No. of total cells.
MS	10 ⁷	2x10 ⁸
LS	10 ⁸	2x10 ⁹
XS	10 ⁹	2x10 ¹⁰

Figure 9 MAC Columns

Column is rinsed with MACs buffer (500 µl for MS, 3 ml for LS) cell suspension is added and elutes are collected into a fresh 50mL tube. Column is washed 3 times with MACS buffer; this is collect into the same tube. These are the CD34-ve cells.

Column is removed from the magnet and MACs buffer added into the column, a plunger is used to flush through the CD34+ve cells into a fresh 50mL tube. The CD34-ve cells are also sorted further, using the same protocol, but to purify for CD11b+ve cells. CD34+ve and CD11b+ve cell suspensions are centrifuged at 300g for 10 minutes and resuspended in 1ml RPMI 1640 (Sigma Aldrich) plus ITS+ per 10^6 cells. The CD34+ve cells are stored in the fridge overnight.

The purity of CD34+ve cells were analysed using Fluorescent activated cell sorting (FACs).

3.3.3. CD34-ve Cell treatment

CD34-ve/CD11b+ve cells are seeded at $\times 10^6$ /mL. 1mL of cell suspension is added into each well on a 48 well plate. The seeded cells were treated with 2ug/ml of rNDK proteins from NM23-H1, *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* as well as an elution buffer (Novagen) control. These are then incubated overnight (~20hours) at 37°C in 5% CO₂ environment.

The following day the treated CD34-ve/CD11b+ve cells are depleted of protein. 100uL of HIS-Select Nickel affinity Resin (Sigma Aldrich) is required per mL of cell suspension. 1mL of conditioned media is removed for each NDK treatment. The remaining CD34-

ve/CD11b+ve treated cells (named sample 1) are left in the incubator for 11 days to study the viability of CD34-ve cells.

3.3.4 Protein Depletion

600uL HIS-Select nickel affinity Resin (sigma Aldrich) is removed from storage buffer, centrifuged at 15,000 RPM for 5 minutes, and washed in 1mL dH₂O, centrifuged at 15,000RPM for 5 minutes. dH₂O is discarded and resin washed in 500uL of equilibrium buffer, (see appendix for recipe) centrifuged at 15,000 RPM for 5 minutes. Supernatant is discarded and resin resuspended in 600uL of equilibrium buffer. 100uL of resin transferred into labelled eppendorfs for each protein, centrifuged at 15,000RPM for 5 minutes and equilibrium buffer removed. Conditioned CD34-ve media is added to eppendorfs corresponding to NDK protein labels. Mix well, and rotate in the cold room for 30 minutes. Resin is removed by centrifugation at 15,000 RPM for 5 minutes. Supernatant is added into fresh eppendorfs labelled for NDK protein. 100uL of depleted conditioned media is mixed with 25ul 4x GLB, to run on acrylamide gel. 20uL of sample is inserted into each well, and run at 120V for 1 hour and 30 minutes.

3.3.5 CD34+ve Cell treatment

CD34+ve cells from the fridge are counted using the haemocytometer. Centrifuge at 300g for 10 minutes and resuspend at $\times 10^6$ /mL in rNDK protein depleted conditioned media. Cell suspension are added into a 96 well plate (200uL per well) and incubated at 37°C, 5% CO₂ for 5 days.

3.3.6 Leukaemic cell treatment

Leukaemia samples are washed in 3x RPMI 1640 media (Sigma Aldrich) with Pen/strep at 374g for 10 minutes. RPMI is discarded and cells resuspended in 15ml of RPMI and centrifuged at 249g for 10 minutes x2. Cells are counted using a haemocytometer, and resuspended in RPMI Media (Sigma Aldrich) with ITS+ and Pen/strep, at $\times 10^6$ cells per mL. Samples are added into 96 well plates, and 200uL added into each well. 2uL of 200ug/mL protein stock are added to wells. Each protein sample added to separate wells, and survival of leukaemic cells are determined after 5 days incubating at 37°C in a 5% CO₂ environment.

3.4 Flow cytometric Analysis

Identification of cell viability is detected using cell surface markers. Antibodies corresponding to primary cells of interest are incubated with cells and then analysed using FACs.

Primary cells are stained for CD34+ve cells using CD34 APC antibody (miltenyl biotec, UK) before they are sorted. Post sorted primary cells are also stained for CD34+ve cells and CD34+ve cells are harvested after 5 days of treatment with NDK protein, and stained for CD34 surface marker. CD34-ve cells are stained for CD34, CD11b, CD19, CD3, CD14, CD14, and CD33 (miltenyl biotec, UK) to identify the viability of cells after culturing with NDK protein for 11 days.

100uL of each protein treated cells are mixed with 5uL FcR block, and 2uL of CD34 APC antibody (miltenyl biotec, UK). Samples are incubated in the dark for 10 minutes, and

then washed in 2mL PBS and centrifuged at 2,000 RPM for 5 minutes. Supernatant is discarded and 200uL FACS Fix is added to stained cells. Samples are transfer into FACS tubes and stored in the cold room, under foil, until processed. This process is repeated for each NDK treated harvested cell suspension, to stain for APC isotype control (miltenyl biotec, UK); 2uL of CD34 APC antibody is replaced with 2uL of APC Isotype (miltenyl biotec, UK).

For the identification of CD34-ve cell viability a cocktail of antibodies are made. 1st cocktail contains 70uL of FcR block, 30uL of CD19 APC, CD3 FITC, and CD14 PE (miltenyl biotec, UK). 2nd cocktail contains 70uL FcR block, 30uL of CD33 PE and CD15 FITC (miltenyl biotec, UK). 3rd cocktail contains 70uL FcR block, 30uL of CD11b PE and CD34 APC (miltenyl biotec, UK). The 4th cocktail contains isotypes, 30uL of APC, PE and FITC and 70uL FcR block (miltenyl biotec, UK).

700uL of CD34-ve cell suspension is harvested for each NDK treatment. These are split into 4 eppendorfs, 200ul into 3 eppendorfs and 100uL into the 4th eppendorf. Each eppendorf is marked corresponding to the cocktails. 11uL of cocktail 1 is added into eppendorf 1, 9uL of cocktail 2 into eppendorf 2, 9uL of cocktail 3 into eppendorf 3 and 11uL of cocktail 4 into eppendorf 4. These are then incubated and protocol followed as above.

Acute lymphoid leukemic cells are stained for CD19 and CD34 surface markers (miltenyl biotec, UK), and Acute myeloid leukemic cells are stained for CD33 and CD34 surface markers (miltenyl biotec, UK), following the protocol as above.

Before FACs analysis, 10uL of cytoCOUNT beads (Miltenyi Biotec, UK) are added into each tube.

3.5 Microscopic Images of CD34-ve treated cells

Before CD34-ve cells are harvested, microscopic images are taken using a camera to capture the viability of cells treated with NDK proteins. After harvesting microscopic images are taken of Jenna Giemsa stained slides to study the morphology of treated and untreated cells.

3.6 Jenna Giemsa Staining

CD34 –ve cells are stained with Jenna Giemsa so that the morphology of the cells can be studied. After 11 days the CD34-ve cells treated with the NDK proteins are harvested. 100uL of cell suspension is added into a cytospin chamber, and centrifuged for 3 minutes at 500 RPM. Slides are left to air dry until the next morning.

The next morning slides are fixed in 50uL of methanol and left to air dry until all methanol has evaporated. Giemsa buffer (see appendix for recipe) is diluted 1 in 25 in dH₂O into 200mL. Jenna stain is made up 1 in 3 in Giemsa buffer into 50mL per coplin Jar. Jenna stain is transferred into the coplin Jar and fixed slides are inserted, and left to stain for 5 minutes at room temperature. Slides are washed thoroughly in dH₂O until colour runs clear. Giemsa stain is diluted 1 in 20 in Giemsa buffer in 50mL per coplin. Giemsa stain is transferred into the coplin Jar, and slides are stained for 5 minutes at room temperature. Slides are washed thoroughly in dH₂O until water runs clear. Slides are left to air dry before mounting in Depex.

4. PART A RESULTS

4.1 Cloning of bacterial NDK DNA into pET15b expression vector

Bacterial NDK DNA from *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were PCR amplified using modified primers (A), specific to the NDK gene. Amplified PCR products (D) were gel purified and digested using Nde1 and BamH1 restriction enzymes along with the pET15b-NM23-H1 plasmid (B), producing an empty vector (C), ready for the ligation of NDK DNA.

The NDK DNA digestion produces blunt ends, which will ligate into the pET15b plasmid. Ligates of pET15b plasmid and NDK DNA were transformed into DH5 alpha competent *E. coli* cells. These were grown over night on ampicillin resistant agar plates. The pET15b vector contains an ampicillin resistant gene, which is used to select colonies which have successfully been ligated. Successfully colony growth indicates the bacterial NDK has been ligated into the pET1b5 plasmid, and therefore grows on an ampicillin resistant plate. Any bacterial containing unsuccessful ligations will not have grown. Colonies were selected and screened for NDK insert, by colony PCR (F).

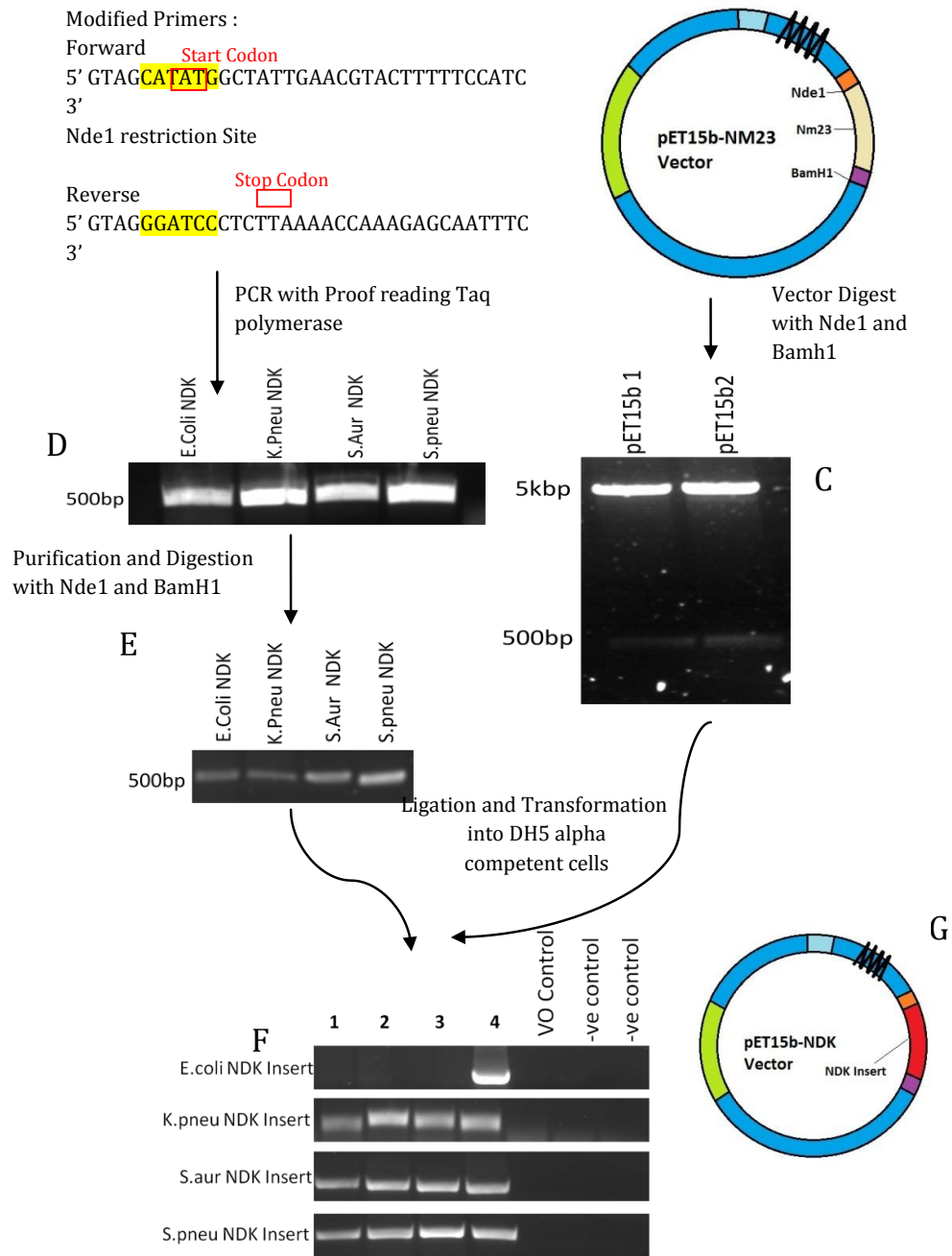


Figure 10 Schematic Diagram of Bacterial NDK Cloning into pET15b Vector

Figure (A) shows modified primers used for amplification of NDK DNA, for full primer sequences see appendix. (B) Shows pET15b-NM23 vector, which is digested with Nde1 and BamH1 restriction enzymes (C). Amplified NDK DNA (D) are digested and purified (E), and ligated with pET15b Vector and transformed into DH5alpha cells. Colony PCR is performed (G) to screen bacterial colonies for NDK insert. Figure (G) shows pET15b-NDK construct.

4.2 Sequence Alignments as confirmation of NDK cloning into pET15b plasmids

pET15b-NDKs were sequenced, and aligned with existing NDK sequences using BLAST to ensure the sequences were 100% correct. These were then translated into their amino acid sequence, using EXPASy translate. Successful protein sequences for the four bacterial strains are shown below.

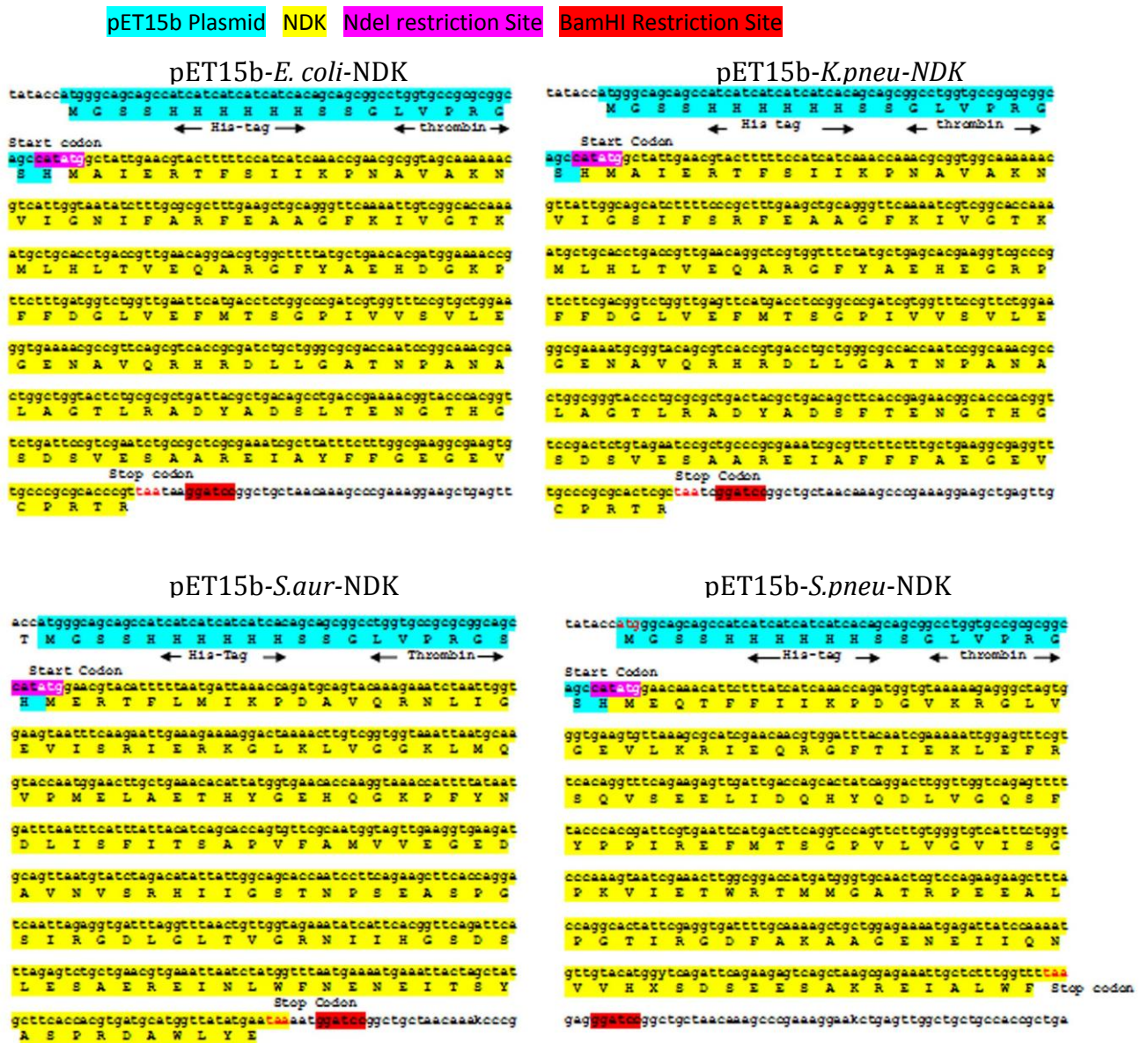


Figure 11 sequence alignments of DH5alpha transformed pET15b-NDK ligates

4.3 Optimisation of IPTG induction of NDK Proteins in BL21 (DE3) expression cells.

pET15b-NDK in DH5 alpha competent cells, were transformed into BL21 (DE3) protein expression cells. Colonies were selected, screened using colony PCR. A successful colony for each bacterial NDK was selected and an overnight culture grown.

Bacterial growth kinetics was done on these cultures, to identify the optimum time for induction with IPTG, for the expression of protein. IPTG induces lac operon. It binds to the lac repressor and deactivates it, allowing transcription of NDK gene to occur. Optimisation of induction allows for the maximum yield of protein.

From the results (figure 12), a time frame between 2-5 hour growth prior to induction, showed to have the best induction affects on BL21(DE3) cells for the expression of NDK protein. Induction at 0 hours showed to increase the lag phase of the bacteria, and induction at 8 hours showed to create a bigger environment change during bacterial growth phase indicated by the dip on the graph. However despite different timed inductions, each bacterial growth reach a similar OD as it reaches stationary phase.

It was decided for experimental purposes that prior to induction bacteria will be left to grow for 4 hours, before induction with 1mmol of IPTG.

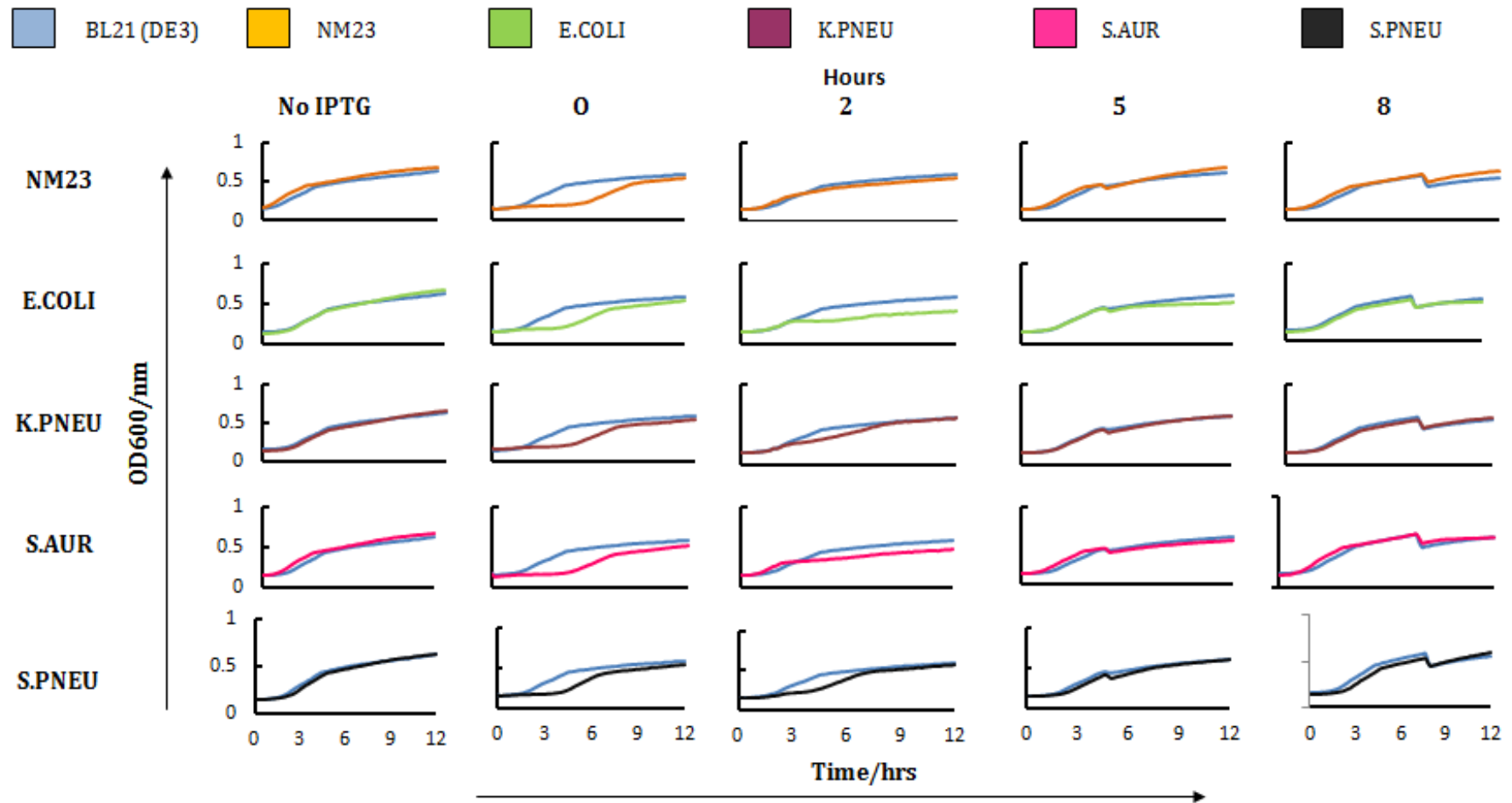


Figure 12 Optimisation of IPTG Induction

Figure 12 shows the induction of IPTG over 5 different time points for BL23 (DE3) NDK proteins from *E. coli*, *K. pneu*, *S. aur* and *S. pneu*. A BL21 (DE3) only induction is used as a control, and is indicated by the blue plot in each graph

4.4 Expression of r-NDK proteins in BL21 (DE3) expression cells

BL21 (DE3) pET15b-NDK plasmids were induced at 4 hours after growth, and at every hour, protein expression was monitored. From the figure below (figure 13) you can observe that BL21 (DE3) continuously expressed protein, up to 18 hours. Results from BL21 (DE3) protein expression, illustrates protein expression is not inhibited, at any point during the time measured, and therefore BL21(DE3) bacteria can be induced and left over night to express protein.

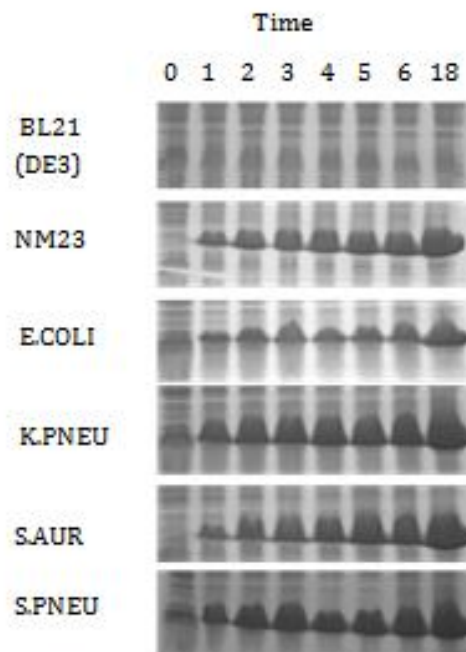


Figure 13 Acrylamide Gels of Protein Expression over 18 Hours

Figure 13 shows the expression of bacterial NDK after induction with 1mmol IPTG, at every hour until 6 hours, and then the next day at 18 hours. Bacterial culture was mixed with GLB, denatured and run on the gel to observe protein quantity, using Coomassie blue staining.

4.5 His-tagged Purification of r-NDK proteins in BL21 (DE3) expression cells

Overnight cultures are diluted 1 in 50, left to grow for 4 hours and induced with IPTG, protein expression occurs over 18 hours. Bacteria are lysed and proteins expressed using his tag affinity chromatography. Purified fractions of protein were run on a gel, to determine quantity of protein within each fraction. From the results below, fraction 1 and 2 were pooled together, and protein determination do

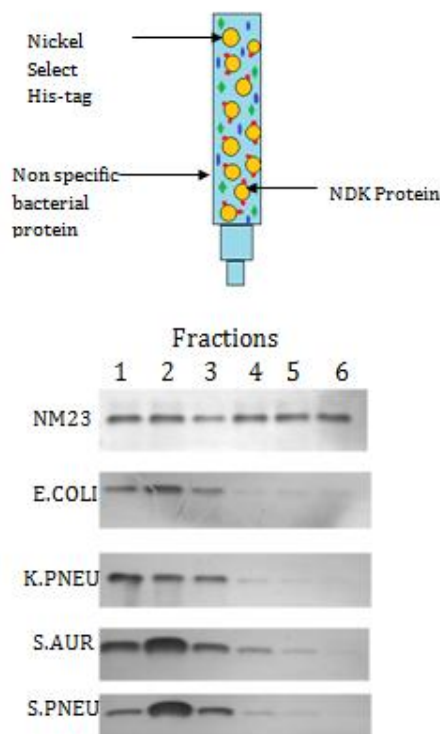


Figure 14 Purified Fractions of NDK Protein

Figure 14 shows the elution of his-tagged NDK protein through the chromatography column. Elution is split into 1ml fractions, where fraction 1 is first off the column and fraction 6 last of the column.

4.6 Western Blot Analysis of NM23 antibody cross reactivity with NDK proteins

Cross reactivity of 3 NM23-H1 antibodies were determined, Santa Cruz biotech C-20, NME1/NDKA Cell Signalling, and Gentex NM23, using western blot analysis. The results below show that both the Santa Cruz and Gentex antibodies cross react with bacterial NDK protein. This allows us to use Gentex or Santa Cruz antibody to determine concentration of bacterial NDK proteins and investigate NDK expression in cells.

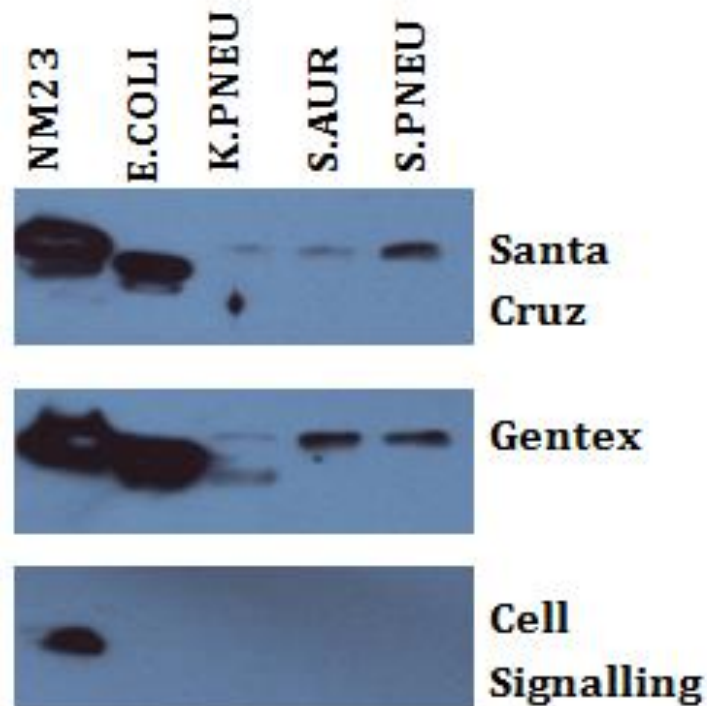


Figure 15 Western Blot analysis to show cross-reactivity of NM23 antibody with bacterial NDK Protein

Figure 15 shows the cross reactivity of bacterial NDK proteins with 3 NM23 antibodies. 1ug of NM23 protein and 5ug of bacterial NDK protein was added. Film was left to exposure for 20 seconds.

4.7 Determining Purity of CD34+ve cell sorts (Pre and post sorts of cord bloods)

Umbilical cord blood samples were sorted for CD34+ve stem cells. A pre and post sort stain/FACs analysis was run to determine the purity of the CD34+ve sorts. The plots below compare side scatter with CD34 APC staining. Side scatter is an indication of the granulation of cell cytoplasm, and this can be used to determine cell maturity, where low granulation occurs in immature cells, and high granulation in more mature cells. CD34 is a stem cell marker, and occurs on haemopoietic progenitor cells. In the post sort CD34+ve APC Plot (figure 16), you can see that CD34-ve cells are also present, indicating that the purity of the cell sorting is not sufficient.

Table 3 shows the percentage purity of CD34+ve cells in the sorts, which range from 43-75%.

Umbilical Cord Blood Samples	% Purity of CD34+ve Cells
CB15	72.11
CB16	43.35
CB17	57.57
CB18	53.55
CB19	48.92

Table 3 Percentage purity of CD34+ve cells

Table 3 shows the percentage of CD34+ve cells in the post sort cell suspension.

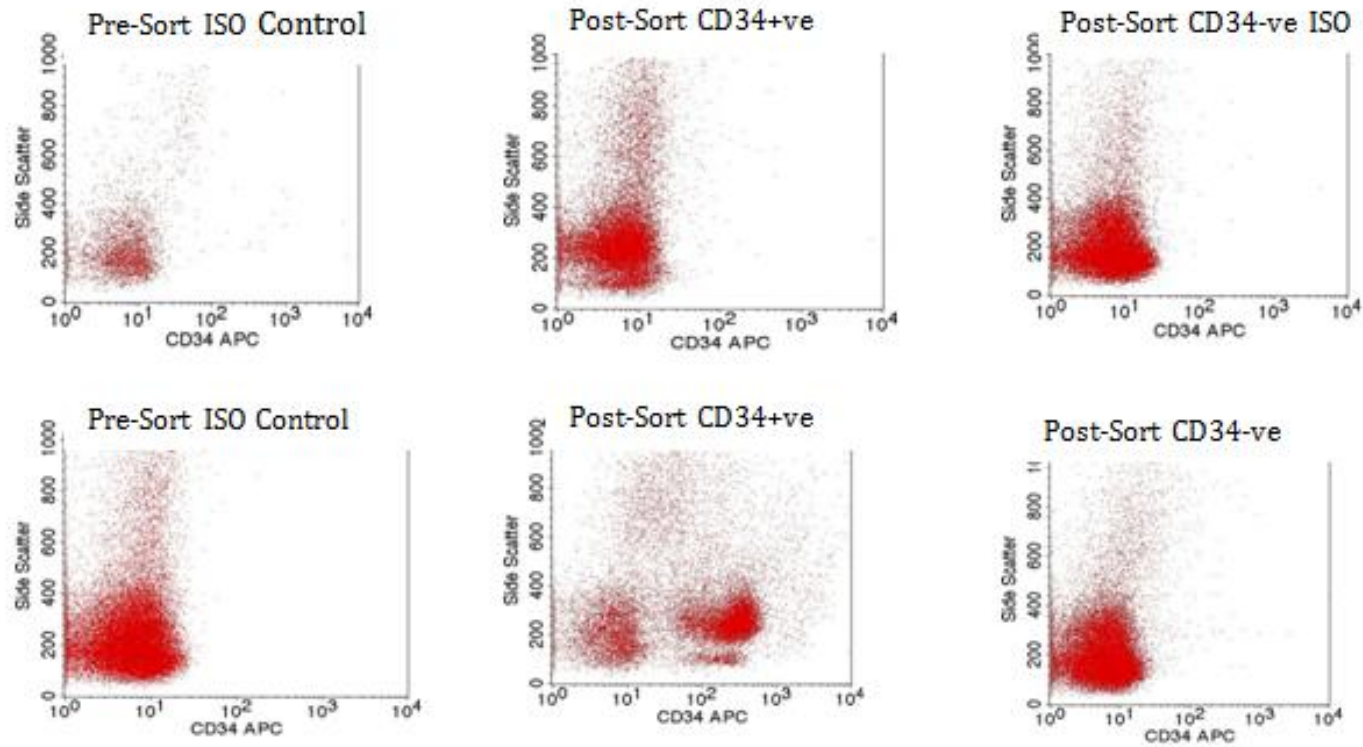


Figure 16 Dot Plot to show purity of CD34+ve Post Sorted Cells

Representation of Pre and Post sort data, of umbilical cord blood samples. FACS analysis of side scatter vs. CD34 APC, shows negative ISO type controls, and shows the presence of CD34+ve cells post sort and Post sort CD34-ve elute shows a nice negative population for CD34+ve cells.

4.8 His-tagged Protein Depleted conditioned media

CD34-ve NDK protein conditioned media, were depleted before the conditioned media was put onto the CD34+ve cells. The depletion of bacterial NDK proteins was checked using western blot analysis. Due to weak cross reactivity of *K. pneu*, *S. aur* and *S. pneu* with 5ug of protein, protein depletion was checked on elution buffer control, NM23 and *E. coli*. Figure 17 below indicates a successful depletion, as no NDK protein was observed in the wells where depleted protein samples were added.

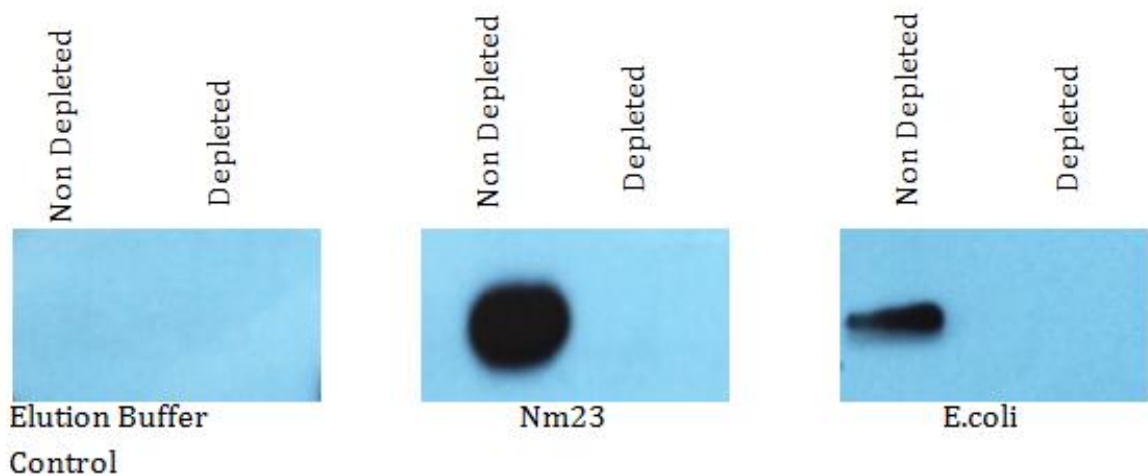


Figure 17 Western Blot Analysis of Depleted and Non Depleted NDK protein

Figure 17 shows the depletion of NM23 and *E. coli*, using 1:1,000 dilution of Santa Cruz biotech NM23-H1 (C-20) antibody. None depleted samples contain less than 0.1ug of protein.

4.9 Bacterial NDK proteins indirectly promotes the survival of CD34+ve cells.

After 5 days of culturing with depleted conditioned media which had been exposed to the NDK proteins. The CD34+ve stem cells were FAC fixed and were screened under fluorescent activated cell sorting, to count the number of viable cells. CD34+ve cell surface markers are representative of primitive progenitor cells of haemopoietic lineages, which are capable of self renewal.

Figure 19, shows the significant difference in CD34+ cell numbers between elution buffer control cultures and the cultures which were exposed to NDK protein from the 4 bacterial species. A clear trend can be seen on the graphs.

If we take into account the significance our the data obtained however, the p value indicates that the only significant result which accepts our hypothesis is that NM23-H1 protein significantly increases the survival of CD34+ve cells compared with the elution buffer control. The p value for *E. coli* NDK protein indicates the significance of the results may be borderline significant, but still not enough to accept the hypothesis. The p value for the other bacterial protein reactions, indicate that the results are not significant. This variability could be due to umbilical cord sample variability.

In the study done by Andrew Lily, he noted that NM23-H1 proteins also promoted the proliferation of CD34+ve cells. However in our experiments, even though we have positively shown extended survival of CD34+ve cells, cells were harvested at less than the numbers they were seeded at.

Umbilical cord blood samples underwent 2 separate protocols. This was because bacterial NDK had not been investigated before and therefore it was decided to develop 2 protocols, one where we sort for CD34+ve cells and then the CD34-ve cells were sorted again for CD11b+ve cells. This was because the study undertaken by (Lilly, et al., 2011) had shown that CD11b+ve cells bind to the human NM23-H1 protein. The other protocol was where CD34+ve cells were sorted and CD34-ve cells were not sorted further but cultured straight with the NDK/NM23 protein. This was to see whether bacterial NDK proteins may bind to other cells within the CD34-ve population or whether they would act in the same way as NM23-H1.

The cord blood samples marked with a dashed line are samples which are not sorted for CD11b+ve cells. Despite it being hard to say; there does seem to be a lower cell number of CD34+ve cells where conditioned media was used from non sorted CD11b+ve cells.

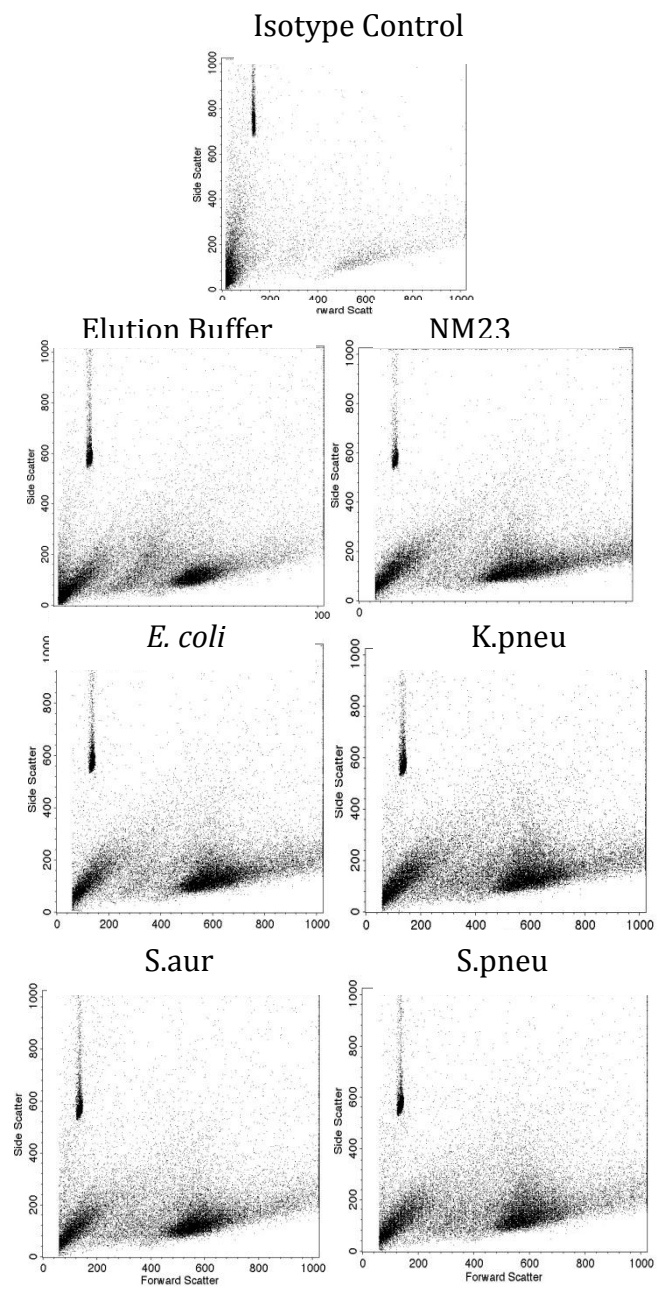
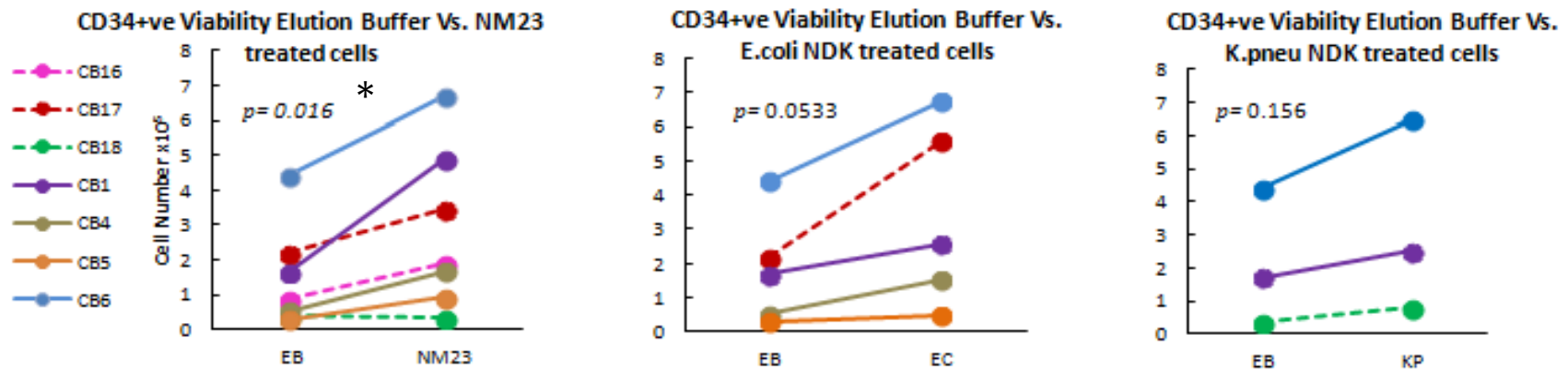


Figure 18 Side Scatter Vs. Forward Scatter of CD34+ve viable cells

Side scatter Vs. Forward scatter shows dead cells, viable CD34+ve cells and cytoount beads. 2uL of antibody were added to each sample.



Please note CB samples 2,3,7-15 did not contain enough CD34+ve stem cells, to be used in this experiment

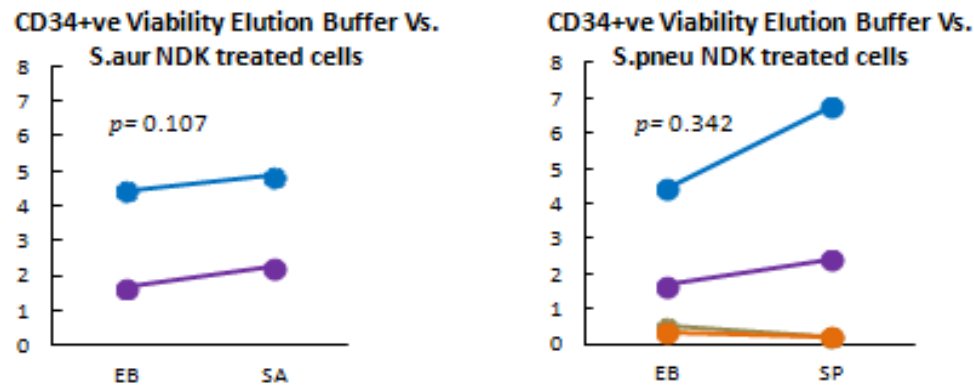


Figure 19 CD34+ve Viability

Cell survival was evaluated after 5 days of culturing with 2ug/mL of NM23-H1 or Bacterial NDK conditioned media or Elution Buffer only. The lines show the viability of treated samples compared to elution buffer control. Cells were seeded at 1×10^6 /mL. Cord blood samples marked with a dashed line, show cord blood samples not sorted for CD11b+ve cells.

4.10 NDK proteins directly promote the survival of CD34-ve cells

CD34-ve cells (sample 1) were cultured with NDK protein for 11 days. CD34-ve cell numbers were assessed and the survival of CD34-ve cells was significantly higher in the cultures that had been incubated with NDK/NM23 protein, compared with elution buffer control. There was a significant difference in cell number, approximately between a 2 to 8 fold increase varying between the different NDK protein treatments. There is also variability between samples which could be due to variability in cord blood samples; and more umbilical cord blood analysis should be undertaken to determine a more significant result.

FACs analysis was done to determine cell viability and cell surface expression for CD34, CD11b, CD3, CD19, CD14, CD33 and CD15, was obtained to determine which cells are surviving in the presence of NDK protein.

From figure 21 you can see the cells surviving after 11 day culture with NDK proteins have significant expression for CD11b, CD3 and CD19. CD11b markers are expressed on many leucocytes, including monocytes, macrophages, natural killer cells, granulocytes and neutrophils. CD3 are expressed on T cells, CD19 on B cells.

The micrographs (figure 23) of cells (sample 1) cultured with NDK proteins show a huge variation in cell population and viability, where cells cultured with elution buffer are scarce and contain mainly dead cells. Jenna Giemsa staining was done on harvested samples at day 11 of culturing to study the morphology of the cells. Cells

cultured with NDK protein, look viable and healthy, with also cell differentiation. It is already been seen from the previous experiments that NDK protein promotes the survival of CD34+ and CD34-ve cells, however from the Jenna Giemsa stained micrographs the presence of red blood cells in NDK protein cultured samples are shown to be healthy and still viable as well.

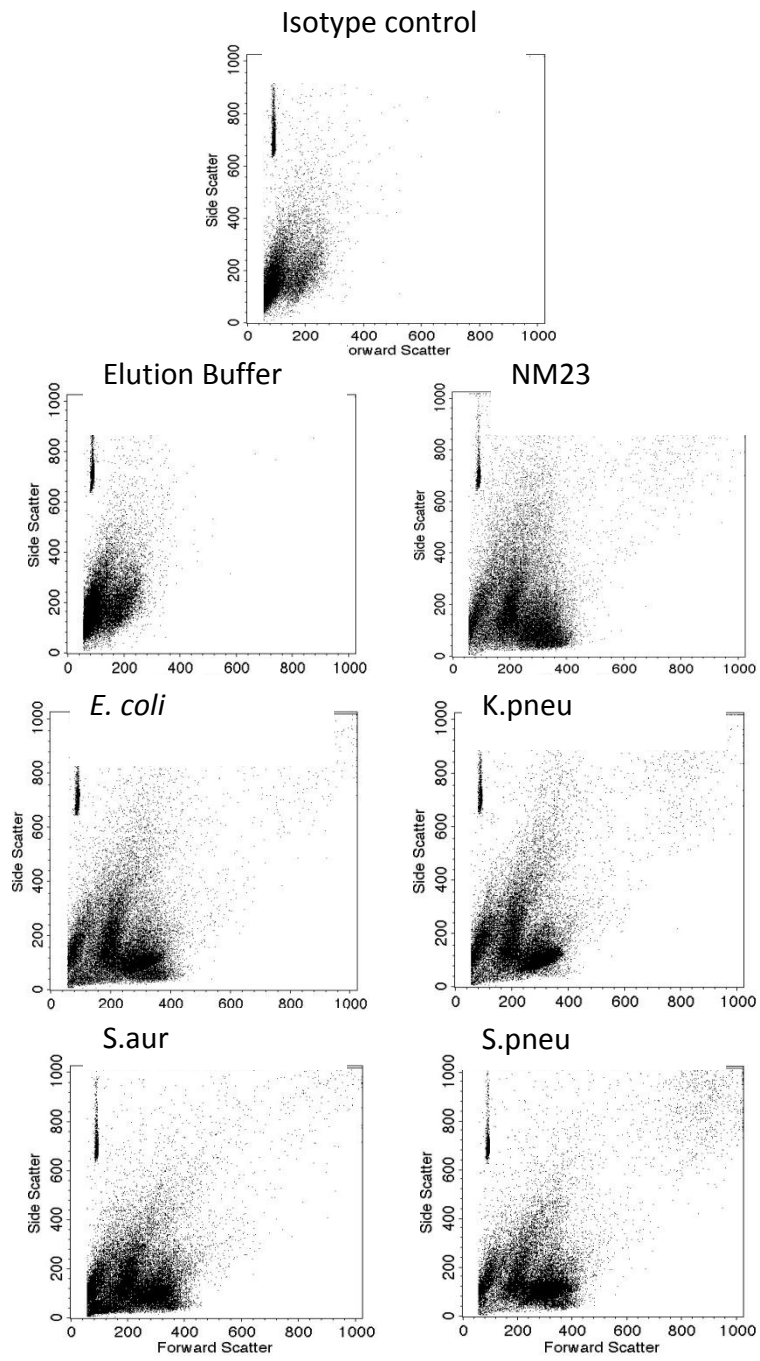


Figure 20 Side Scatter Vs. Forward Scatter of CD34-ve viable cells

Side scatter Vs. Forward scatter shows dead cells, viable CD34-ve cells and cytoquant beads. 2uL of antibody were added into each sample

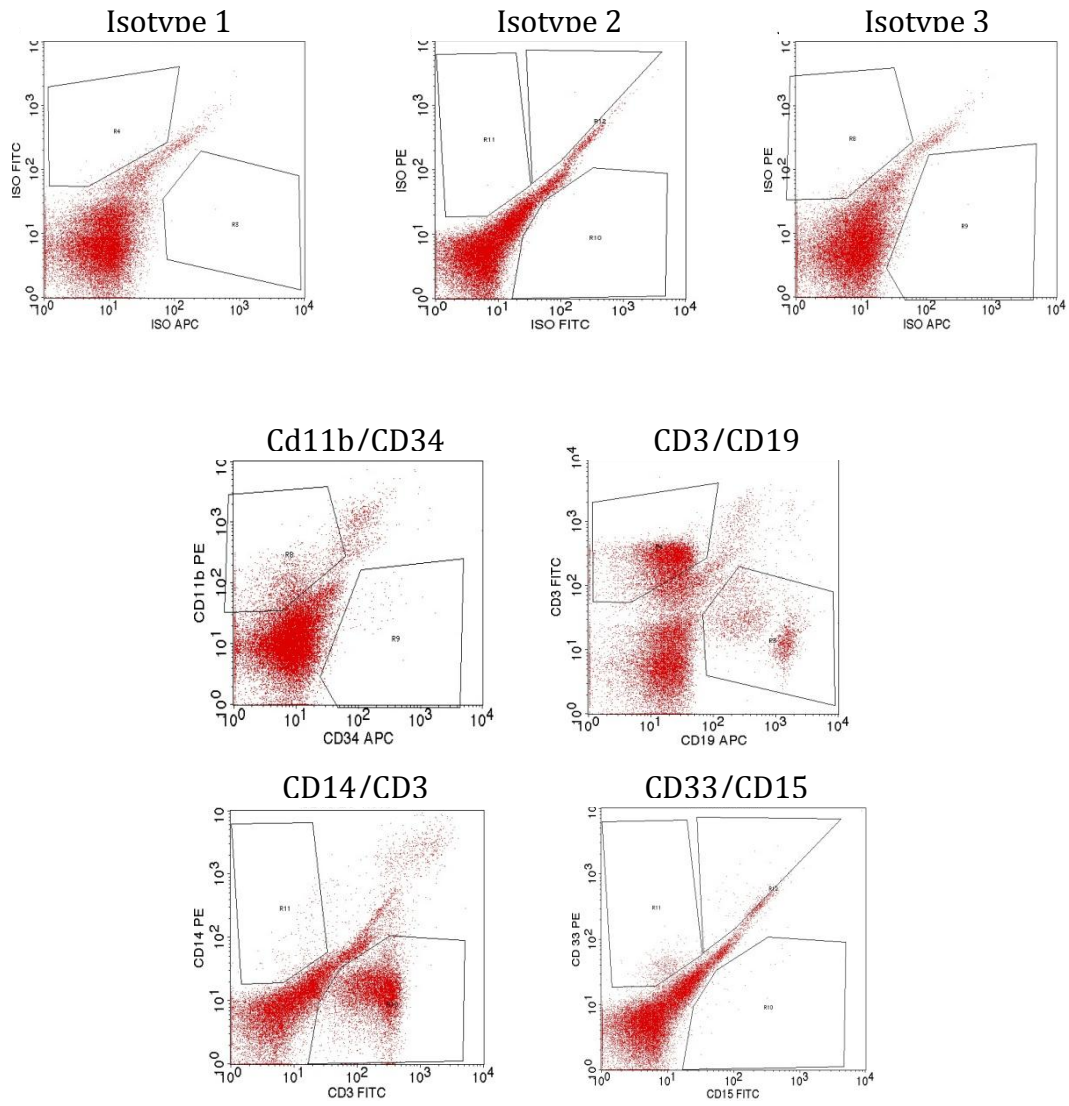
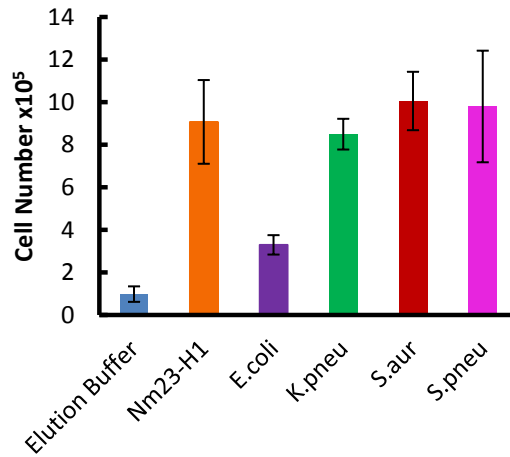


Figure 21 FACS Analysis of CD expression markers in CD34-ve cell populations

Dot plot analysis shows cell viability and expression for CD11b, CD34, CD33, CD3, CD15, CD14, and CD19. Isotype controls for each fluoro-chrome. 2uL of antibody were added into each sample.

Cord Blood 16 - CD34-ve Cell Viability in Elution Buffer and NDK protein treated cells



Cord Blood 17 - CD34-ve Cell Viability in Elution Buffer and NDK protein treated cells

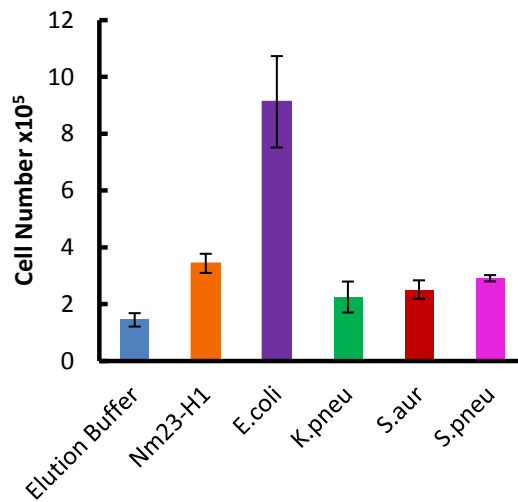


Figure 22 CD34-ve Viability of NDK treated cells

Cell survival was evaluated after 11 of in culture with 2ug/mL of NM23-H1, Bacterial NDK or Elution Buffer only. Each column shows the viability of treated samples compared to elution buffer control. Cells were seeded at 1×10^6 /mL

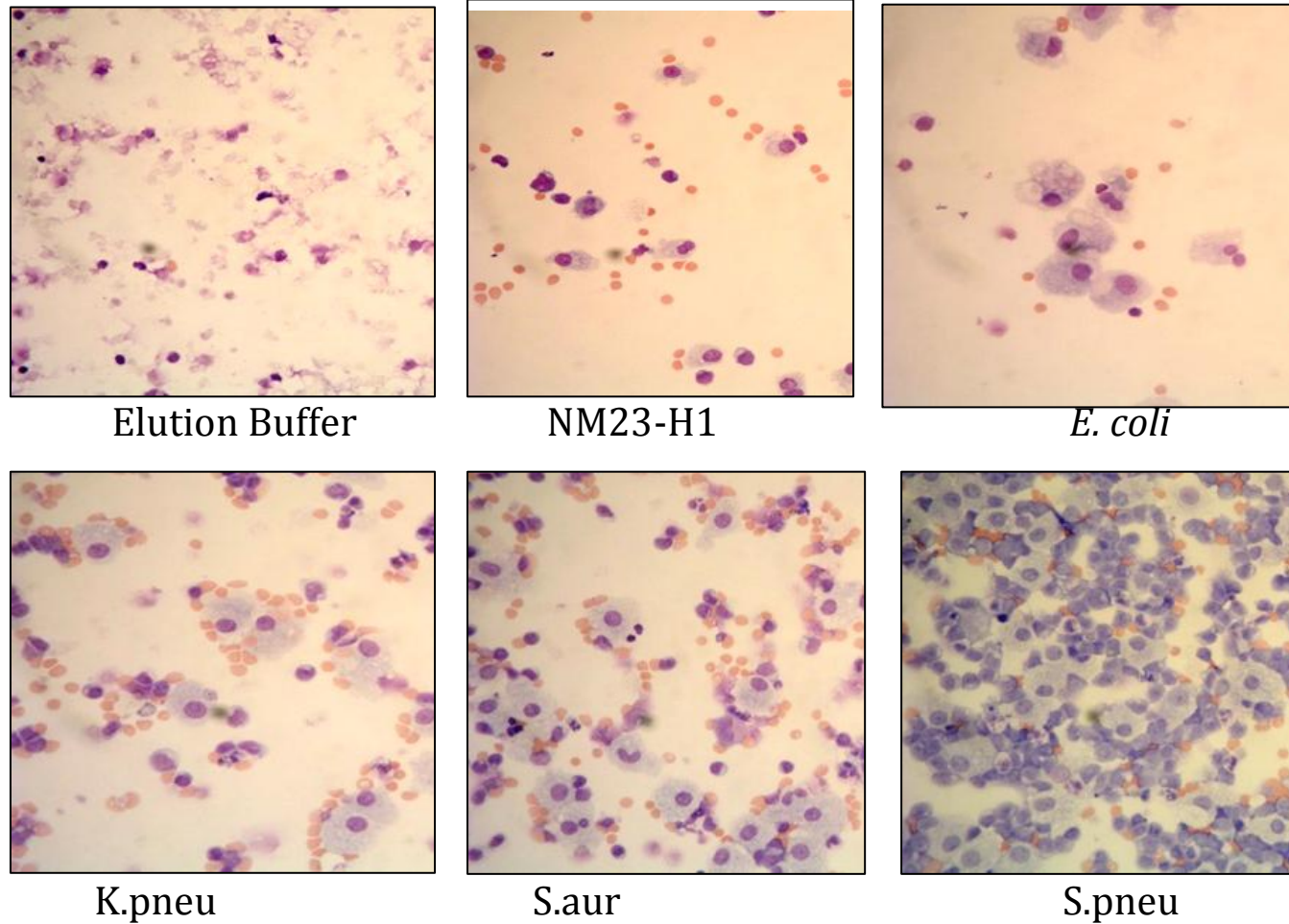


Figure 23 Micrographs of Jenna Giemsa stained CD34-ve cultured cells

Micrographs of cells (sample 1) stained with Jenna Giemsa of cultured samples with elution buffer and NDK proteins, taken at x20 magnification.

4.11 The survival of Leukaemic blast cells is varied amongst NDK protein treatment.

Acute myeloid leukemic cells are cultured in ITS+ media with 2ug/mL of NDK protein, or elution buffer and harvested after 11 days. Without NDK protein the survival of cells in elution buffer only, were significantly lower compared with cell survival of cells treated with NDK protein. AML samples treated with *E. coli* and *S. pneu* NDK protein show an 8 fold increase in cell survival compared with elution buffer cultured cells, and cells treated with NM23-H1 and *S.aur* show about a 5 fold increase.

Acute lymphoid leukemic cells were also cultured in ITS+ media with 2ug/mL of NDK protein or elution buffer, and harvested after 5 days. Cell survival of NDK treated samples were not very significant compared with elution buffer control. And if compared to survival of other samples, the cell numbers are significantly lower.

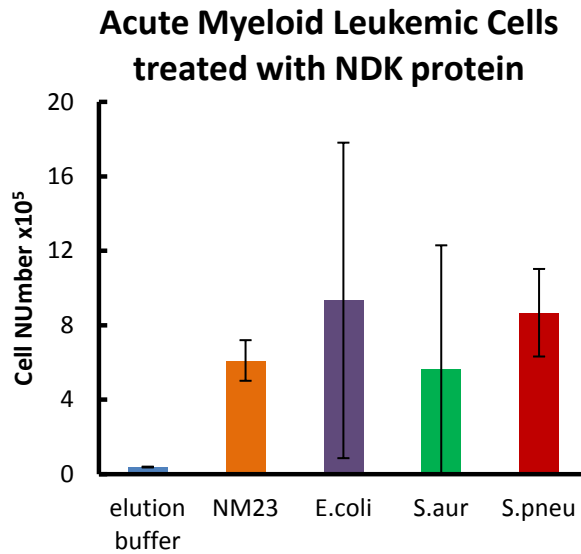


Figure 24 AML Cell Survival

Cells treated with 2ug/mL NDK protein or elution buffer control, harvested at day 11. Each column shows the viability of treated samples compared to elution buffer control. Cells were seeded at 1×10^6 /mL. (n=3 for treatments with each NDK protein, however only 1 AML sample used)

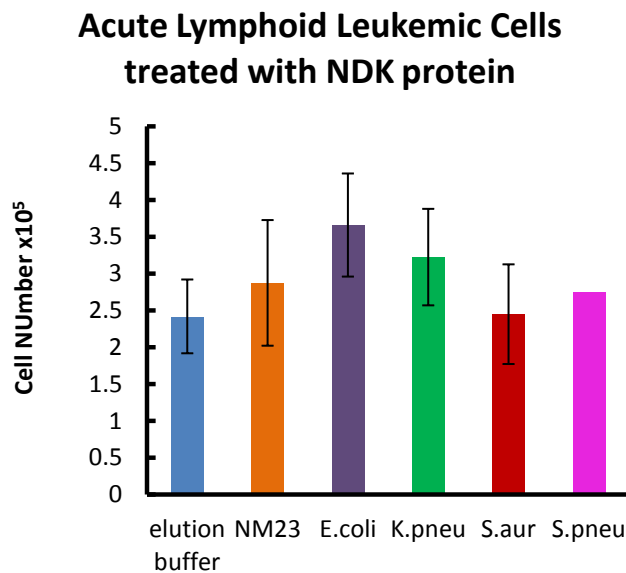


Figure 25 ALL Cell Survival

Cells treated with 2ug/mL NDK protein or elution buffer control, harvested at day 11. Each column shows the viability of treated samples compared to elution buffer control. Cells were seeded at 1×10^6 /mL. (n=3 for treatments with each NDK protein, however only 1 AML sample used)

5. PART A CONCLUSION

The results obtained from the indirect NDK protein assay with conditioned media from human NM23 protein, suggest that we were able to reproduce results that the human NM23-H1 protein does act as a survival factor for CD34+ve haemopoietic stem cells, as seen in the work by (Lilly, et al., 2011).

The results obtained from the indirect NDK protein assays suggest a slight increase in the amount of viable stem cells present after 5 days of culturing with NDK protein conditioned media, and this is enough to indicate that with further work and optimisation of the purity protocols, with increased repeats on fresh cord bloods, that significant results may be obtainable.

Previous studies have indicated how and also the importance of the human NM23-H1 protein in cell survival, cell development and differentiation (Lombardi, et al., 2000). It could be suggested that they may use bacterial NDK proteins in the same manner. This increase in viability of CD34+ve cells could have some therapeutic advantages as well as research advantages.

The results obtained on the indirect leukaemic cell assays suggest that for the cell assays containing acute myeloid leukaemic cells cultured with NDK proteins from human NM23-H1 and bacterial NDK, show an increased survival compared with elution buffer control. However for the stem cell assays for acute lymphoid leukaemic cells cultured with NDK proteins, the results are quite different, and do not show a survival increase compared with elution buffer control.

6. PART B INTRODUCTION

Part B of this research project focuses on the characterisation of bacterial NDK proteins and determines whether point mutations or deletions within the conserved protein domains of NDK could affect the function of the protein.

6.1 Previous Studies

Previous literature has shown the NDK family, contain a number of conserved sites within the protein which play a role in function and structure of the NDK protein. NDK proteins from *Mycobacterium tuberculosis* were isolated and its hexameric structure is shown to play an important role in thermal stability (Georgescauld, et al., 2013). In a study by (Sundin, et al., 1996) 4 mutations within the NDK protein of *Pseudomonas aeruginosa* were isolated and results indicated a loss in biochemical activity. In an *Escherichia coli* strain knockout, NDK bacteria were isolated and results indicated a greater number of mispaired bases within the cell indicating a role for NDK in DNA replication. Double mutants lacking both the NDK gene have base substitutions 15 fold higher than wild type (Miller, et al., 2002). These higher levels of base substitutions and mispairs; and the secretory ability of certain bacteria to release NDK proteins out of its cell into host environments leads to the question as to whether bacterial NDK like the human NM23 homology has an association with cancer and whether bacterial NDK proteins can interact with human DNA, as the NM23 protein does.

6.2 Aims of the project

The aim of this study is to characterise bacterial NDK proteins from species commonly known to cause infections in cancer patients. NDK KO *Escherichia coli* will be produced and site directed mutagenesis will be used on several conserved domains within the protein structure in *Escherichia coli* to determine the importance of the function and structure of certain conserved domains of NDK protein.

7. PART B METHODS AND MATERIALS

7.1 cDNA Production

7.1.1 Primer Design

A web tool Primer 3 was used to automatically construct a forward and reverse primer set for *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* NDK proteins, from each bacterial NDK coding sequence. Conditions were set at; melting temperature 60°C, GC ratio 50%, primer size optimum 20bp, and product size range between 200 and 500bp. Primer designs can be found in appendix

7.1.2 Growth Kinetics

Growth kinetics analysis was done to determine mid -logarithmic and stationary phase of each bacteria. Overnight cultures were made for each bacterial species, by inoculating 5 mL LB broth with *Escherichia coli*, *pseudomonas aeruginosa* and 5mL MH broth with *Staphylococcus aureus*. Overnight cultures were diluted 1 in 100 the next day and 200 uL of diluted bacterial were added in replicate to a 96 well plate and fluostar was set up to read absorbance ever 10 minutes.

	1	2	3	4	5	6	7	8
A	Blank control for LB broth	EC 1	EC 2	EC 3	Blank control for MH broth	SA 1	SA 2	SA 3
B								
C		PA 1	PA 2	PA 3				
D								
E								
F								
G								
H								

EC = *Escherichia coli*

PA = *Pseudomonas aeruginosa*

SA= *Staphylococcus aureus*

Table 4 Plate layout for Growth Kinetics

7.1.3 RNA Extraction

Overnight cultures for each bacterial species are incubated at 37°C in a shaking incubator. Overnight cultures were done in Lysogeny Broth (LB) broth for *Escherichia coli* and *Klebsiella pneumoniae*, in Muller Hinton (MH) broth for *Staphylococcus aureus* and Brain Heart Infusion (BHI) Broth for *Streptococcus pneumoniae*. *Streptococcus pneumoniae* was also grown in a static incubator.

The following day these were diluted 1 in 25 into 5mL and the remainder of the overnight culture was stored in the static incubator. These were then left to grow for approximately 2 and ½ hours or until their OD was between 0.4-0.6. This information was sought from the growth kinetics analysis data.

Once we have bacterial growth to mid-logarithmic and at stationary phase, 4mL of each culture was added to clearly labelled falcon tubes, decipherable between mid-logarithmic and stationary phase cultures and species. 1mL of phenol (5%): ethanol (95%) was added, and the mixture was incubated on ice for 30 minutes. Samples were then centrifuged at 4°C at maximum RPM for 10 minutes. Supernatant was discarded into phenol waste, and pellet was resuspended in remaining liquid

N.B. *Streptococcus pneumoniae* was not grown overnight, due to them undergoing autolysis at stationary phase. *Streptococcus pneumoniae* was grown for 6 hours. At 3 hours the OD of the cultures were checked. When an OD of 0.2 was reached (beginning of stationary phase) 4mL of culture was added to phenol (5%): ethanol (95%) and incubated on ice for 30minutes. The cell suspension was centrifuged at 4°C at maximum RPM for 10 minutes and supernatant discarded. The pellet was stored at -80°C overnight. At 6 hours, when an OD of 0.9 has been reached, 4mL of culture was added to phenol (5%): ethanol (95%) and incubated on ice for 30minutes. Suspension was pelleted and supernatant discarded. The Pellet was stored at -80°C overnight. RNA extraction was obtained following the protocol explained in detail below.

Samples were transferred into labelled eppendorfs and centrifuged using a desk top centrifuge for 60 seconds at maximum speed. Remaining fluid was discarded and pellet was resuspended in 100uL TE buffer with 50mg/mL lysosyme.

N.B. for *Staphylococcus aureus* lysostaphin enzyme (Sigma Aldrich) was used to break open the cell walls. A Sonication step was added to aid in the lysing process and RNA was extracted using RNeasy mini kit (Qiagen) following manufacturer's instructions.

Samples were incubated at RT for 5 minutes. 75ul lysis reagent was added and gently mixed by inversion. 350uL RNA dilution buffer added, mixed by pipetting up and down. Samples were added to a heat block at 70°C for 3 minutes. Samples were vortexed and centrifuged for 10 minutes at maximum RPM. A white pellet formation was an indication that bacteria have been lysed. Samples were transferred into fresh eppendorfs, ensuring no white pellet formation was transferred over. 200uL of 95% ethanol was added and samples mixed by pipetting up and down.

Samples were transferred into a spin column and centrifuged for 30 seconds, eluates were discarded. 600uL of wash buffer was added and samples were centrifuged for 30 seconds at maximum RPM. Eluates were discarded and 10uL of commercially bought DNase mix containing $MnCl_2$, DNase core buffer and DNase was added to the spin column and incubated at RT for 15 minutes. 200uL DNase stop solution was added and samples centrifuged for 30 seconds. 600uL wash buffer was added to the samples, centrifuged and eluates discarded. 250uL wash buffer was added, and samples centrifuged, and eluates discarded. Samples were spun dry, and then spin columns were placed into a fresh eppendorfs. 100uL RNase free H_2O was added, and left to stand for 1 minute. Samples were centrifuged for 2 minutes at maximum RPM. And RNA extracted was stored in the freezer.

5uL of each sample was run on a 1% agarose gel at 100V for approximately 30 minutes. This was to quantify the amount of RNA extracted, for cDNA synthesis.

7.1.4 cDNA synthesis using Superscript III

The following reagents were added into a nuclease free micro centrifuge tube; 2ug RNA in RNase free water, 1uL 50ng/uL random primers, and 1uL 10nM dNTP mix,

The RNA for each bacterial species was quantified using gene tool, based on the gel separation results, determining the volume or RNA in solution required to make up 2ug.

For example:

	Quantity in ng	Quantity to 2ug	Volume to 11uL
<i>E. coli</i> mid-logarithmic 1	572.7313	2000/572.7313	11 - (2000/572.7313)
<i>E. coli</i> mid-logarithmic 1	717.1652	2000/717.1652	11-(2000/717.1652)
<i>E. coli</i> mid-logarithmic 1	678.5955	2000/678.5955	11- (2000/678.5955)
<i>E. coli</i> Stationary	288.7863	2000/2.88.7863	11- (2000/2.88.7863)
<i>E. coli</i> Stationary	518.9769	2000/518.9769	11- (2000/518.9769)

Table 5 Quantification of cDNA

The mixture was then heated at 65°C on a heat block for 5 minutes, and incubated on ice for 1 minute. A master mix containing; 4uL 5x first strand buffer, 1uL 0.1 M DTT,

1uL RNase OUT, and 1uL superscript III RT was added per sample. 7uL of master mix was added to each sample, mixed gently up and down and incubated for 5 minutes at RT. Samples were incubated on a heat block at 50°C for 1 hour, and reaction inactivated by heating at 75°C for 15 minutes.

7.1.5 Real Time PCR for cDNA amplification

cDNA was then amplified by Real-Time PCR. cDNA was diluted 1 in 10 and 1 in 1,000. Each PCR mix contains; 22.5uL of commercially ready PCR mix, 0.75uL of a 25uM working stock of Forward and 0.75uL of Reverse primers, and 1uL of cDNA template.

Primers used were specific to each species: 16s gene Primers (strain control) and NDK gene Primers were used.

N.B. for *S. pneumoniae* control primers rpoB gene primers were used as a control to show efficient expression of cDNA.

PCR Conditions:

95°C	-->	5 minutes (denaturation)	} x 30 Cycles.
95 °C	-->	30 seconds (denaturation)	
50 °C	-->	30 seconds (primer annealing)	
72 °C	-->	30 seconds (primer extension)	
72 °C	-->	10 minutes (final extension)	
4 °C	-->	hold.	

Amplify cDNA in neat form, 1 in 10 dilution and 1 in 1,000 dilution were check by gel electrophoresis to determine cDNA expression.

NDK and bacterial control cDNA from each bacterial species was quantified using gene tool, to determine whether NDK DNA was expressed all the time and whether they differ in quantity at mid-logarithmic and at stationary phase.

7.2 Construction of NDK Knockout in *Escherichia coli* using Datsenko and Wanner Method

7.2.1 Primer Design

A web tool Primer 3 was used to automatically construct knock out forward and reverse primer set for *Escherichia coli* from the bacterial NDK coding sequence. On the end of the NDK KO primers, a sequence matching the Kan cassette was added.

Conditions were set at; melting temperature 60°C, GC ratio 50%, primer size optimum 20bp, and product size range between 200 and 500bp. Primer designs can be found in appendix

7.2.2 GeneJet Plasmid miniprep of pkd46 and pkd4

The pkd46 plasmid which contains an ampicillin resistance gene was used as a antibiotic resistance marker within the knockout production, and the pkd4 plasmid which contains a kanamycin cassette and was used as a PCR template to replace the NDK gene.

Any strain containing pkd46 plasmid, in this case (L642) and pkd4 plasmid in this case, (I633) was grown overnight on LB plates containing 50ug/mL of ampicillin, and LB plates containing 50ug/ml of kanamycin respectively.

The following day L642 and I633 were grown in (separate) 5mL LB broth with 50ug/mL AMP, and LB broth with 50ug/ml kanamycin respectively at 37°C in the shaking incubator overnight.

The overnight cultures were centrifuged at 4°C for 10 minutes at 3000 RPM, and the pkd46 and pkd4 plasmids were purified out of the L642 *Salmonella* strain and the I633 *E. coli* strain the next day, using thermo scientist GeneJet Plasmid miniprep kit, following manufacturer's instructions. Purified plasmid DNA was stored at -20°C.

5uL of sample was run on a 1% agarose gel for 40 minutes at 100V to check purity and quantity of plasmid prep.

7.2.3 Transformation of recipient strain I364 with pkd46

I364 was a laboratory strain of K-12 *E. coli*,

I364 wild type were transferred into 5mL of LB broth and grown at 37°C until logarithmic phase (OD 0.4) was reached. The culture was then centrifuged at 3000 RPM at 4°C for 10 minutes. It was crucial that everything was done on ice. Glycerol was kept on ice prior to resuspension. Supernatant were discarded and the pellet resuspended in 1mL of 15% glycerol, step repeated and final pellet was resuspended in 500uL of 15% glycerol. Into a pre chilled electroporation cuvette 50ul of cell suspension was add as well as 2uL of pkd46 purified plasmid. Sample was electroporated at 2.5Kv. 1mL of LB broth was added into the cuvette after electroporation and placed into a universal to revive cells. Competent cells were left in the incubator at 37°C for 1 hour. 200uL of competent cells were streaked onto an LB

plate and onto an LB plate with 50ug/mL ampicillin and left to grow at room temperature for 2 days.

Streaking competent 1823 cells onto an LB plate was used to check that the cells were still alive. Streaking on to an LB plate with ampicillin was used to check that the competent cells have incorporated the pkd46 plasmid, as pkd46 contains an ampicillin resistant marker, and therefore should only grown on the ampicillin resistant plate if they have incorporated the pkd46 plasmid.

Transformation was checked by purifying plasmid using GeneJet miniprep kit, following manufacturer's instructions.

7.2.4 Kanamycin cassette PCR amplification

Pkd4 plasmid used as DNA template for NDK knockout production. The NDK knockout primers were designed for the amplification of the kanamycin cassette within the pkd4 plasmid. The NDK knockout primers contain a complement sequence for the kanamycin cassette and compliment sequence for the NDK gene, which will be used to encourage the 'swapping' of the NDK gene with the kanamycin cassette when electroporated into the recipient knockout strain.

NDK knockout primers (shown in appendix) were reconstituted in RNase free water, and a working stock of 25uM used in the following experiment.

Each PCR mix contains; 22.5uL of commercially ready PCR mix, 0.75uL of a 25uM working stock of Forward and 0.75uL of Reverse primers, and 1uL of pkd4 template.

PCR Conditions:

95°C	-->	5 minutes (denaturation)	
95 °C	-->	30 seconds (denaturation)	} x 30 Cycles.
50 °C	-->	30 seconds (primer annealing)	
72 °C	-->	1 minute and 30 seconds (primer extension)	
72 °C	-->	10 minutes (final extension)	
4 °C	-->	hold.	

Amplified pkd4 plasmid was separated on a 1% agarose gel to check amplification of plasmid has worked.

7.2.5 Electroporation of I823 and I365 *E. coli* with pkd4 KO constructs

I823 and I365 containing pkd46 plasmid were transferred in 5mL LB broth containing 50ug/ml ampicillin and incubated over night at 30°C. The following day 10mM arabinose, 50ug/mL AMP was added to 10mL of LB media and inoculated with 400 uL of overnight culture and incubated with aeration at 30°C for 4 hours, until an OD600 was reached. Cells were harvested by centrifugation at 3,000 x g for 10 minutes at 4°C, supernatant was discarded and bacterial pellet resuspended in 1ml of 15% ice cold glycerol and centrifuge at 3000 x g for 10 minutes at 4°C, this step was repeated twice. Final pellet was re-suspended in 500uL of 15% ice cold glycerol. Competent cells were electroporated using a range of KO construct quantities. See table below.

Competent cell volume (ul)	KO construct quantity (ng)
50	2
50	5
50	10

Table 6 Knockout Construct Quantities

50uL of competent cells were added to a pre-chilled 0.2cm electroporation cuvette, into 3 separate electroporation cuvettes, either 2ng, 5ng or 10ng of KO construct was added. The electroporator was set to 2.5kV and samples were pulsed with electricity for approximately 5 seconds. To recover cells, the electroporated cells were incubated in 1mL of LB media for an hour at 37°C. 400uL of recovery mixture was plated onto LB plates containing 50ug/mL kanamycin. Plates were incubated for 2 days at 37°C. This allows the for the loss of the pkd46 plasmid which was temperature sensitive.

The NDK knockout production by Datsenko and Wanner method failed on 3 attempts, and due to time constraints it was decided that the p86 F6 NDK KO from the culture collection would be sequenced and transduced into I364 E. coli background for our use.

7.3 Transduction of p86 F6 NDK Knockout into I364 using p1 phage

7.3.1 Production of p2 bacteriophage

Prior to P2 bacteriophage production with p86 F6 NDK knockout. Phage assay was carried out to determine the ability of phage to lyse and infect the I364 *E. coli* strain. This was carried out by spreading I364 *E. coli* on to a an LB plate. 5uL of P1 phage was added to the plate along with 5uL of P22 Salmonella bacteriophage as a control (this should not lyse and infect *E. coli*) and left to grow overnight at 37°C. The following day the formation of a plaque indicates successful lysis of bacteria.

p86 F6 NDK KO in 5mL of LB media was grown overnight at 37°C. The following day culture was diluted 1:100 into 5mL of fresh LB media containing 10mM of MgSO₄ 5mM CaCl₂ and aerated for 30minutes at 37°C. 5uL of P1 bacteriophage was added and culture was grown overnight at 37°C in shaking incubator. The following day overnight was transferred into 50mL falcon tube and 1mL of chloroform was added. Suspension vortexed well for 15 seconds, and then centrifuged at 4,000 RPM at 4°C for 10 minutes. Supernatant was transferred into a glass bottle (as chloroform attacks plastic); a further 200uL of chloroform was added and stored at 4°C.

Transductions were screened by PCR and 1 successful colony was cultured on a plate and stored on protect beads.

7.3.2 P2 Transduction into I364

I364 *E. coli* was grown up in 5mL LB media at 37°C. The following day the cultures were spun at 4,000 RPM at room temperature for 5 minutes and resuspended in 1mM MgSO₄ and 5mM CaCl₂. Transductions were set up using different volumes of phage lysates. See table below.

Vol. Cells (ul)	Vol. Phage lysate (ul)
100	0
100	5
100	10
100	100

Table 7 Transduction Quantities

In 4, 1.5mL Eppendorfs add 100ul of I364 cells and add the different quantities of phage lysates to consecutive tubes. Tubes were incubated for 15 minutes at 37°C in heat block. 100uL of 1M sodium citrate and 1mL LB media was added and cells incubated for a further 45minutes at 37°C. 100uL of cell suspension was plated out onto LB plates containing 50ug/mL kanamycin to select for transductants. Plates were incubated for 2 days at 37°C.

To check for successful transduction of NDK KO into I364 background strain, colonies were streaked onto a fresh plate, and the following day, were amplified by PCR, using NDK KO check primers. PCR reaction mix was made up with 22.5uL Reddy mix, 0.75uL Forward and Reverse primers and 1uL of template. Negative control containing no

template and a positive control using pET15b NDK EC BI21 DE3 used as a positive control for NDK.

PCR Conditions:

95°C --> 5 minutes (denaturation)

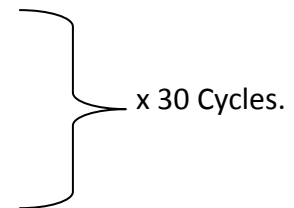
95 °C --> 30 seconds (denaturation)

50 °C --> 30 seconds (primer annealing)

72 °C --> 2 minute (primer extension)

72 °C --> 10 minutes (final extension)

4 °C --> hold.



x 30 Cycles.

Successful transducts were stored in protect beads at -20 °C

Transductions were screened by PCR and 1 successful colony was cultured on a plate and stored on protect beads.

7.4 Quikchange II Site Directed Mutagenesis

7.4.1 Primer Design

A web tool Quikchange mutagenesis Primer design was used to automatically construct a forward and reverse primer set for point mutations and deletions in the NDK gene of *Escherichia coli*. Conditions were set at; melting temperature 60°C, GC ratio 50%, primer size optimum 20bp, and product size range between 200 and 500bp.

Primer designs can be found in appendix

7.4.2 Mutant Strand synthesis reaction

pET15b plasmid was purified using GeneJet Plasmid miniprep kit (Thermo Scientific) following manufacturer's instructions. The pET15b plasmid was used as the template plasmid for site directed mutagenesis.

Conditions for the synthesis of 2 complimentary oligonucleotides of *Escherichia coli* NDK with desired mutations according to manufacturer's instructions were as follows;

Control reaction: 5ul 10x Reaction buffer, 2ul (10ng) pWhitescript 4.5kb control plasmid, 1.25ul (125ng) control forward and reverse primers, 1uL dNTP mix, 38.5ul dH₂O and 1uL *Pfu*Ultra HF DNA polymerase.

Sample reaction: 5ul 10x Reaction buffer, 1ul (100ng) pET15b 45.7kb plasmid, 1.25ul (125ng) pET15b mutant forward and reverse primers, 1uL dNTP mix, 38.5ul dH₂O and 1uL *Pfu*Ultra HF DNA polymerase.

Please note the site directed mutants desired in the following protocol consist of changes at the following sites:-

MAIERTFSII **KP** NAVAKNVIGNIFARF EAAGFKIVGTKMLHLTVEQARGFY
AEHDGKPPFDGLVEFMTSGPIVVSVLE **G** ENAVQRHRDLLGATNPANALAG
T **LRA** DYADSLTENGTHGSDSVESAAREIAYFFGEGEVCPRT

KP to AA a31g_a32c_c34g

G to A g236c

LRA – deletion del307_315

PCR Conditions according to manufacturer's instructions –

95°C	-->	30 seconds (denaturation)	}	x 18 Cycles.
95°C	-->	30 seconds (denaturation)		
55°C	-->	30 seconds (primer annealing)		
68°C	-->	6 minute (primer extension)		
4°C	-->	hold.		

After thermal cycling samples were placed on ice for 2 minutes so that the reaction can be cooled to approximately 37°C before proceeding to the next step.

N.B. In the production of Site Directed Mutagenesis the thermal cycling conditions were optimised to the following;

95°C	-->	30 seconds (denaturation)	}	x 25 Cycles.
95°C	-->	30 seconds (denaturation)		
68°C	-->	10 minutes (primer annealing)		
68°C	-->	6 minute (primer extension)		
4°C	-->	hold.		

Annealing temperature was changed to 68°C and time changed to 10 minutes. The number of cycles was also changed to 25.

7.4.3 DpnI Digestion of amplified PCR product

According to manufacturer's instructions, the amplimers were digested by adding 1uL of Dpn I restriction enzyme to each reaction mix. Each reaction was mixed by gently

pipetting up and down, and then spun down in a bench top centrifuge at 15,000 RPM for 1 minute and immediately incubated at 37°C for 1 hour. This allows for the parental DNA plasmid to be digested.

N.B. In the production of Site Directed Mutagenesis the digestion protocol was optimised by adding 2ul of Dpn I restriction enzyme and increasing the incubation time to 2 hours.

7.4.4 Electroporation into alpha-select top 10 electrocompetent cells

The alpha select top 10 electrocompetent cells were gently thawed on ice. 0.2ml electroporation cuvettes were pre-chilled on ice and 50ul of competent cells and 2uL of Dpn I digested DNA was added to the cuvettes. This was done for each mutagenesis reaction required. Electroporation of cells at 2.5kV were then recovered in 1mL of LB broth and incubated with aeration for 1 hour. A puC plasmid was also electroporated as a control as above. 400uL of the recovery mixture was plated onto ampicillin resistant plates and incubated at 37°C for 24 hours.

Transductions were screened by PCR and 1 successful colony was cultured on a plate and stored on protect beads.

7.4.5 Electroporation of SDM into I364 and I364 KO background

I364 *E. coli* and I364 knockout *E. coli* were transferred and grown overnight in 5ml LB broth at 37°C with aeration. I364 knockout *E. coli* was grown in LB with 50ug/mL kanamycin. The following day 100uL of the overnight cultures were diluted into 10mL

of fresh LB containing 50ug/mL kanamycin (in knockout strain only) and grown until mid-logarithmic phase was reached (OD 0.4). Once the cultures have reached mid-logarithmic phase, the cultures were spun at 4°C for 10 minutes at 3,000 x g. Supernatant was discarded and pellet resuspended in 1mL of 15% glycerol. The cell suspension was spun down at 3,000 x g for 10minutes at 4°C and pelleted resuspended in 15% glycerol. The following step was repeated and the pellet was resuspended in a final volume of 500uL of 15% glycerol.

Site directed mutants were purified using GeneJet miniprep kit, (thermo scientific) following manufacturer’s instructions. 50uL of the competent cell suspension was added into a 0.2ml electroporation cuvette, and 2uL of purified SDM plasmid, according to the table below.

Competent Cells (50ul)	SDM plasmids (2ul)
I364	a31g_a32c_c34g
I364 KO	a31g_a32c_c34g
I364	g236c
I364 KO	g236c
I364	del307_315
I364 KO	del307_315

Table 8 Site Directed Mutants

Competent cells were electroporated at 2.5kV and recovered in 1mL of fresh LB broth and incubated at 37°C with aeration for 1 hour. 400uL of recovered cells were plated onto ampicillin (50ug/ml) LB plates and incubated at 37°C for 24 hours.

The following days 4 colonies were picked at random from each plate and screened. The electroporated SDM plasmid in the I364 background was purified using GeneJet miniprep kit (thermo scientific) following manufacturer's instructions and purified plasmids (5ul) were mixed with DNA loading buffer (2ul) were run on a 1% agarose gel to determine the presence of the plasmid. The electroporated SDM plasmid in the I364 KO background was screened using PCR using *E. coli* NDK primers to amplify NDK mutant within the pET15b plasmid only.

Reaction mix x1: 22.5uL PCR Reddy Mix, 0.75uL forward and reverse primers, 1uL template.

PCR conditions:

95°C	-->	5 minutes (denaturation)	} x 30 Cycles.
95 °C	-->	30 seconds (denaturation)	
50°C	-->	30 seconds (primer annealing)	
72 °C	-->	1 minute (primer extension)	
72°C	-->	10 minutes (final extension)	
4 °C	-->	hold.	

5ul Amplimers were run on a 1% agarose gel to determine the presence of NDK mutant in pET15b plasmid. The presence of the NDK mutant in pET15b plasmid on the gel indicates a successful electroporation.

7.5 Protein expression of r-NDK mutants

The following cells were cultured overnight in 5mL of LB broth I (containing 50ug/ml appropriate antibiotic) at 37°C with aeration

Mutant/Control	Background	Antibiotic
pET15b EC NDK <i>a31g_a32c_c34g</i>	I364	Ampicillin
pET15b EC NDK <i>a31g_a32c_c34g</i>	I364 KO	Ampicillin
pET15b EC NDK <i>g236c</i>	I364	Ampicillin
pET15b EC NDK <i>g236c</i>	I364 KO	Ampicillin
pET15b EC NDK <i>del307_315</i>	I364	Ampicillin
pET15b EC NDK <i>del307_315</i>	I364 KO	Ampicillin
pET15b EC NDK	BL21 (DE3)	Ampicillin
<i>E. coli</i> Wild Type	I364	None.
NDK knockout	I364	Kanamycin
NDK knockout	P86 F6	Kanamycin

Table 9 rNDK mutants for protein expression

The following day samples were diluted 1:50 into fresh LB broth including appropriate antibiotic (10uL in 5ml). 2x pET15b EC NDK (one to be induced and one none induced) and incubated with aeration at 37°C until an OD 0.4 was reached. Once an OD of 0.4 was reached 1mmol of IPTG was added to the following cultures and incubated at 37°C with aeration for 20 hours.

Mutant/Control	Background
pET15b EC NDK <i>a31g_a32c_c34g</i>	I364
pET15b EC NDK <i>a31g_a32c_c34g</i>	I364 KO
pET15b EC NDK <i>g236c</i>	I364
pET15b EC NDK <i>g236c</i>	I364 KO
pET15b EC NDK <i>del307_315</i>	I364
pET15b EC NDK <i>del307_315</i>	I364 KO
pET15b EC NDK (induction)	BL21 (DE3)

Table 10 rNDK mutants for IPTG induction

The following cultures were not induced but were incubated under the same conditions as the induced samples.

pET15b EC NDK (no induction)	BL21 (DE3)
<i>E. coli</i> Wild Type	I364
NDK knockout	I364
NDK knockout	P86 F6

Table 11 rNDK non induced protein samples

The following day 1mL of each culture was pipette into a clean universal, clearly labelled and spun down at 4°C for 10 minutes at 3,000 x g. 150ul of supernatant was pipette into a clean eppendorf and mixed with 50uL of 4xGLB (recipe can be found in the appendix). The remaining supernatant was discarded and the bacterial pellet (containing the expressed protein) was mixed with 200uL of 1x GLB. The re-suspended

pellet was then denatured at 91°C using the heat block for 5 minutes. Once the bacterial cells have been denatured to release the intracellular protein, the samples were spun down for 1 minute on a bench top centrifuge at 15,000 RPM to pellet the cell debris.

Pre-bought BOLT Bis-Tris 4-12% Protein Gels (Life technologies) were loaded with 20uL of each intracellular protein sample and supernatant. 5uL of protein ladder was added to the first well. The Gel was run at 150V for approximately 40minutes to separate proteins according to mass, until GLB has reached the bottom line on the gel. After the proteins have been separated, the gels were washed in 20mL of ultra pure water in a square petri dish, placed on bench top shaker at 20RPM, for 5 minutes. This was repeated 3 times, before gels were stained in safe stain (20ml) for 1 hour. Gels were then washed in 20ml ultra pure water, for 1 hour, then fresh ultra pure water (20ml) was added and gels were washed overnight. The following day gels were analysed.

7.6 Protein Modelling using Swiss Modelling software.

SWISS model, Swiss institute of Bioinformatics used to predict models for NDK by using the protein alignments of the SDM mutants and wild type *E. coli* for the following.

NDK Wild Type

MAIERTFSIIKPNNAVAKNVIGNIFARFEAAGFKIVGTKMLHLTVEQARGFYAEHDGKPFDDGLVEFMTSGPIV
VSVLEGENAVQRHRDLLGATNPANALAGTLRADYADSLTENGTHGSDSVESAAREIAYFFGEGEVCPRTR

Mutant 1: SDM a31g a32c c34g

MAIERTFSIIAANAVAKNVIGNIFARFEAAGFKIVGTKMLHLTVEQARGFYAEHDGKPFDDGLVEFMTSGPIV
VSVLEGENAVQRHRDLLGATNPANALAGTLRADYADSLTENGTHGSDSVESAAREIAYFFGEGEVCPRTR

Mutant 2: SDM g236c

MAIERTFSIIKPNNAVAKNVIGNIFARFEAAGFKIVGTKMLHLTVEQARGFYAEHDGKPFDDGLVEFMTSGPIV
VSVLEAENAVQRHRDLLGATNPANALAGTLRADYADSLTENGTHGSDSVESAAREIAYFFGEGEVCPRTR

Mutant 3: SDM del307-315

MAIERTFSIIKPNNAVAKNVIGNIFARFEAAGFKIVGTKMLHLTVEQARGFYAEHDGKPFDDGLVEFMTSGPIV
VSVLEGENAVQRHRDLLGATNPANALAGTDYADSLTENGTHGSDSVESAAREIAYFFGEGEVCPRTR

First gland by Jmol was used to highlight the conserved domains that were mutated. This was done by uploading the PDB file created by SWISS model, and then using the find tool to find the mutant substitution, which was then automatically highlighted.

8. PART B RESULTS

8.1 Growth Kinetics

Bacterial growth kinetics was done on *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* was done prior to RNA extraction to determine when the bacterium has reached mid-logarithmic growth phase. Using fluostar growth kinetics analysis this can be determined both in time and in optical density.

Streptococcus pneumoniae has not tested as *S. pneumoniae* autolyse itself when it gets to stationary phase.

Looking at the growth kinetics results shown in figure 1, mid-logarithmic growth was achieved at approximately 2 ½ hours (OD 0.2-0.4) for *E. coli*, 1 ½ hours (OD 0.2-0.4) for *K. Pneumoniae* and 4 and ½ hours (OD 0.2-0.4) for *S. aureus*

Growth Kinetic Analysis of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*

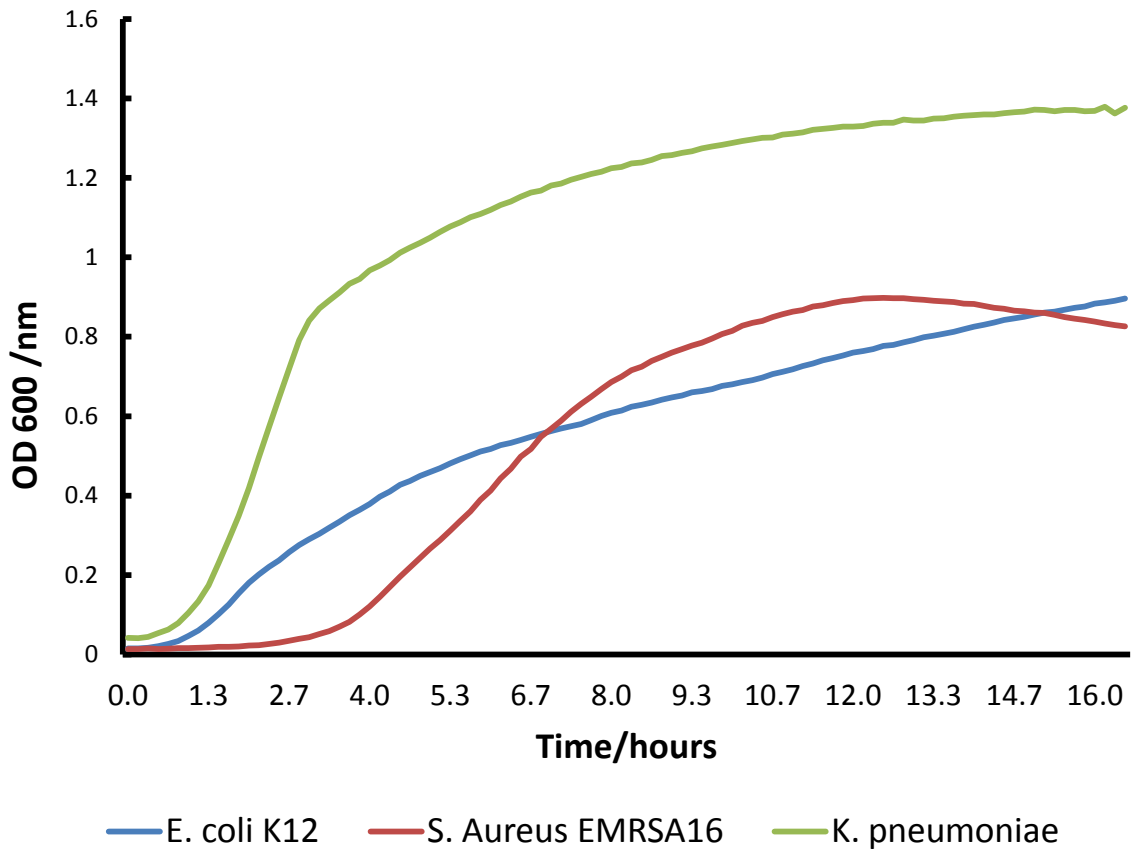


Figure 23 Growth Kinetic Analysis of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*

Figure 27 shows the growth of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* in LB media, grown over a 16.5 hour period with an absorbance reading was taken every 10 minutes.

8.2 Gene Expression

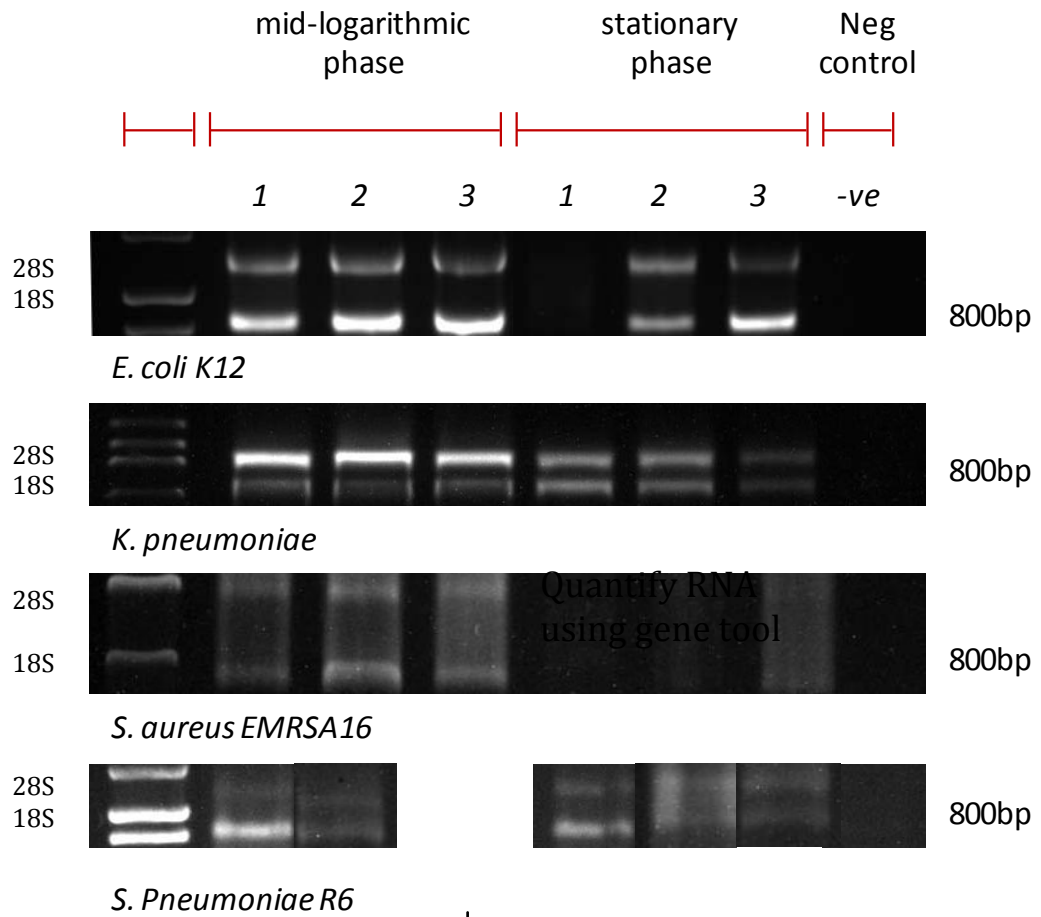
To determine NDK expression at mid-logarithmic and stationary growth phase, RNA was extracted from the 4 bacterial species from overnight cultures. Figure A shows the presence of RNA extracted from the 4 bacterial species, the 2 bands were indicative of 28S and 18S ribosomal RNA species. The 28S band should appear brighter, however if the 18S bands appears brighter it could be due to the presence of excess DNA within the sample. This can be seen in RNA extraction of *E. coli* and *S. pneumoniae*. A smudge through the well was indicative of messenger RNA, which can be seen quite strongly in the RNA extraction of *S. aureus*. The RNA extraction gel of *S. aureus* appears to not have any bands of ribosomal RNA; however the quantification shows that ribosomal RNA has been extracted just in a smaller quantity. Regardless of the differences in quantity (shown in figure B) of RNA extracted from the 4 different species of bacteria, we were able to obtain enough RNA to perform cDNA synthesis.

Figure B shows the quantification of RNA, using gene tool, Syngene, and the conversions to determine the quantity of RNA extracted required in a 2 μ g concentration of a final volume of 11 μ l. RNA extraction was done in replicates; this was to ensure a successful quantity of ribosomal RNA for cDNA synthesis. The rows highlighted in blue, indicated an insignificant amount of RNA extracted, and therefore these samples were not used in the synthesis of cDNA.

Figure C shows cDNA synthesis of 16S cDNA, and NDK. 16S was a house keeping gene found in most bacteria, it plays a structural role, in defining the position of ribosomal

proteins. It was therefore useful as a positive control to ensure the synthesis protocol was working sufficiently. A *rpoB* gene was used as a positive control in *S. pneumoniae*, only because we had a primer set available for use. The *rpoB* gene codes for a beta-subunit of bacterial RNA polymerase and was therefore a house keeping gene found in most bacteria, and therefore useful as a genetic marker.

A RNA Extraction from *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*



Quantify RNA
using gene tool

B Quantification of RNA using Gene Tool.

<i>Escherichia coli</i>		Quantity/ng	Vol (ul) in 2ug 2000/quantity	Vol of dH ₂ O final Vol = 11ul
mid-log'	1	572.73	3.49	7.51
	2	717.17	2.79	8.21
	3	678.60	2.95	8.05
Stat	1	16.24	123.12	-112.12
	2	288.79	6.93	4.07
	3	518.98	3.85	7.15

<i>Klebsiella pneumoniae</i>		Quantity/ng	Vol (ul) in 2ug 2000/quantity	Vol of dH ₂ O final Vol = 11ul
mid-log'	1	676.96	2.95	8.05
	2	786.54	2.54	8.46
	3	570.01	3.51	7.49
Stat	1	325.26	6.15	4.85
	2	289.78	6.90	4.10
	3	0.00	-	-

<i>Staphylococcus aureus</i>		Quantity/ng	Vol (ul) in 2ug 2000/quantity	Vol of dH ₂ O final Vol = 11ul
mid-log'	1	405.36	4.93	6.07
	2	5941.74	0.34	10.66
	3	16775.79	0.12	10.88
Stat	1	227.40	8.80	2.20
	2	256.65	7.79	3.21
	3	248.21	8.06	2.94

<i>Streptococcus pneumoniae</i>		Quantity/ng	Vol (ul) in 2ug 2000/quantity	Vol of dH ₂ O final Vol = 11ul
mid-log'	1	62.61	3.19	7.81
	2	18.08	11.00	0.00
	3	-	-	-
Stat	1	32.48	6.16	4.84
	2	32.33	6.19	4.81
	3	35.82	5.58	5.42



cDNA production

C cDNA synthesis using superscript III and random primers.

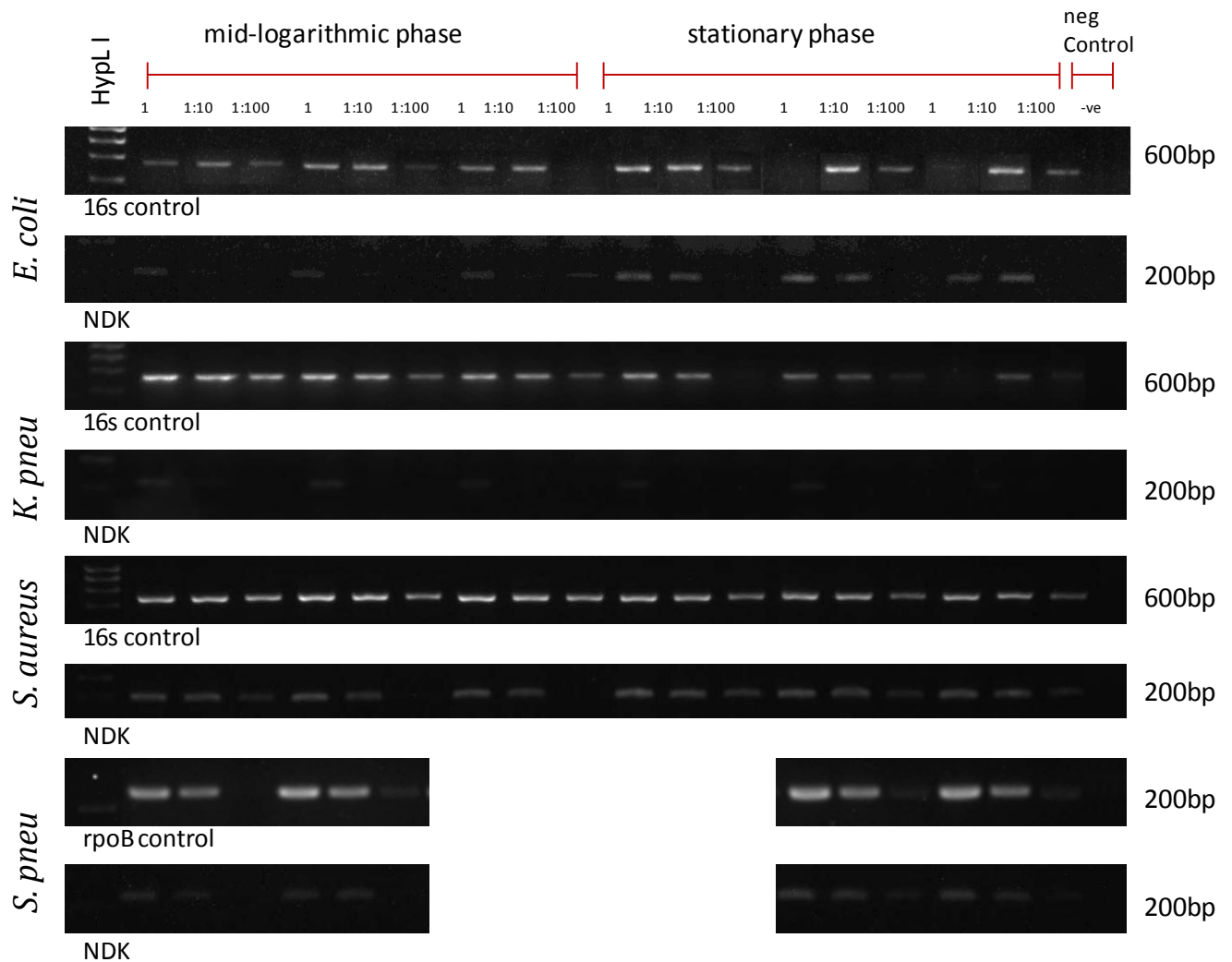


Figure 24 NDK Gene Expression of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*

Figure 28 shows the NDK gene expression at mid-logarithmic and at stationary bacterial growth phase compared with control gene expression. (A) RNA extraction, 5ul of sample separated on 1% agarose gel. (B) RNA quantification using gene tool, to determine the volume of each sample required in 2ng of RNA for cDNA production. (C) cDNA expression of control gene and NDK gene at mid-logarithmic and stationary phase, neat, 1 in 10 and 1 in 100 dilution, to ensure cDNA quantification was obtained from unsaturated bands.

8.3 Mid-logarithmic and Stationary phase cDNA expression of NDK

cDNA synthesis of NDK for the 4 species of bacteria was obtained from RNA at mid-logarithmic and at stationary phase. In figure 3 the expression of NDK at mid-logarithmic and at stationary phase were compared, expression values were corrected for using the control cDNA quantities and results were shown as a percentage, where mid-logarithmic growth was marked at 100% and stationary phase was calculated to be either higher or lower depending on the cDNA quantification values. Values were only obtained from unsaturated bands, to determine an accurate cDNA value. In 3 of the 4 bacterial species dilution 1:10 was used to determine the percentage expression of cDNA at stationary phase. In *K. pneumoniae* expression was calculated from neat. The graph shows quite clearly that NDK was expressed nearly 3 fold more at stationary phase than at mid-logarithmic phase in *e. coli*, and nearly 2 fold at stationary phase in *S. aureus*, however in both *K. pneumoniae* and *S. pneumoniae* there was no significant difference.

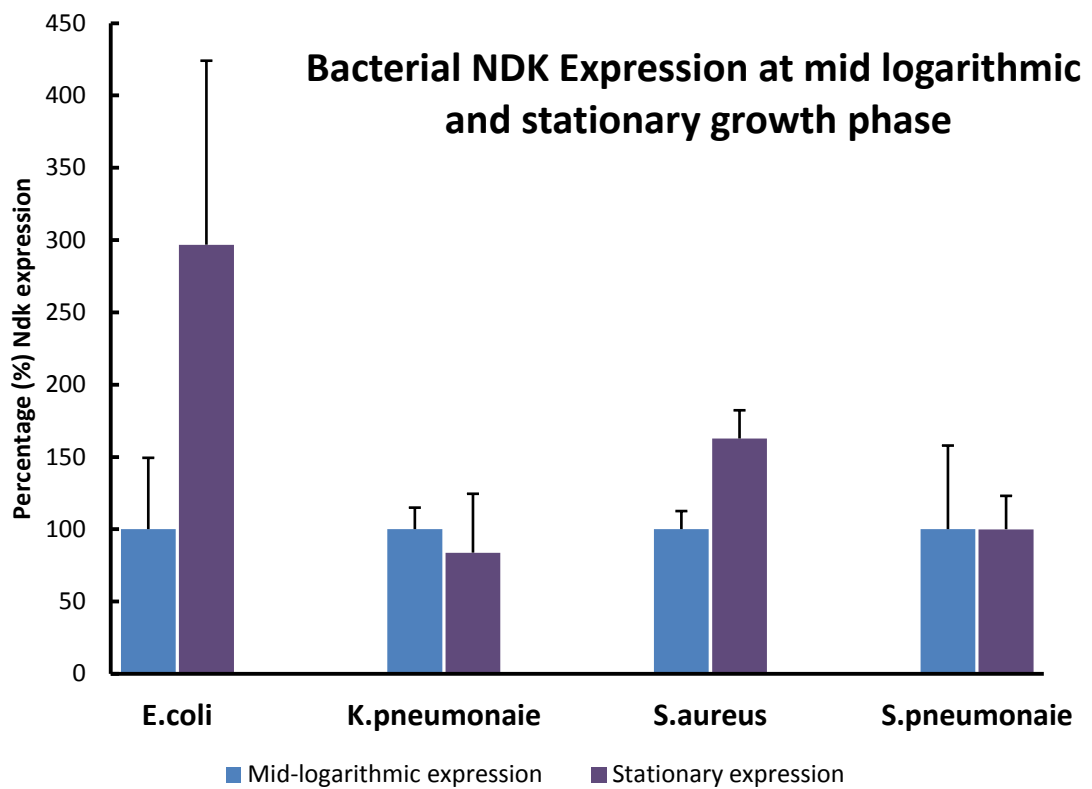


Figure 25 Bacterial NDK Expression at mid-logarithmic and stationary growth phase

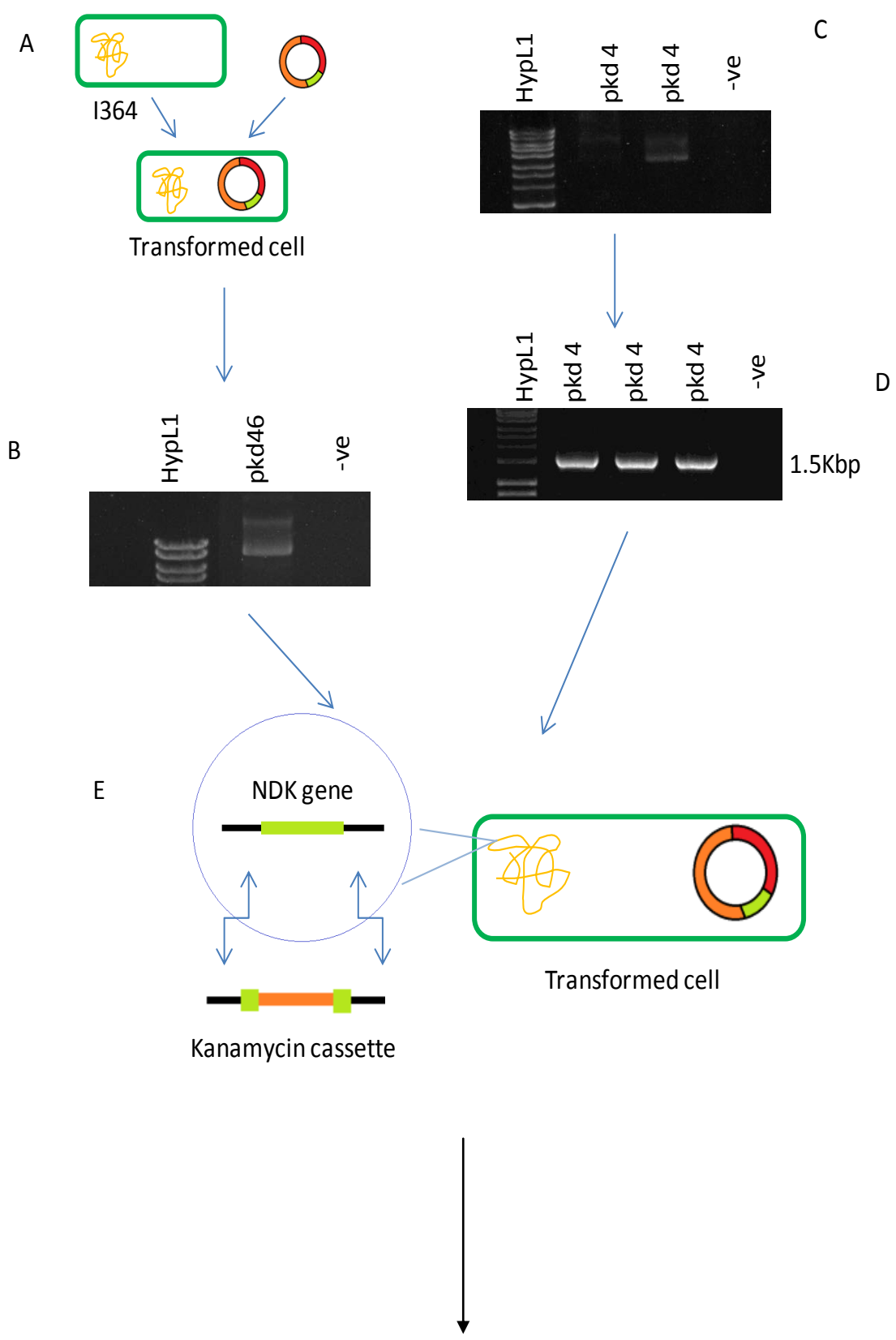
Figure 29 shows the expression of NDK of *E. coli*, *K. pneumoniae*, *S. Aureus* and *S. Pneumoniae* at mid-logarithmic and at stationary growth phase. NDK expression was calculated by assuming NDK expression at mid logarithmic phase was calculated as 100% and the stationary phase expression was calculated as a difference in percentage between mid logarithmic and stationary phase. *Please see appendix 3 for workings for NDK expression.*

8.4 NDK Knockout construction in *Escherichia coli* using Datsenko and Wanner Method

To construct NDK gene knockout, I364 *E. coli* cells were prepared by transformation of pkd46 plasmid (figure A) which contains lambda recombinase, which helps in the targeted genetic changes in *E. coli*, i.e. 'swapping' of the NDK gene with a kanamycin cassette. The successful transformation of pkd46 plasmid into I364 *E. coli* cells was shown in figure B. Because the plasmid was super coiled it does not pass through the gel. Figure C shows the purification of pkd4 plasmid, which was isolated from a *salmonella* strain. Using primers complimentary to the kanamycin cassette, with an end sequence complimentary to the NDK gene, the kanamycin cassette within the pkd4 plasmid was amplified, (figure D) the amplified kanamycin cassette will have a tail nucleotide sequence which was complimentary to NDK; this will help with the gene knockout process.

The kanamycin cassette was electroporated into the I364 *E. coli* with pkd46 plasmid shown in figure E, and grown over night in kanamycin LB plates. Successful colonies were screened using PCR with Knockout check primers, designed with a nucleotide sequence either side of the kanamycin cassette, thus the presence of a kanamycin cassette was indicative of a successful knockout. However the presence of NDK gene indicates unsuccessful gene knockout, which was shown in figure F. The knockout production was repeated several times. Primers were also used to determine whether the kanamycin cassette could have been inserted into another region of the bacterial genome, this was done by using primers specific to nucleotides inside the kanamycin

cassette. Figure G shows that this was not the case as there was no presence of the kanamycin cassette.



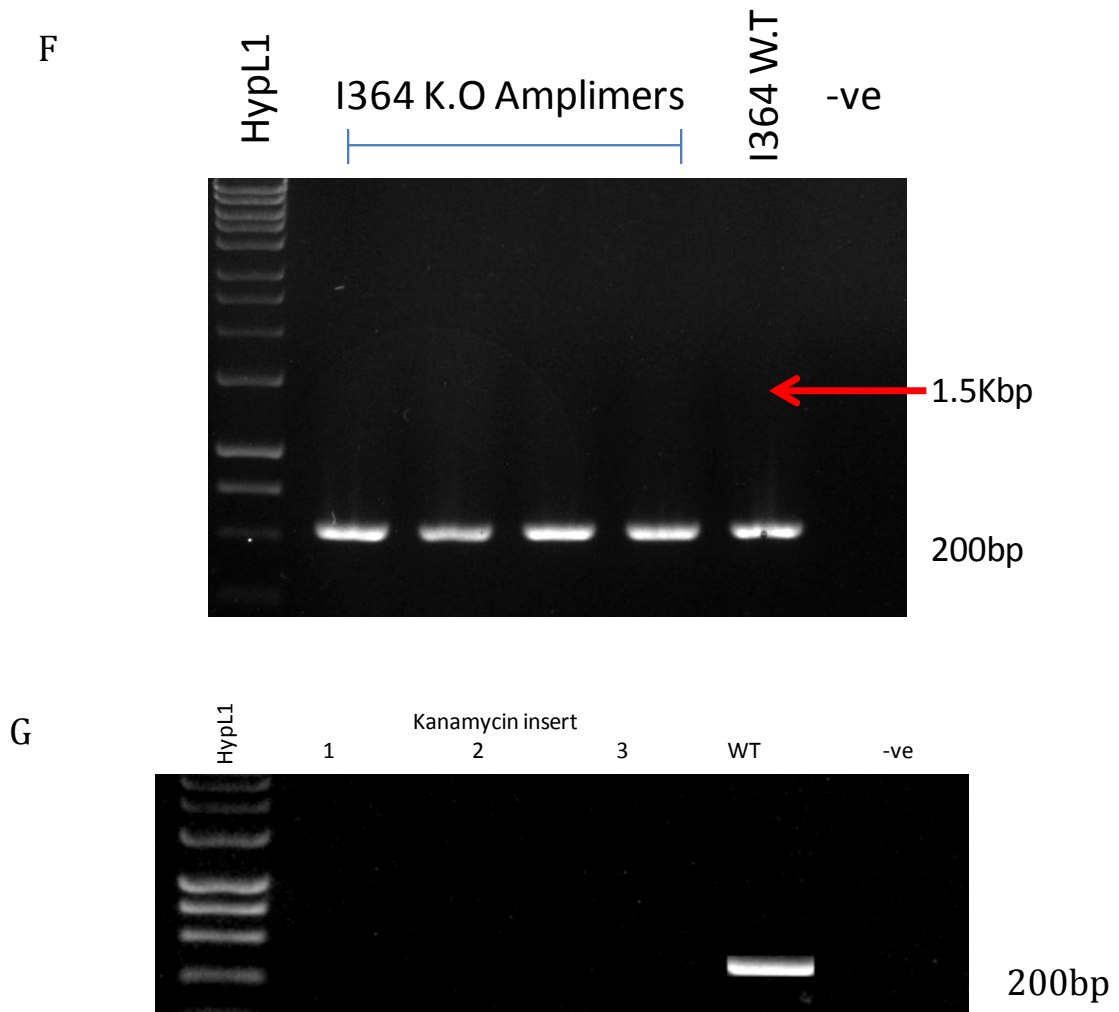
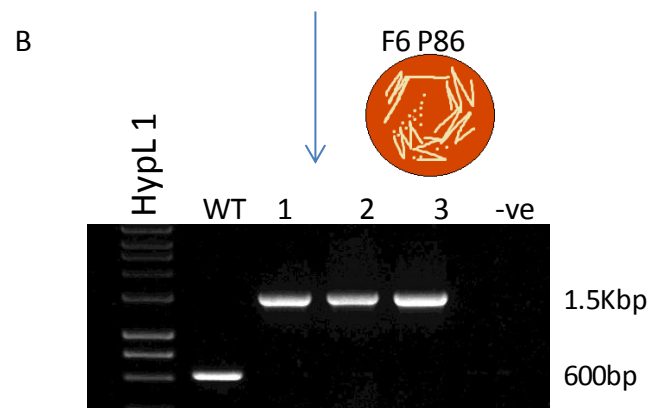
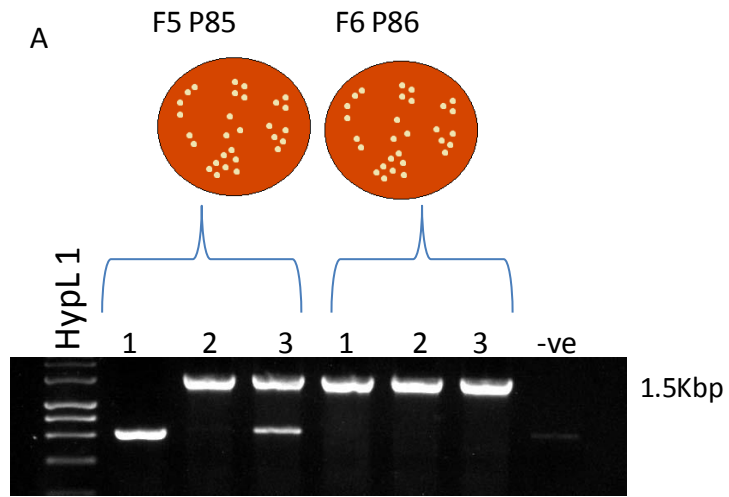


Figure 26 Schematic diagram of *E. coli* I364 K12 NDK knockout Construction

Figure (A) shows the transformation of *pkd46* (containing lambda red recombinase) plasmid into I364 *E. coli* cell. Figure (B) shows the purification of *pkd46* plasmid from *E. coli* I364 to show successful transformation. Figure (C) shows the purification of *pkd4* plasmid, and figure (D) shows the PCR amplimers of the kanamycin cassette from the *pkd4* plasmid. Figure (E) indicates the 'swapping' of NDK gene with Kanamycin cassette (knockout construct) during electroporation of background I364 strain containing *pkd46* plasmid with knockout construct. Figure (F) shows amplimers of electroporated colonies grown on selective antibiotic (kanamycin) plates. Figure (G) shows the amplification of the kanamycin cassette using primers specific to the inside of the cassette

8.5P86 F6 NDK Knockout

The production of NDK gene knockout was not successful. A F6 p86 NDK gene knockout from a knockout library was obtained. To determine that the NDK knockout was a true knockout, PCR was done on selected colonies of 2 NDK knockout strains, using the knockout check primers, designed for the purposes of the previous experiment., shown in figure A. In figure B, bands were separated by gel electrophoresis, and it can be seen that for F5 p85 are a mixture of wild type and knockout, whereas F6 p86 were all true knockouts, shown by the band at 1.5Kbp (kanamycin cassette). For the purposes of this project, all our work was done in *e. coli* I364 and therefore the F6 p86 NDK knockout was transduced into I364 cells, shown in figure C and D. Figure E shows the successful transduction of F6 p86 into I364, by the presence of a band at 1.5Kbp representative of the kanamycin cassette.



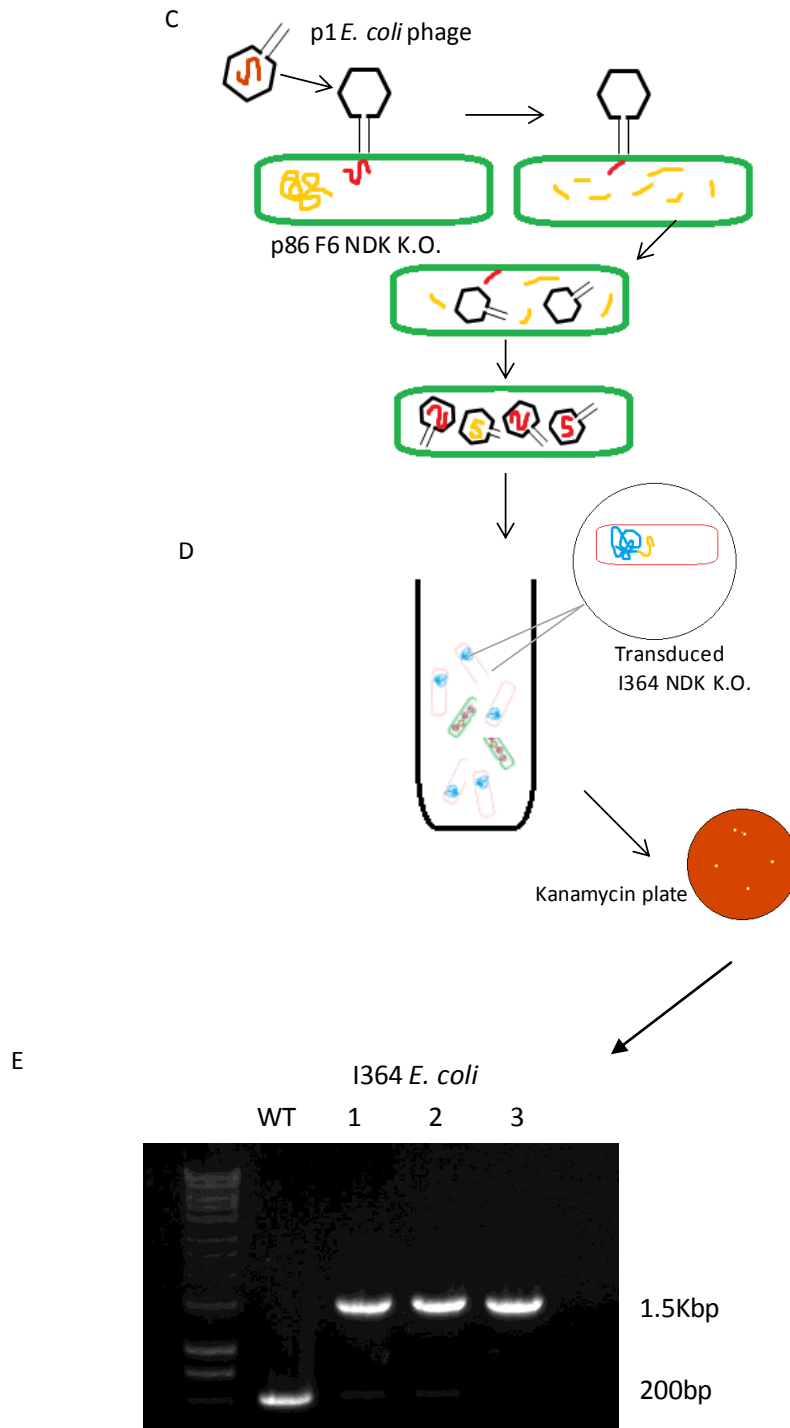


Figure 27 schematic diagram of p86 F6 NDK KO transduction into I364

Figure (A) screen of F5 p85 and F6 p86 NDK knockouts from knockout library to identify 'true' knockouts. Figure (B) freshly streaked F6 p86 onto kanamycin plates re-screened using kanamycin cassette check primers. Figure (C) production of p1 *E. coli* bacteriophage with F6 p86 Knockout DNA. Figure (D) transduction of p1 *E. coli* bacteriophage with I364 background strain. Figure (E) PCR amplification of selected colonies to show successful transduction.

8.6 NDK Knockout Growth Kinetics

Bacterial growth kinetics was done on *Escherichia coli wild type* and both knockout strains, to see whether the knockout of NDK influences the growth of *E. coli*. An absorbance reading was taken every 10 minutes for 16.5 hours. Figure 6 shows that knocking out the NDK gene, does not have an effect on the growth of *Escherichia coli*, the only notable difference was that the lag phase of the knockout bacterium was slightly increased, however all 3 strains grow to the same levels quite happily.

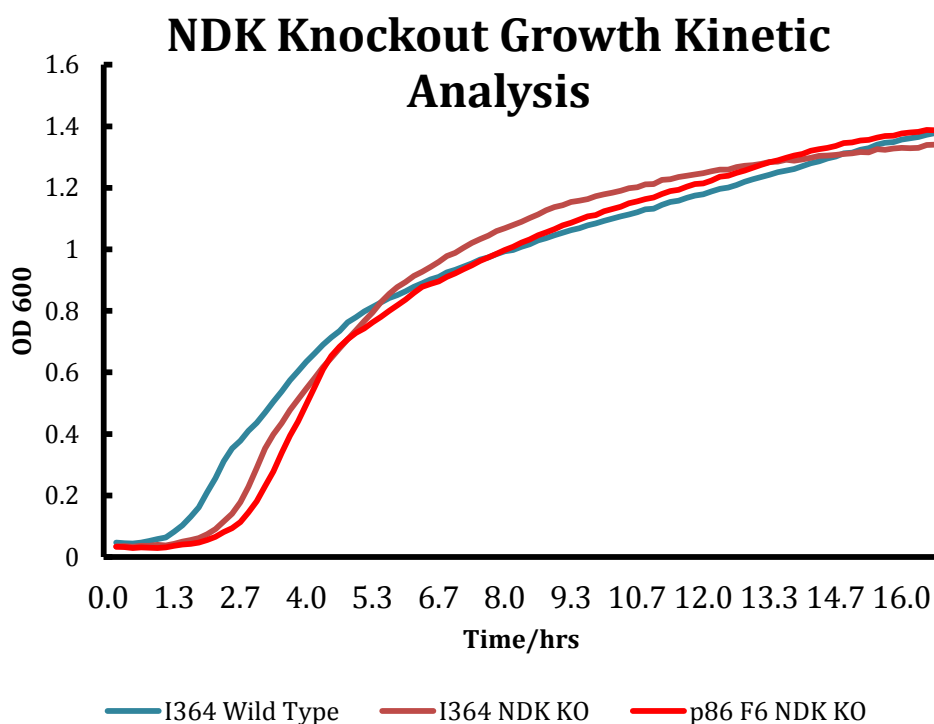
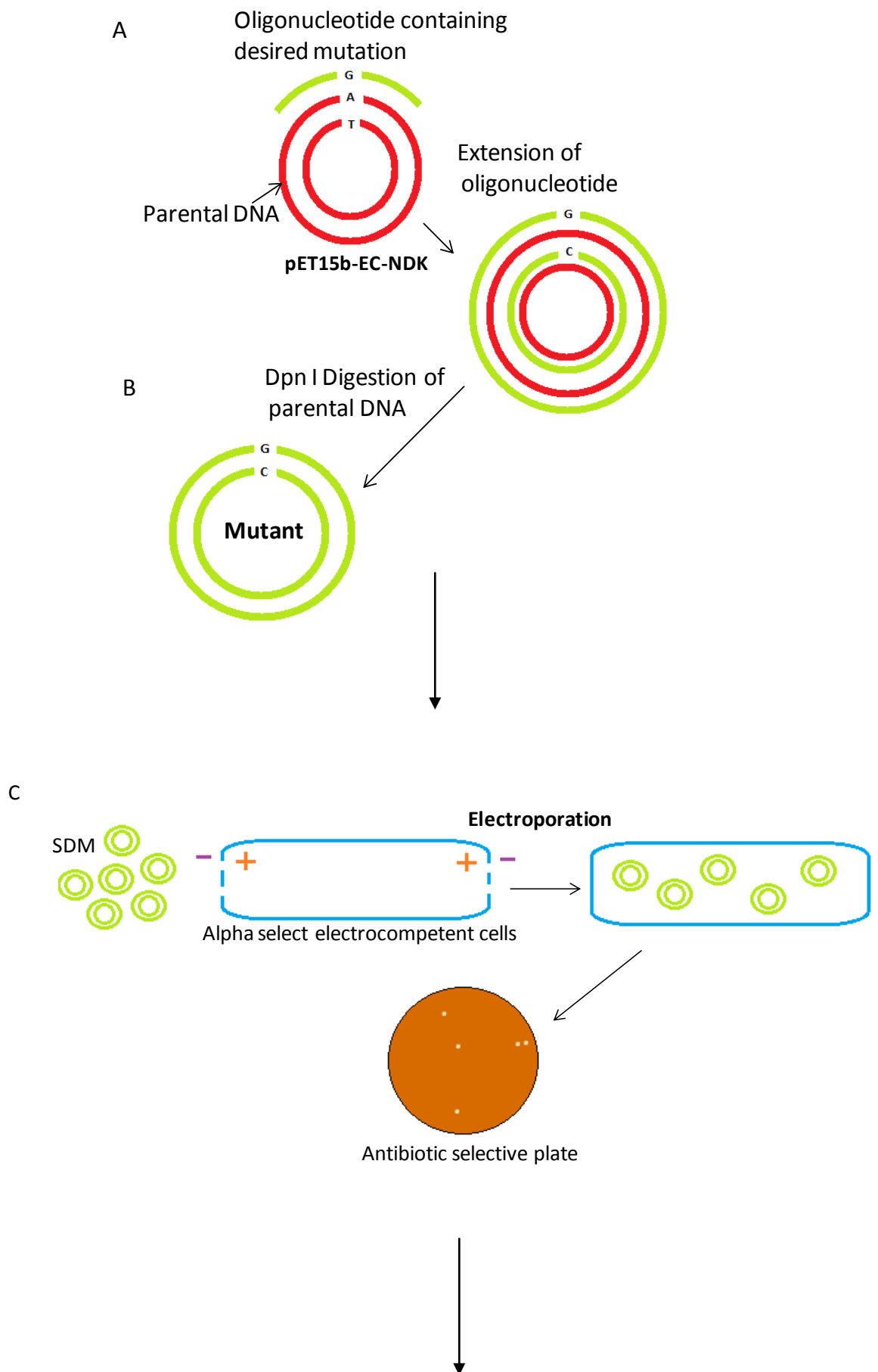


Figure 28 Growth Kinetics for I364 NDK Knockout, p86 F6 NDK knockout compared with I364 Wildtype.

Figure 32 shows the growth of *E. coli* I364 wild type and knockout mutants, to determine any differences in growth. Grown over a 16.5 hour period with an absorbance reading taken every 10 minutes.

8.7 Site Directed Mutagenesis

To produce a site directed mutant, primers were designed with a single nucleotide change within the NDK sequence where the mutation was desired. These primers were used to synthesis 2 complimentary oligonucleotide sequences containing the desired mutation, Shown in figure A. A pET15b plasmid was used which already contains the NDK gene, ready to be mutated. The parental DNA was digested using Dpn 1 restriction enzymes, show in figure B. The mutants were electroporated into alpha select competent cells and grown on a selective antibiotic LB plate. The pET15b plasmid contains an ampicillin resistant gene, and therefore electroporation of the plasmid will be indicative of bacterial growth on ampicillin plate (figure C). Colonies that grow on the ampicillin plates were sequenced to determine whether they were true mutants. Most plates only grew a couple of colonies, but sometimes these can be false positives. Figure D shows the sequence results of 3 successful mutants. The nucleotide substitutions/deletion was highlighted in each sequence, and also highlighted in wild type NDK



D

Wild type NDK

```
atggctattgaaagctacttttccatcatcaaaacggaacgaggttagcaaaaaagctaat
M A I E R T F S I I K P N A V A K N V I
ggtaataatcttggcggcttgaagctgcaagggttcaaaatgttggcaccacaaatgctg
G N I F A R F E A A G F K I V G T K M L
cactgacoggtgaaacaggaacogtggctttatgctgaacaogtaggaaaaacogtctttt
H L T V E Q A R G F Y A E H D G K P F F
gatggtctgggtgaaatcatgacctctggccogatactgggttccogtctggaaagtgaa
D G L V E F M T S G P I V V S V L E E E
aacgcoggtcagcgtcaccogcgtctgctgggogcgaacaaacogcaaacgcactggct
N A V Q R H R D L L G A T N P A N A L A
ggtactctggcggctgattacogtgacagcctgacogaaaaogtaccacoggttctgat
G T L R A D Y A D S L T E N G T H G S D
tccgtogaaatctgocogctogogaaatogcttattcttggogaaggogaagtgtgocog
S V E S A A R E I A Y F F G E G E V C P
cgcaccogttaa
R T R -
```

Substitution KP11,12 – AA11,12

```
gtagcatatggctattgaaagctacttttccatcatcgcagcgaacgaggttagcaaaaaac
- H M A I E R T F S I I A A N A V A K N
gtcaatggtaataatcttggcggcttgaagctgcaagggttcaaaatgtcggcaccacaa
V I G N I F A R F E A A G F K I V G T K
atgctgacogtgaacoggtgaaacaggaacogtggctttatgctgaacaogtaggaaaaacog
M L H L T V E Q A R G F Y A E H D G K P
ttcttggatggtctgggtgaaatcatgacctctggccogatactgggttccogtctggaa
F F D G L V E F M T S G P I V V S V L E
ggtgaaacgcoggtcagcgtcaccogcgtctgctgggogcgaacaaacogcaaacgcactgg
G E N A V Q R H R D L L G A T N P A N A
ctggctggtaactctgocogctgattacogtgacagcctgacogaaaaacogtaccacoggt
L A G T L R A D Y A D S L T E N G T H G
ctgatccogtogaatctgocogctogogaaatogcttattcttggogaaggogaagtgtgocog
S D S V E S A A R E I A Y S W X K A E R
```

Deletion AA 103-105

```
tggtagcatatggctattgaaagctacttttccatcatcaaaacggaacgaggttagcaaaaa
W - H M A I E R T F S I I K P N A V A K
aacgtaattggtaataatcttggcggcttgaagctgcaagggttcaaaatgtcggcacc
N V I G N I F A R F E A A G F K I V G T
aaaaatgctgacogtgaacaggaacogtggctttatgctgaacaogtaggaaaa
K M L H L T V E Q A R G F Y A E H D G K
cogtcttggatggtctgggtgaaatcatgacctctggccogatactgggttccogtctgg
P F F D G L V E F M T S G P I V V S V L
gaaggtgaaacgcoggtcagcgtcaccogcgtctgctgggogcgaacaaacogcaaac
E G E N A V Q R H R D L L G A T N P A N
Gcactggctggtaactctgattacogtgacagcctgacogaaaaaggtaccacoggttct
A L A G T D Y A D S L T E X G T H G S
gatccogtogaatctgocogctogogaaatogcttattcttggogaaggogaagtgtgocog
D S V E S A A R E I A Y S L R R S N R
```

Substitution G79 – A79

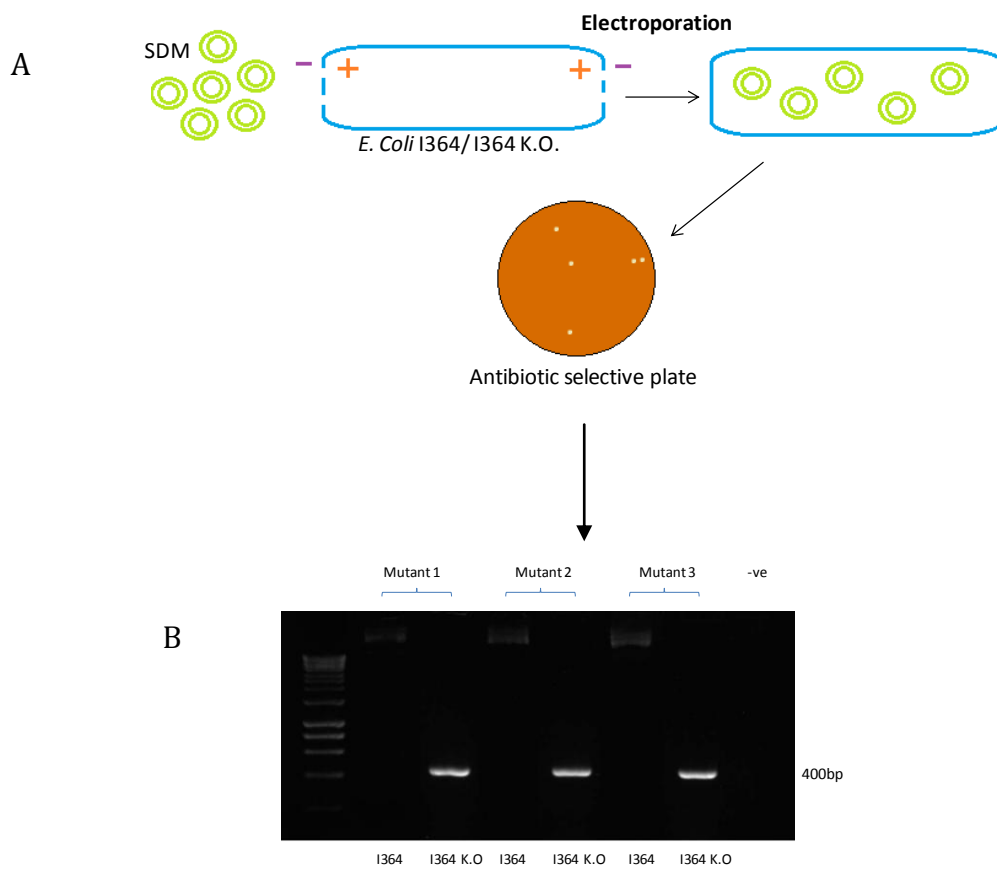
```
gtagcatatggctattgaaagctacttttccatcatcaaaacggaacgaggttagcaaaaaac
- H M A I E R T F S I I K P N A V A K N
gtcaatggtaataatcttggcggcttgaagctgcaagggttcaaaatgtcggcaccacaa
V I G N I F A R F E A A G F K I V G T K
atgctgacogtgaacoggtgaaacaggaacogtggctttatgctgaacaogtaggaaaaacog
M L H L T V E Q A R G F Y A E H D G K P
ttcttggatggtctgggtgaaatcatgacctctggccogatactgggttccogtctggaa
F F D G L V E F M T S G P I V V S V L E
cctgaaacgcoggtcagcgtcaccogcgtctgctgggogcgaacaaacogcaaacgcactgg
A E N A V Q R H R D L L G A T N P A N A
ctggctggtaactctgocogctgattacogtgacagcctgacogaaaaacogtaccacoggt
L A G T L R A D Y A D S L T E N G T H G
ctgatccogtogaatctgocogctogogaaatogcttattcttggogaaggogaagtgtgocog
S D S V E S A A R E I A Y L G E R R X
```

Figure 29 Schematic diagram of Site Directed Mutant production

Figure (A) thermal cycling and formation of complimentary oligonucleotides containing desired mutations. (full primer designs can be seen in appendix) Figure (B) Dpn I digestion of parental DNA. Figure (C) Electroporation of Dpn I treated mutant DNA into alpha-select competent cells. Figure (D) sequence results of successful site directed mutagenesis.

8.8 Electroporation of SDM into I364 and I364 KO background

For the purpose of this project, all our work was done in *Escherichia coli* I364 strain, therefore all the mutants were electroporated into like strain, for an accurate comparison on further experimentations. Figure A shows the electroporation of SDM into competent I364 and I364 knockout backgrounds. Electroporated cells were grown on ampicillin resistant plates, and colonies grown were screened to determine whether the mutant has been successfully electroporated. The I364 KO background containing potential mutants, were checked by PCR using the *E. coli* NDK primer set to amplify the NDK gene, a positive result will indicate a successful electroporation, because the I364 KO strain will not contain the NDK gene within its chromosomal DNA, as it has been knocked out, shown in figure B. The I364 strain, because it contains its own NDK gene in its chromosome, cannot be screened in the same way. Therefore miniprep was done to purify the plasmid. A positive band for the plasmid indicates a successful electroporation, shown in figure B.



Mutant 1 = a31g_a32_c34g, Mutant 2= g236c, Mutant 3 = del307_315

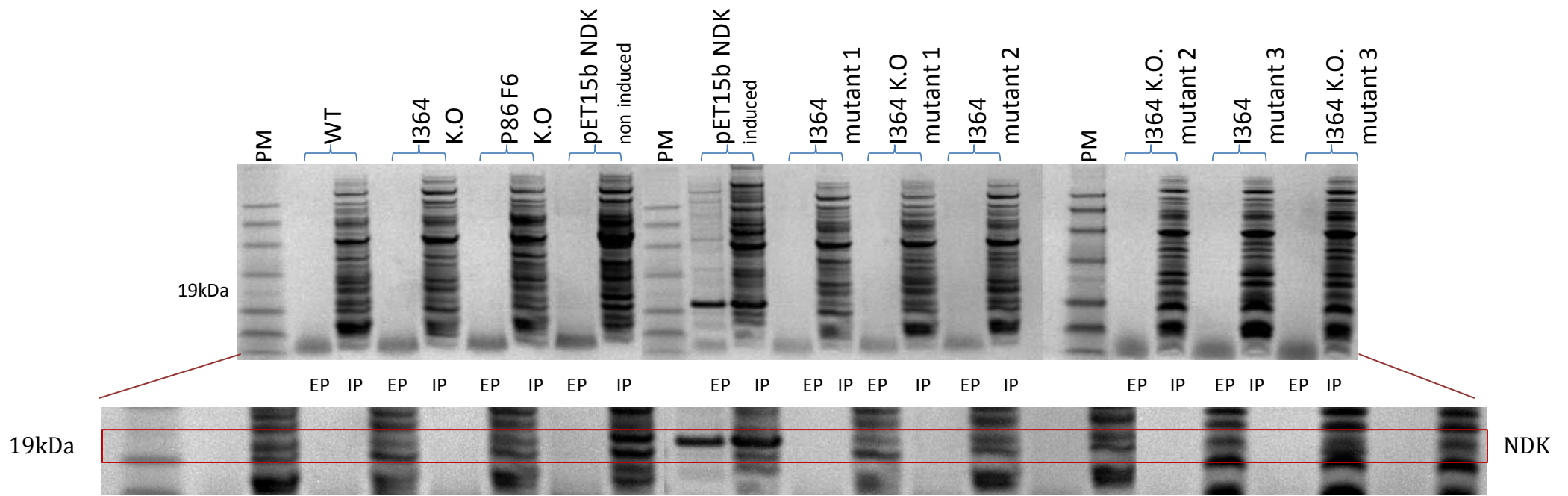
Figure 30 Electroporation of SDM into I364 and I364 KO background

Figure A, electroporation of purified site directed mutants into I364 and I364 knockout strains. Figure B shows the successful electroporation of mutants into both background stains.

8.9 Protein expression of *E. coli* wild type and *E. coli* NDK mutants

The expression of NDK protein from *E. coli* wild type and the *E. coli* mutants were compared. Cultures containing the Site Directed Mutants within the pET15b plasmid were induced along with the *E. coli* pET15b NDK wild type plasmid, with 1mmol of IPTG. The *E. coli* knockout and wild type was left to grow under the same conditions as the site directed mutant cultures, however without induction with IPTG, (because they're not inducible with IPTG). After induction cultures were spun down and pelleted, cells were denatured to release intracellular protein and mixed with gel loading buffer. 20ul of supernatant containing any extracellular protein and denatured cell lysate containing intracellular protein were run on a 4-12% Bis-Tris Bolt protein gel.

The mass of NDK falls approximately at 20kDa. From Figure 9 you can see clearly that the pET15b *E. coli* NDK which over expresses NDK, shows a definite band at approximately 20kDa, indicative of NDK. I364 wild type, you can see a faint band, indicative of NDK. It was not easy to establish whether the knockouts lack the NDK banding at 20kDa or not. It can also be observed that the site directed mutants contained within the pET15b plasmid, which should over express NDK when induced, like the pET15b NDK wild type, does not express NDK to the same levels as the wild type does.



Mutant 1 = a31g_a32_c34g, Mutant 2= g236c, Mutant 3 = del307_315

Figure 31 Protein Expression of *E. coli* Wild type and *E. coli* NDK mutants

Figure 35 shows the differences in expression of *E. coli* wild type NDK, pEt15b over expressed *E. coli* NDK, *E. coli* NDK knockout mutants and *E. coli* site directed mutants. 5ul of intracellular and extracellular protein samples were run on 4-12% Bolt Bis-Tris protein gels.

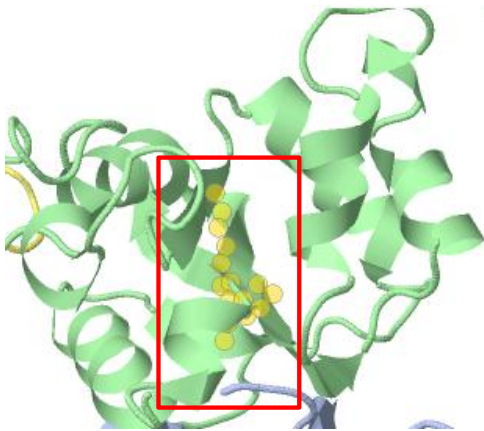
8.10 Protein Models of NDK wild type and

Protein modelling was used to determine whether there were any structural differences between wild type and the mutated NDK proteins. SWISS computer modelling was used to predict models of the structures for *E. coli* wild type and *E. coli* mutant NDK proteins.

Protein alignments for each mutant and wild type were entered into an online database, used to calculate the structure of each protein.

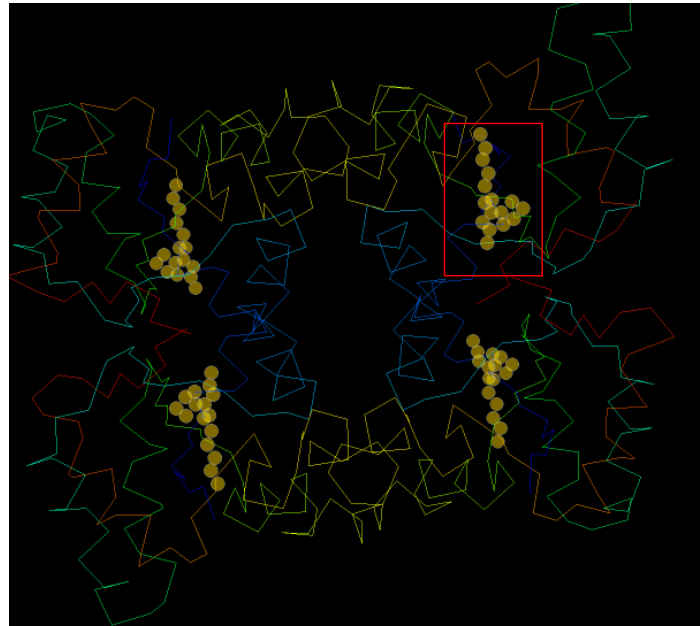
Mutant 1 and 2 show no structural differences between wild type and mutant NDK. This was as expected as these were just amino acid substitutions. Mutant 3 however, where 3 amino acid residues AA103 to AA 105 had been deleted shows a structural difference within the conserved KPN loop region, as seen in figure the last diagram of figure 10.

Wild type 1

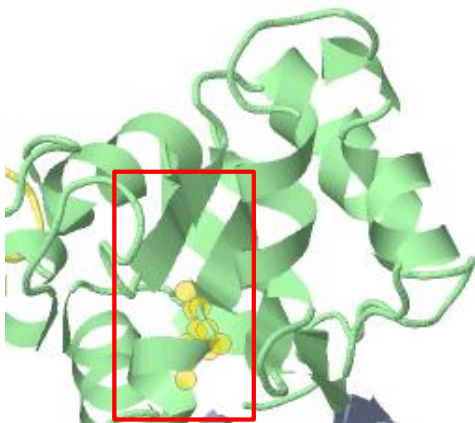


Lysine11 (9 atoms) and
Proline12 (7 atoms)

NDK *E. coli* Wild type
monomer

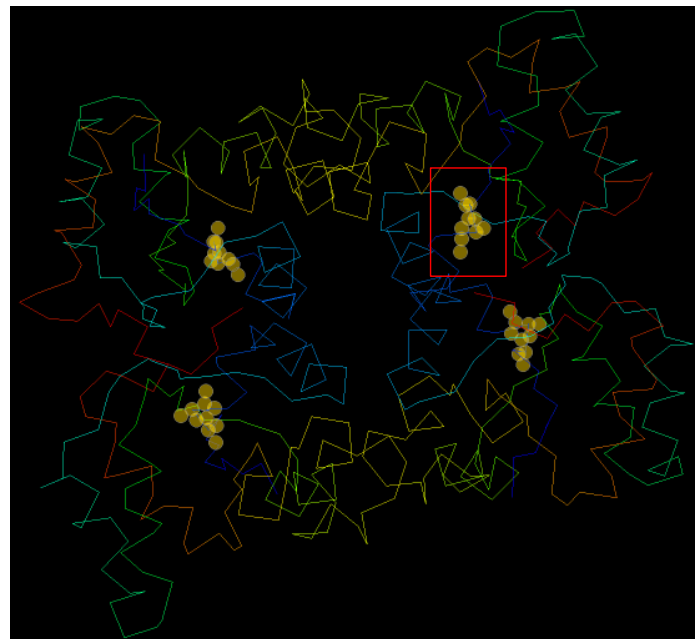


Mutant 1

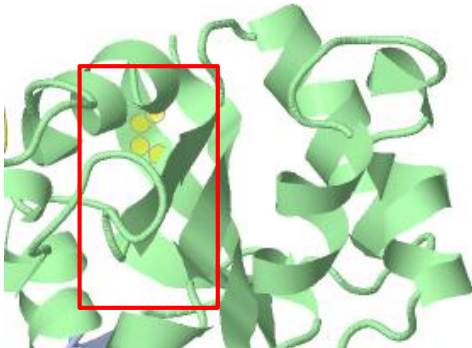


Alanine11 (5 atoms) and
Alanine12 (5 atoms)

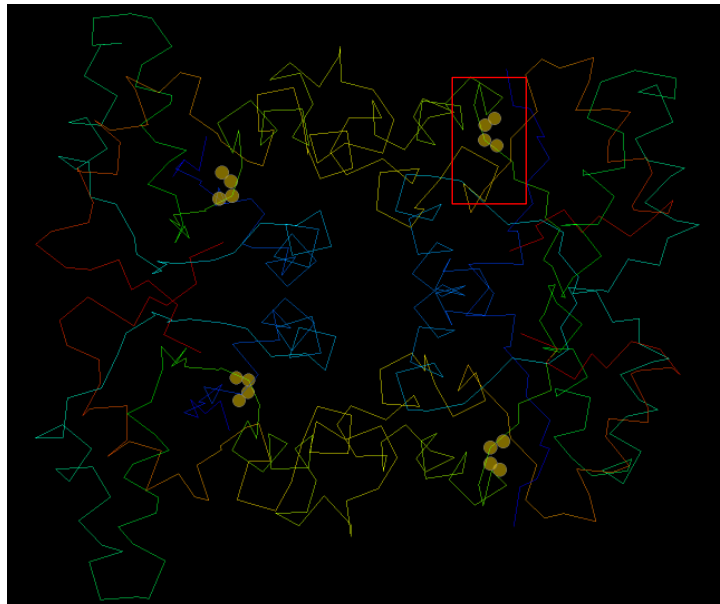
NDK *E. coli* mutant 1
monomer



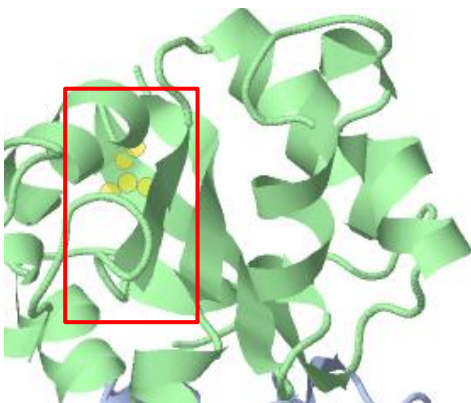
Wild type 2



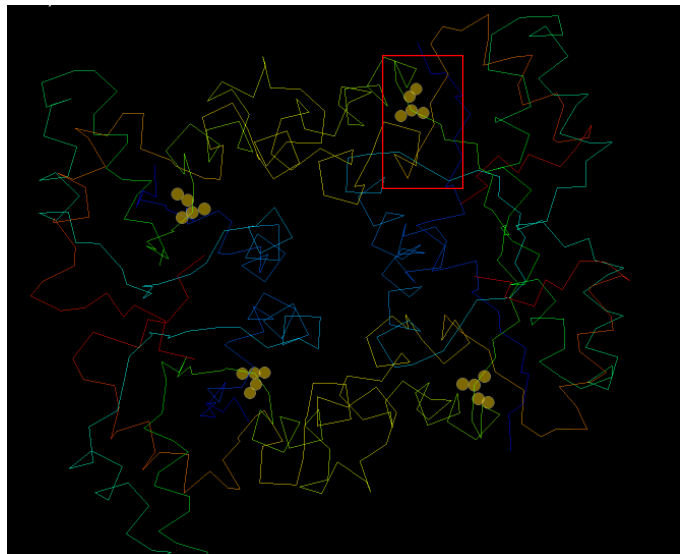
Glycine79 (4 atoms)
NDK *E. coli* wild type
monomer



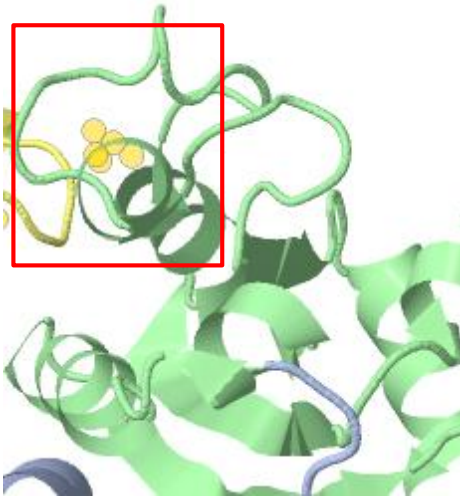
Mutant 2



Alanine79, (5 atoms)
NDK *E. coli* mutant 2
monomer

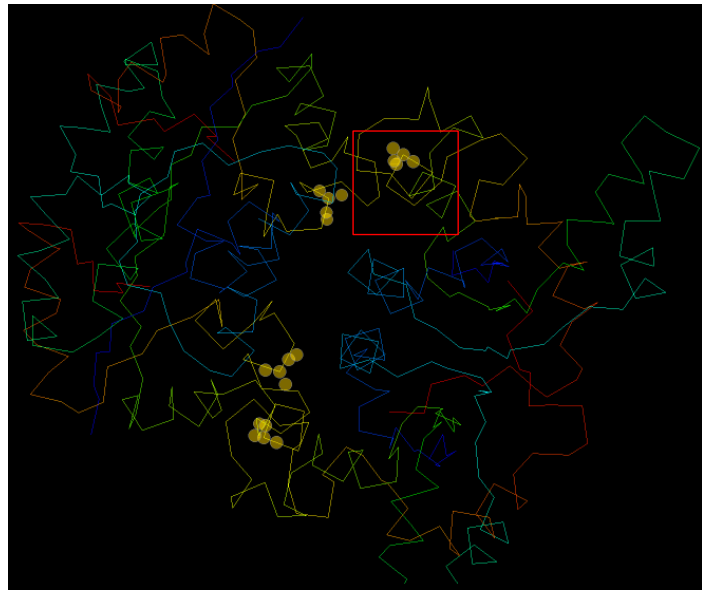


Wild type 3

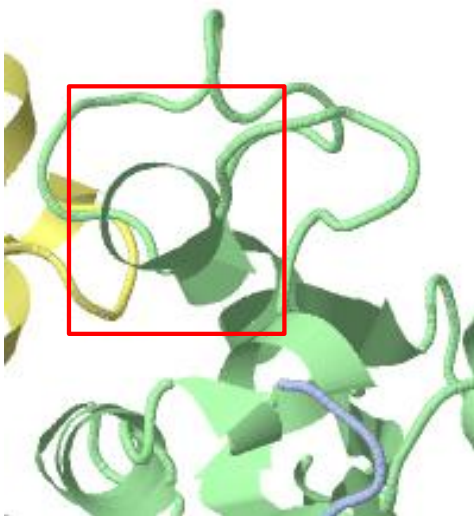


Alanine103-105, (5 atoms)

NDK *E. coli* wild type 3 monomer

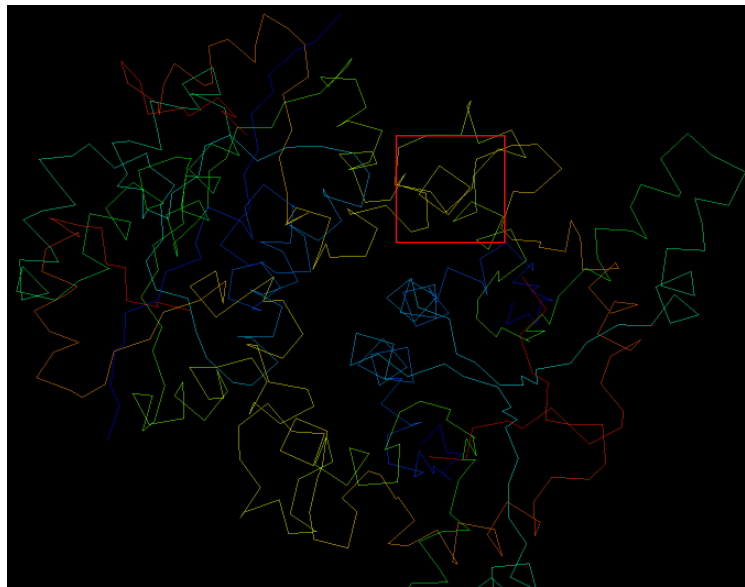


Mutant 3



Deletion - Alanine103-105, (5 atoms)

NDK *E. coli* mutant 3 monomer



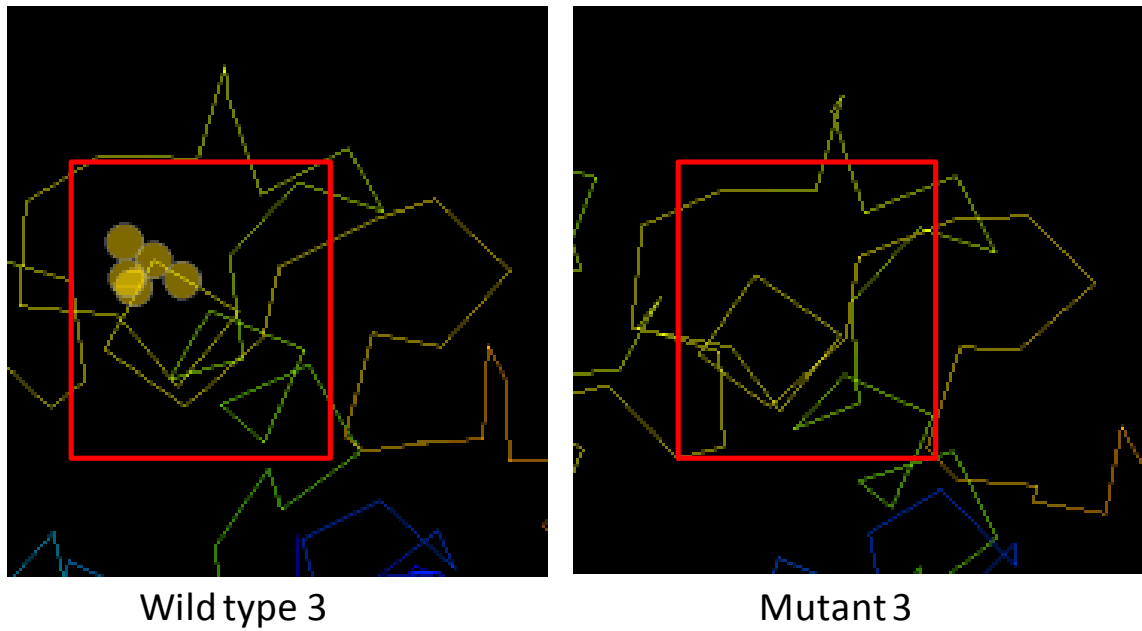


Figure 32 Predicted Protein Models of wild type and mutant NDK proteins

Figure 36 shows predicted models for the 3 SDMs compared with wild type I364 NDK protein. Predicted models were made using SWISS model and mutated domains were highlighted using first glance in Jmol.

9. PART B CONCLUSION

This research project has shown that it was successfully possible to obtain NDK knockout mutant, as well as site directed mutants, over several conserved domains. It was possible to optimise protocols, over the problems that we faced, in obtaining these mutations. Mutated proteins were expressed and predicted structures for these proteins using computational modelling software were obtained. The protein expression gel shows the differences in the expression of induced and non induced NDK proteins of the different mutations, indicating that the mutation in the NDK conserved domains may play a role in the expression of the protein.

Using the SWISS modelling software to predict the structure of the mutants, the only mutant to show a change in structure was the Kpn loop deletion mutant, this was a conserved domain shown to play an important role in structural formation of NDK.

10. DISCUSSION

10.1 Can 'normal' stem cells use bacterial NDKs to promote their survival?

To investigate the affects of bacterial NDK on 'normal' stem cells, recombinant bacterial NDK protein from *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were cultured with umbilical cord blood CD34-ve cells, and the conditioned media depleted of NDK protein, were then cultured with CD34+ve cells.

Previous studies show that NM23-H1 bind to more mature clones and not CD34+ve stem cells themselves. The culture of CD34-ve cells with NM23-H1 causes NM23-H1 to bind with CD11b+ve cells and the release of cytokines, which stimulate survival. (Lilly, et al., 2011) Taking this into consideration; we based our technique on the same principle that bacterial NDK proteins will bind to more mature CD11b+ve cells and release cytokines which will be cultured with CD34+ve stem cells to promote survival.

However because bacterial NDK have not been investigated previously it was decided to not sort for CD11b+ve cells from umbilical cord blood, but to culture all CD34-ve cells with NDK protein; and observe viability. A second batch of umbilical cord blood samples were sorted for CD11b+ve cells, which where cultured with NDK protein.

The results suggest that both techniques allow for a slight increase in viability; however this increase in survival was not significant through all the bacterial species, and reproducing the results would indicate whether these results can be significant enough to accept the hypothesis, of ***"I hypothesise that bacterial NDK proteins will indirectly promote the***

survival and proliferation of umbilical cord blood stem cells and acute leukaemic progenitor cell.”

The results obtained for the stem cell assay using NM23-H1 protein, showed to be significant, this confirms and reproduces results that have been seen before, (Lilly, et al., 2011) For the survival of CD34+ve stem cells, however it was noted that in the stem cell assays, it was not possible to reproduce the results that Lilly et al, had produced to show the proliferation of stem cell growth not just increased survival. There were a few reasons why this could have been.

The samples which were not sorted for CD11b+ve had a lower viability rate than those that were sorted. This could be due to an ‘overcrowded’ population of cells which do not bind NDK protein, making it harder for CD11b+ve cells to bind. It was also observed while culturing that; samples that had been sorted for CD11b+ve cells had formed clusters of cells when treated with NDK; this observation was seen at a slower rate with samples cultured with full CD34-ve cell populations. One could also argue that culturing with a full CD34-ve population was closer to the microenvironment within a host, and therefore more likely to be an accurate representative model of what would occur in vivo.

To analyse purity of our sorted samples, pre and post stains for CD34 was done on umbilical cord blood cells. Our FAC analysed post sort results show that our purity of CD34+ve samples, were not as pure as we would have liked. This could have been due to samples being ready for collection only towards to end of the day, and samples being kept in the fridge overnight and prepared only the morning after. With this suggested, consecutive cord blood samples were prepared the same day they were retrieved on. However results still indicate an impure population of CD34+ve cells. The bags in which the cord bloods were

retrieved in; contain a fixed amount of anti-coagulant, however this does not take into consideration the volume of cord blood received, and therefore if anti-coagulant was insufficient in volume in relation to the volume of cord blood, cells will start to become 'sticky' and clot, making the sort for CD34+ve cells harder. This could be overcome however, by doing a double sort, although this would put the cells under more stress; and therefore might cause a change in the viability of our CD34+ve cells.

The results obtained suggest that we were able to reproduce results that the human NM23-H1 protein does act as a survival factor for CD34+ve haemopoietic stem cells. However we were unable to produce significant results of whether bacterial NDK proteins do promote the survival of CD34+ve cells however the preliminary results, were enough to suggest that the stem cell assays with the bacterial NDK proteins were following a similar pattern to that of the human NM23-h1, and therefore a great repeat in stem cell assays, and optimisation of the purification of the stem cells, would help to produce significant results.

Previous studies show that normal haemopoietic stem cells use human NM23-H1 protein for cell survival, development and differentiation. (Lombardi, et al., 2000) And it could be suggested that they may use bacterial NDK proteins in the same manner. This increase in viability of CD34+ve cells could have some therapeutic advantages as well as research advantages.

The increased survival of CD34+ve cell in research, means that cells could be kept viable for longer, so that ongoing research can be done on 1 sample, this could also be an advantage of increased viability in leukaemic cells.

An important advantage of increased survival of CD34+ve stem cells was in a therapeutic setting, for stem cell transplantation. Finding a stem cell match has been a challenge, with fewer than 30% of leukemic patients having HLA antigen sibling matches. An increased public population of bone marrow and stem cell donors were always pleaded for, and matches were always sought as close to the patient as possible, due to the low survival rate of stem cells in vitro. Increasing the survival of stem cells in culture media with conditioned CD34-ve cell media (treated with recombinant bacterial NDK) was a cheap way of keep cell alive. High concentrations of pure recombinant bacterial NDK proteins were easily produced in the laboratory. The results do suggest that not all bacterial NDK cause a significant increase in survival of stem cells; however *E. coli* NDK and *K. pneumoniae* NDK showed consistent increased survival similar to that of NM23-h1.

After culturing of CD34-ve populations with NDK protein; samples were depleted of protein and cultured with CD34+ve cells. The remaining CD34+ve cells were left in the incubator. It was then discovered that cell survival of the CD34-ve population were still viable even after weeks. Investigations were done to look at the morphology of the cells surviving, cell surface markers were used to identify which cells were still viable, and viability counts were also done. The result showed a strong increase in viability compared with elution buffer controls. The Jenna Giemsa stained cells showed a healthy population of both differential leucocytes and erythrocytes, in samples cultured with NDK protein, and dead matter was mainly seen in elution buffer controlled samples. The cell surface marker investigations suggested that the viable cells consisted of macrophages, monocytes, neutrophils, T cells and B cells. It would be of interest to find out whether these cells had immune function; and whether they would be of therapeutic value.

10.2 Does bacterial NDK contribute to the survival of leukaemic cells?

To investigate the affects of bacterial NDK on leukaemic cells, recombinant bacterial NDK protein from *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were cultured with acute myeloid cells and separately with acute lymphoid leukaemic cells.

Previous research has shown that NM23-H1 found in the blood aids in the proliferation and survival of AML cells. AML samples have been shown to have an increased expression of NM23-H1 compared with normal control samples and studies have shown that only 60% of AML cells actually bind NM23-H1. It was not understood fully why not all leukaemic clones bind NM23. (Okabe-Kado, et al., 2009) Early progenitor cells express NM23-H1 cells; more so than more mature haemopoietic cells. The cycle of leukaemic survival could be suggested by a similar mechanism to that of normal haemopoietic stem cell survival. The increased proliferation of leukaemic blast cells into the blood causes an increased expression of NM23-h1, this over expression of NM23-H1, bind to more mature CD11b+ve cells causing the release of cytokines and therefore promoting the survival of AML CD34+ve cells.

The results suggest that acute myeloid leukaemic cells cultured with NDK protein, show an increased survival compared with elution buffer control, the error was quite high. This was due to the lack of repeats as we only obtained one acute myeloid leukaemia sample, therefore repeating this work would decrease the error of the results.

The acute lymphoid leukaemic cells cultured with NDK protein don't show a significant increase, this was as predicted, because as far as literature suggests, NDK proteins only bind to more mature CD11b progenitor cells (CD34-ve cells), which were cells from the myeloid

lineage, hence the increase in survival of acute myeloid leukaemic cells and not acute lymphoid leukaemic cells. However it was hard to comment on the reliability and accuracy of these results because only one sample from each leukaemic was treated at this point. Even though repeats were done within each sample, the standard deviation for acute myeloid leukaemia was quite high, which was suggestive of large variability amongst the repeats.

It could be suggest that bacterial NDK do have an effect on the survival of leukaemic stem cells. This is due to the similarities between the structure and function of the NDK protein and the human N23-H1 protein. With the results from (Lilly, et al., 2011) which show that NM23-H1 does have an effect on the survival of leukaemic stem cells, suggests that with further work, we should get a better idea of the effect of NDKs on leukaemic stem cells.

10.3 NDK expression at mid-logarithmic and at Stationary growth phase.

Figure 29 shows the expression of NDK from 4 species of bacteria. The results suggest that NDK was expressed at a higher level at stationary phase in *E. coli* and in *S. aureus*. Bacteria spend majority of their life cycle at stationary phase. From the literature we know that NDK proteins were used in maintaining the NTP and dNTP pools and play a role in bacterial growth. (Chakrabarty, 1998) NDK were also known to have a secretory function, and play a role in the apoptosis of macrophages (Zaborina, et al., 1999). NDK proteins also play a role in the pathogenicity of bacteria. The high amount of energy required for bacterial pathogenicity can be gained from NDK, when eATP was used from the site of inflammation. It has been suggested that eATP was used by the NDK to favour the survival of the pathogenic bacteria (Hussain, et al., 2011). Both *E. coli* and *S. aureus* were capable of

secreting proteins after infecting the host cell. The higher levels of NDK expressed at stationary phase, suggest that this could be a mechanism for pathogenicity. The roles played by NDK increase the survival chances of *E. coli* and *S. aureus* at stationary phase, as well as increasing the pathogenicity of the bacteria into the host cell (A Latifi, 2008) (Mangan, 2006).

The expression of NDK for *K. pneumoniae* and *S. pneumoniae* show no significant change at mid- logarithmic or at stationary phase. This suggests that NDK may not have a strong pathogenic role in *K. pneumoniae* and *S. pneumoniae*.

10.4 NDK gene knockout

Using the Datsenko and Wanner method it was attempted to knockout the NDK gene from *Escherichia coli*. During the protocol we had successfully transformed our recipient strain 1364 *E. coli* cell with pkd46 plasmid and amplified the kanamycin cassette from pkd4 plasmid as shown in figure 30. However when screening the colonies, which had been electroporated and grown on selective kanamycin plates, they had always shown to be unsuccessful. During the electroporation phase of the protocol the NDK gene within the 1364 *E. coli* strain, should have been knocked out and replaced by the kanamycin cassette in the presence of lambda red recombinase. The amplimer of the kanamycin cassette were tagged with nucleotides complimentary to the end sequence of NDK, to enable 'swapping' to occur more readily. However after several attempts a NDK gene knockout was not successful.

It has been stated that the Lambda red recombinase based protocol was dependent on several factors, one being the removal of as much primer from the PCR reaction, as primers and primer dimers can compete with the cassette. To ensure that as much primer was

removed as possible, an extra step could be implemented after the amplification of the kanamycin cassette, using a PCR clean up kit. The PCR cleanup kits will also remove salt from the PCR reaction, which will allow electroporation of higher DNA concentration without arcing. Another way of optimising the chances of gene knockout would be to increase the concentration of the amplicon DNA; this can be done by using a butanol extraction protocol. (Katrina Bepple, 2004) The NDK protein was highly conserved across eukaryotes, eubacteria and archaea, (Bilitou, Watson, Gartner, & Ohnuma, 2009) and therefore might not be as readily knocked out as other genes, even though the knockout of NDK gene was possible, it might only be possible within rigid protocol methods. Given the time, these optimisation techniques would have been trialled in the attempt of obtaining a successful knockout. However due to time restrictions, a NDK gene knockout was sought from the knockout library.

10.5 NDK knockout growth kinetics

Growth kinetics analysis was done on the NDK knockout constructs in *E. coli* I364 and F6 p86. From figure 32, you can clearly see that the NDK knockout does not affect the growth of *E. coli*, I364 Knockout and F6 p86 knockout both grew to a similar optical density as wild type I364. The only difference in the knockout mutants was that their lag phase was slightly increased, but without any major consequences to the growth of the bacteria. The results obtained within the growth kinetics experiment were what were expected. NDK proteins play a role in bacterial growth, however was not an important protein in the growth of bacteria, and therefore knocking out the NDK gene should not have an adverse effect on bacterial growth.

10.6 Optimisation of SDM protocol

The quikchange II site directed mutagenesis kit, Agilent Technologies, was used to produce the desired mutations. During the production of site directed mutagenesis, a few stages of the protocol had to be optimised, around the instructions given by the manufacturer to allow for efficient mutant products. Using the instructions given for thermal cycling, it produced a low yield of colonies, and those sequenced were false positives. No protocol for mutant production will be sufficient for every site directed mutation desired. Allot depends on the area being mutated, how conserved it was and how important the gene was within the protein. To increase yield efficiency, and increase true mutant production, the annealing temperature was increased to 68°C and timing increased to 10 minutes and the thermal cycling number was increased to 25 cycles, this would allow sufficient annealing of the complimentary mutant oligonucleotides. The Dpn I restriction enzyme was also doubled, and incubation time was also doubled, to allow for sufficient digestion of parental DNA. With these changes, 3 site directed mutations, 2 substitutions and 1 deletion, were successfully produced.

10.7 Expression and modelling of mutant NDK proteins

Whole intracellular and extracellular protein from wild type *E. coli*, wild type pET15b NDK, site directed and knockout mutants were separated on a BOLT 4-12% Bis-Tris Protein Gel, to observe any differences in the expression of NDK. The NDK protein was approximately 19kDa. Shown in figure 35, both the NDK knockout mutants, show a fainter band at 19kDa compared with I364 wild type. Because we separated the whole protein from the bacteria, we cannot conclusively say whether the knockouts lack NDK or not, the presence of a faint band at 19kDa could be from another protein with a similar molecular weight to NDK.

Genetically these mutants were NDK knockouts, as shown in figure 30. To determine the absence of NDK, western blot analysis was required, using specific antibodies for NDK.

Comparing the pET15b NDK non-included protein expression with wild type I364, you'd expect the NDK band to only be expressed from the bacterial chromosomal DNA and not the plasmid, however sometimes these plasmid vectors can leak, and therefore a small amount of protein expression may occur from the plasmid too, which explains the stronger band present at 19kDa compared with wild type.

The pET15b induced protein expressed shows a very prominent band at 19kDa both intracellularly and extracellularly. All 3 of the site directed mutants, don't seem to have expressed NDK protein as expected. All the site directed NDK mutants were incorporated within the pET15b plasmid, same as the pET15b NDK wild type, therefore once induced with IPTG, it was expected that the gene inserted within the cloning region was transcribed into RNA and then translated. What perhaps was occurring was that there was no reason to doubt that the plasmid was not being induced sufficiently, and therefore the gene was being transcribed into RNA, however perhaps because of the mutant it was not being translated and therefore not being expressed. To determine whether the NDK gene was being transcribed, real time PCR will need to be performed using primers specific for NDK. Comparing the SDM in I364 strain and in I364 knockout strain, there was no noticeable difference between the bands at 19kDa. The SDM's within the knockout strain will not express any NDK from the bacteria's chromosomal DNA, but the SDM's within the I364 strain should also express NDK from the chromosomal DNA.

SWISS model, an online protein modelling tool was used to model the wild type and the 3 SDM mutant NDK proteins and first glance in Jmol was used to show the conserved domain

that was mutated and compared with wild type to determine whether the mutations had caused any structural differences. As shown in figure 36, the only mutation to show a structural difference was mutant 3 where AA 103 to 105 were deleted, these amino acids were part of the KPN loop, a conserved domain, which has been shown to play an important role in structural formation of NDK, where the lack of this KPN loop prevents the formation of hexameric structural formations. Mutant 1 and 2 where substitutions were made, show no structural differences.

10.8 Future Work

Further investigations could be done on the cytokines released when CD11b+ve cells bind bacterial NDK, to discover how they may cause the survival of CD34+ve stem cells. This could be done by using a immunoassays or bioassays, however these could be time consuming and the sensitivity of the assays are sometimes not great. However *in situ* hybridisation, which is a direct cellular approach, can be used to give high sensitivity in detecting cytokines produced when CD11b+ve cells bind bacterial NDK. The binding receptor for NM23-H1 and bacterial NDK can be investigated; this could be done by computer modelling, circular dichroism or x ray crystallography.

CD34+ve cell purity techniques need to be reconsidered to obtain optimum purity. A double sort could be considered, but cell survival might be hindered. Apart from using high affinity microbeads and sorting kits, other techniques could be used. These include; Fluorescent Activated Cell sorting (FACs), panning, where anti-CD34 antibodies are bound to the surface of the sorting flask. Once the suspension is added to the flask the CD34+ve cells stick to the surface and the remaining CD34-ve cells remain in the suspension. High affinity

chromatography could also be used to determine a high purity sort. This is dependent on biotin-avidin interactions (Carlo, et al., 1995).

Literature and previous research suggests similarity between NM23 proteins and NDK proteins, with identical binding sites. The protein data bank only contain crystal structures for *Escherichia coli* and *Staphylococcus aureus*, it would be interested to find out how similar other bacterial NDK proteins were to NM23-H1 and other bacterial NDK. NM23-H1 and NM23-h2 were thought to form fusion proteins; could bacterial NDK proteins bind to NM23? What would the affect be on the survival of normal and leukaemic stem cells? Also would a combined assay containing NM23 and bacterial NDK convey altered survival of leukaemic and/or 'normal' haemopoietic stem cells?

The project was undertaken to develop a further understanding and characterisation of Bacterial NDK proteins. Current literature suggests that within the NDK protein, several conserved domains, which have already been mutated, have shown to influence the structural stability of the NDK protein, (Sundin, et al., 1996) as well as a larger percentage prone to mis-pairment. (Miller, et al., 2002). This project looked at 4 bacterial species which were known to cause infections in cancer patients, to determine whether there was a difference in expression of NDK between these bacteria, and whether a mtation in the conserved domains of NDK influence the structure, expression and function of bacterial NDK protein, as literature may suggest.

The work undertaken in this project has provided us with the tools to further the research into bacterial NDK and their affects on the survival of leukemic and haemopoietic stem cells. The preliminary work undertaken on wild type bacterial NDK had suggested an increase in

the survival of haemopoietic and leukaemic stem cells, however more repeats need to be done to determine an accurate and reliable result.

The protein expression results have suggested that the biology of the mutants has been disrupted, however further analysis was required to determine where this disruption has occurred. Performing real-time PCR on the SDM's to determine whether the NDK gene was being transcribed into RNA, will determine whether the defect was within transcription or in translation and expression of the NDK protein.

The protein modelling has suggested that mutant 3 has affected the structure of NDK by disrupting the KPN loop. To further conclude this finding, undertaking circular dichroism to determine the structure of the mutant by looking at the difference in left and right circular polarised light, compared with wild type NDK protein, would backup the findings already obtained. (Giartasia, et al., 1996) (Sandra Jeudy, 2009)

Once the structure and biology of the mutants were confirmed, further analysis can be done to determine how these mutants affect haemopoietic and leukaemic stem cells, by carrying out survival assays, similar to those done on wild type NDK proteins. This will give an indication whether the mutations have affected the survival mechanism of NDK proteins.

Once the characterisation and survival mechanisms have been proven in vitro, in vivo testing was necessary to determine the affects of bacterial NDK proteins within a real-time setting. Myelodysplastic mice have been produced, which were pre-disposed to pre-leukaemia, an observation can be made to see whether the addition of bacteria containing over expressed NDK could cause this myelodysplastic condition to progress into a leukaemic stage.

The survival of leukaemic cells by bacterial NDK could be a significant factor in patient treatment and therapy. With bacterial infections being a high possibility within leukaemic patients, and the suggestive increase in survival of leukaemic cells by bacterial NDK, can it be possible that bacterial infections may have a bigger impact on leukaemic patients, and then first thought.

11. APPENDICES

11.1 Appendix 1. Recipes and Buffers

4x Gel Loading Buffer

3.7 ml dH₂O

1.25 ml 0.5 M Tris HCl pH 6.8

2.5 ml glycerol (40%)

2.0 ml 10% SDS (2%)

0.5 ml 2-β-mercaptoethanol-Brilliant blue

1% Agarose Gel

1 g agarose dissolve in 100ml 1×TBE buffer.

Heat to dissolve.

Cool and add 3 μl ethidium bromide

Lysogeny Broth (LB)

20 g dissolve in 1L dH₂O

Lysogeny Agar

1.2% agar in 1L of LB-Broth

Acrylamide Gel- Resolving Layer

3.2 ml dH₂O

2.5 ml 1.5 M Tris HCl pH 8.8

4.2 ml ProtoGel (30% (w/v) acrylamide: 0.8% (w/v) Bis-acrylamide stack solution (37.5:1)

100 µl 10% SDS

50 µl 10% Ammonium persulphate

3.3 µl TEMED

Acrylamide Gel – Stacking layer

2.03 ml dH₂O

830 µl 0.5 M Tris HCl pH 6.8

440 µl ProtoGel (30% (w/v) acrylamide: 0.8% (w/v) Bis-acrylamide stack solution (37.5:1)

66 µl 10% SDS

16.7 µl 10% Ammonium persulphate

1.7 µl TEMED

10x Running Buffer

30 g Tris (250 mM)

144 g Glycine (1.92 M)

10 g SDS (1%)

Dissolve in 1L dH₂O

1.5M TRIS pH 8.8

90.8 g TRIS

Dissolve in 400 ml dH₂O pH to 8.8

Make up to 500 ml

0.5M TRIS pH6.8

30.3 g TRIS

Dissolve in 400 ml dH₂O, pH to 6.8

Make up to 500 ml

1x TBE Buffer

10.8 g TRIS

5.5 g Boric acid

4 ml 5 M EDTA pH 8.0

Make up to 1L H₂O

Transfer Buffer

7.575 g TRis (25 mM)

36 g glycine (192 mM)

500 ml methanol (20%)

Make up to 2.5 L dH₂O

TBS-T Buffer

40 ml 1.0 M tris HCl pH 7.6

16 g NaCl

2 ml Tween20

Make up to 2L dH₂O

Coomassie blue stain

50% (v/v) methanol

10% (v/v) acetic acid

0.05% (w/v) Coomassie Brilliant blue

Equilibrium buffer

50mM sodium phosphate pH 8

0.3M sodium chloride

10mM imidazole in H₂O

Macs Buffer

2 mM EDTA

1% FBS

500mL PBS

FAC Fix

1% formaldehyde (v/v)

2% FBS (v/v)

500 ml PBS

Giemsa Buffer pH 7

8 mM KH_2PO_4

6 mM Na_2HPO_4

11.2 Appendix 2. cDNA expression Primer Designs

Sequences of *ndk*

>ndk (*Escherichia coli* str. K-12 substr. MG1655) plus 200 bp. upstream
ggatgatggatgacgtggggcaaatcgcgacagtgaaatgtcatgcaatagtctgtttt
gtccctgattggtgctaaaactcattttattttaaaaaaatgttaccttcatcaatagtc
aacggccctggttgcattataatccgcgccatctcgtacgctggtacagacaacaacag
aacaatttacagaggtaaaa ATGGCTATTGAACGTACTIONTTTTCCATCATCAAACCGAACG
CGGTAGCAAAAACGTCATTGGTAATATCTTTGCGCGCTTTGAAGCTGCAGGGTTCAAAA
TTGTTGGCACCAAAATGCTGCACCTGACCGTTGAACAGGCACGTGGCTTTTATGCTGAAC
ACGATGGAAAACCGTTCTTTGATGGTCTGGTTGAATTCATGACCTCTGGCCCGATCGTGG
TTTCCGTGCTGGAAGGTGAAAACGCCGTTACGCGTCACCGGATCTGCTGGGCGCGACCA
ATCCGGCAAACGCACTGGCTGGTACTCTGCGCGCTGATTACGCTGACAGCCTGACCGAAA
ACGGTACCCACGGTTCTGATTCCGTCGAATCTGCCGCTCGCGAAATCGCTTATTTCTTTG
GCGAAGGCGAAGTGTGCCCGCGCACCCGTTAAtaatttcgtaaatagcccgcgtgcaaactg
ggcatccgtgcccagaatgtgacaatgcagcgcggccggacgagcagccgctcaccgg
ggcgtttcttttttcaaccctccagggggccataacgtgtaataacgaggccggaatagca
tatgtctgaacaattagtcacacctgaaaacgtcaccacgaaaagatggaaaa

MAIERTFSIIKPNVAKNVIGNIFARFEAAGFKIVGKMLHLTVEQARGFYAEHDGKPF
DGLVEFMTSGPIVSVLEGENAVQRHRDLLGATNPANALAGTLRADYADSLTENGTHGSD
SVESAAREIAFFFEAGEVCPRT

RT primers

Forward. ACCGTTCTTTGATGGTCTGG
Reverse. GATTCGACGGAATCAGAACC

201 bp 50 C annealing.

>ndk (*Klebsiella pneumoniae* 342) plus 200 bp. upstream
ggctgggcaaacggcgccagtttaccctgcaataaccagggtaaatgtgtgtg
gcggtttgtgtttccaatactcatcttattttaaaaaaatgttaccttcatccgtaggca
tcgcccagcgttgcattataatgcccgcagccatacagatcgatgcccgaacaacaga
acatttaattagaggtaatcATGGCTATTGAACGTACTIONTTTTCCATCATCAAACCAACG
CGGTGGCAAAAACGTTATTGGCAGCATCTTTCTCGCTTTGAAGCTGCAGGGTTCAAAA
TCGTGCGCACCAAAATGCTGCATCTGACCGTTGAGCAGGCTCGCGGTTTCTATGCTGAGC
ACGAAGGTCGTCCTTTCTTCGATGGTCTGGTTGAATTCATGACCTCCGGCCCGATCGTGG
TTTCCGTCTGGAAGGCGAAAATGCGGTTACGCGTCACCGTGACCTGCTGGGCGCCACCA
ACCCGGCGAACGCTCTGGCCGGTACCCTGCGCGCTGACTACGCTGATAGCTTCACTGAGA
ACGGCACCCATGGTTCCGATTCTGTAGAATCCGCTGCTCGCGAAATCGCGTTCTTCTTTG
CTGAAGGCAGGTTTGGCCGCGCACTCGCTAAtcctcgtcgtcttttcgaccgtcgcggc
ggtggctgcccgcgttcaccccggtcacatagttatctatgctcccggggattcacttgc
ttgccgccttgccgacaatcgaaaagccttagaggattaagcatgcccgttatgattttttt
atcattgactgcccgcagcagcagcgttccatgcccagaatgtgagaatat

MAIERTFSIIKPNVAKNVIGSIFSRFEAAGFKIVGKMLHLTVEQARGFYAEHEGRPF
DGLVEFMTSGPIVSVLEGENAVQRHRDLLGATNPANALAGTLRADYADSLTENGTHGSD
SVESAAREIAFFFEAGEVCPRT

RT primers

Forward. CGTTCTTCGATGGTCTGGTT
Reverse. GCCTTCAGCAAAGAAGAACC

237 bp 50 C annealing

>ndk (*Staphylococcus aureus* subsp. aureus MW2) plus 200 bp. upstream
ttcgaagtatttaagtaaagctttggatttgatttctgagttaccagatggacatccgaa
atcactacttttaagtttgacgaaaaaatgggttcaagaaacacgtagtatttatgaaa
agtattgaaagcgctttaccaacctgtaatatataatagtaatatataacataaacctatta
aacacagggggatataaaaGTGGAACGTACATTTTTAATGATTAAACCAGATGCAGTAC
AAAGAAATCTAATTGGTGAAGTAATTTCAAGAATTGAAAGAAAAGGACTAAAACCTTGTCG
GTGGTAAATTAATGCAAGTACCAATGGAACCTTGCTGAAACACATTATGGTGAACACCAAG
GTAACCATTTTATAATGATTTAATTTCAATTTATTACATCAGCACCAGTGTTCGCAATGG
TAGTTGAAGGTGAAGATGCAGTTAATGTATCTAGACATATTATTGGCAGCACCAATCCTT
CAGAAGCTTCACCAGGATCAATTAGAGGTGATTTAGGTTTAACTGTTGGTAGAAAATCA
TTCACGGTTCAGATTCATTAGATTCTGCTGAACGTGAAATTAATCTATGGTTAATGAAA
ATGAAATTACTAGCTATGCTTCACCACGTGATGCATGGTTATATGAATAAaatttaaact
gtaaacctttacgattttattataaaggtgagaaagggttttggtatgtggttagtcatta
tgattatacataacaaggcccgtttttatggtgtagtaaattacttgaaaaattttata
gtttttttgtaacacgtattaaaaagagaggaatattctttatcaaatgaaactaaaca
gagagaaggg

MERTFLMIKPDVQRNLI GEVISRIERKGLKLVGGKLMQVPMELAEETHYGEHQKPFYND
LISFITSAPVVFAMVVEGEDAVNVSRHIIGSTNPSEASPGSIRGDLGLTVGRNIIHGSDSL
DSAEREINLWFNENEITSYASPRDAWLYE

RT primers

Forward. CGCAATGGTAGTTGAAGGTG
Reverse. GCATCACGTGGTGAAGCATA

222 bp 50 C annealing

>ndk (*Streptococcus pneumoniae* R6) plus 200 bp. upstream
aatcctccagtagaggaagaagaaatgaagaaacaacagaagtagttgtggatactgcc
gttgaaactgtggaagaacagtagaataagagagaatgagagaacccgagggttctctc
attctctcttctgaaaactttctccattttccacgaaatgtggtaaaataaaagaaaga
agctgacaaaggagacgggtATGGAACAAACATTTCTTTATCATCAAACCAGATGGTGTA
AAAGAGGGCTAGTGGGTGAAGTGTAAAGCGCATCGAACACGTGGATTTACAATCGAAA
AATTGGAGTTTCGTTACAGGTTTCAGAAGAGTTGATTGACCAGCACTATCAGGACTTGG
TTGGTCAGAGTTTTTACCCACCGATTTCGTGAATTCATGACTTCAGGTCCAGTTCTTGTGG
GTGTCATTTCTGGTCCCAAAGTAATCGAAACTTGCGGACCATGATGGGTGCAACTCGTC
CAGAAGAAGCTTTACCAGGCACTATTCGAGGTGATTTTGCAAAAGCTGCTGGAGAAAATG
AGATTATCCAAAATGTTGTACATGGTTTCAGATTCAGAAGAGTCAGCTAAGCGAGAAATG
CTCTTTGGTTTTAAgagtggttggtcaatcaattgggttaaagctcatttgaatagaa
agtatagtcaattagtttaagacatgacgcatgatatacaaaccttttttagtttttgatg
gtgcggttttttgattaagtgactattagtttgctcgcctccgtaggtgcgagacaaaaa
aaacaccgctatgcggtgtgctgcaaggtta

MEQTFFIKPDGVKRLVGEVLKRIEQRGFTIEKLEFRSQVSEELIDQHYQDLVGQSFYP
PIREFMFTSGPVLVGVISGPKVIETWRTMMGATRPEEALPGTIRGDFAKAAGENEIIQNVV
HGSDSEESAKREIALWF

RT Primers

Forward. TGAAGTGTAAAGCGCATCG
Reverse. GCTTCTTCTGGACGAGTTGC

257 bp 50 C annealing

11.3 Appendix 4. Bacterial NDK expressions at mid logarithmic and stationary growth phase calculation

E.coli

		1in10				1in1000						
16s	ndk	16s	ndk	16s	ndk	16s	ndk	16s	ndk	16s	ndk	
Log	log	Stat	stat	Log	log	Stat	stat	Log	log	Stat	stat	
	69.2205	125.800	124.428	55.1145	15.3446	115.279	47.7368	26.4811	0.30882	53.1560	14.3272	
	33.6978569	6	8	8	7	3	2	6	6	9	3	4
	50.0617	2.74741	155.154	85.0532	11.1419	130.319	96.3889	17.4159	0.26251	47.8668	3.68875	
	82.57421112	4	4	8	6	3	8	4	7	9	6	4
	57.6285	4.84058	83.6057	74.5658	28.4552	108.588	123.066	2.43155	0.43898		3.39407	
	60.22824478	9	8	3	4	3	6	7	6	7	38.5574	8
	58.9702	125.800	121.063	71.5778	18.3139	118.062	89.0641		0.33677	46.5267	7.13669	
Av	71.40122795	9	8	1	9	3	5	6	15.4429	9	6	1
	9.64962		35.8930	15.1913	9.03051	11.1297	38.1953		0.09149	7.39100	6.22894	
SD	15.80098433	4		7	5	4	6	5	12.1456	4	4	2
percent error	0.16363		0.29648	0.21223	0.49309		0.42885	0.78648	0.27167	0.15885	0.87280	
	0.221298496	5	0	2	5	5	0.09427	2	5	3	5	5

K.pneumoniae

		1in10				1in1000						
16s	ndk	16s	ndk	16s	ndk	16s	ndk	16s	ndk	16s	ndk	
Log	log	Stat	stat	Log	log	Stat	stat	Log	log	Stat	stat	
	36.7670	420.772	16.7047	597.074	6.52088	306.682				25.0330	1.97948	
	646.3381958	1	2	2	3	6	6	-	449.903	-	5	2

		42.7384	254.633	28.4976	484.444		192.355	0.72564	254.983	0.10942	85.4742	0.71457
	568.1751709	6	3	6	6	0.53528	8	1	8	6	2	9
		31.7258	4.28426	10.6803	391.073		176.635	1.21454	197.621		69.7783	0.15845
	477.1807556	2	8	8	4	-	8	8	4	-	9	3
			337.702	18.6275	490.864	3.52808	225.224	0.97009		0.10942	60.0952	0.95083
Av	563.8980408	37.0771	8	9	1	3	8	5	300.836	6	2	8
		5.51286	117.477	9.06294	103.150	4.23246	70.9811	0.34570	132.243			
SD	84.65979137	5	9	3	4	2	3	9	4	-	31.3625	0.93322
percent		0.14868	0.34787	0.48653		1.19964	0.31515	0.35636	0.43958	#VALUE		0.98147
error	0.150133154	7	4	3	0.21014	9	7	6	6	!	0.52188	1
	<i>S.aureus</i>											
					1in10				1in1000			
	16s	ndk	16s	ndk	16s	ndk	16s	ndk	16s	ndk	16s	ndk
	Log	log	Stat	stat	Log	log	Stat	stat	Log	log	Stat	stat
		306.645	625.737	475.392	603.185	300.524	549.936	346.200	515.360	145.090	349.789	240.475
	546.7540894	5	7	9	5	3	4	7	7	6	6	1
		367.837	566.569	391.934	676.978	234.075	504.910	353.790	425.985	2.46241	273.362	147.193
	746.1793213	4	5	7	1	9	9	6	1	5	9	4
		349.970	500.274		647.858	264.802	401.399	282.262	526.159	0.49100	206.128	116.060
	737.8336792	6	9	380.504	8	5	5	6	9	3	4	1
		341.484		415.943	642.674	266.467	485.415		489.168	49.3480		167.909
Av	676.9223633	5	564.194	8	2	6	6	327.418	6	1	276.427	5
			62.7651	51.8006	37.1685	33.2554	76.1633	39.2893	54.9842	82.9213	71.8796	64.7429
SD	112.8062369	31.4662	5	3	3	6	5	8	4	9	3	2
percent		0.09214	0.11124	0.12453	0.05783	0.12480	0.15690	0.11999	0.11240	1.68033	0.26003	0.38558
error	0.166645753	5	7	8	4	1	3	8	3	9	1	2
	<i>S.pneumoniae</i>											
					1in10				1in1000			
	rpob	ndk	rpob	ndk	rpob	ndk	rpob	ndk	rpob	ndk	rpob	ndk

	Log	log	Stat	stat	Log	log	Stat	stat	Log	log	Stat	stat
		62.6569	558.026	112.903	279.674	35.5088	346.539	81.0344		0.31445	53.1645	30.9089
	405.729126	7	2	6	1	4	3	3	-	4	8	9
		52.0480	528.199	96.9368	357.685	57.3716	304.359	58.6796	59.7523	1.82981	53.6377	14.9691
	513.3250732	7	1	8	2	1	3	3	5	3	5	7
		92.9122		0.64718		112.214		0.42792		22.8240		0.35446
	-	2	-	4	-	3	-	2	-	2	-	1
		69.2057	543.112	70.1625	318.679	68.3649	325.449	69.8570	59.7523	8.32276	53.4011	15.4108
Av	459.5270996	5	6	6	7	1	3	3	5	2	6	7
		21.2045	21.0909	60.7291	55.1621	39.5167	29.8257	15.8072		12.5812	0.33458	15.2820
SD	76.08182394	9	4	1	8	2	6	3	-	9	3	5
percent error	0.165565478	9	0.03883	0.86554	0.17309	0.57802	0.09164		#VALUE	1.51167	0.00626	0.99164
			3	9	6	6	5	0.22628	!	3	5	1
		<i>EC_{1in10}</i>		<i>KP_{neat}</i>		<i>SA_{1in10}</i>		<i>SP_{1in10}</i>				
		<i>E.coli</i>		<i>K.pneumoniae</i>		<i>S.aureu</i>		<i>S.pneumoniae</i>				
		Log	Stat	Log	Stat	Log	Stat	Log	Stat			
Control		74.6	108.6	563.9	337.7	642.7	485.4	318.7	325.5			
NDK		28.5	123.1	37.1	18.6	266.5	327.4	68.4	69.8			
		0.68692		1.66982		1.32406		0.97910				
CF		4		5		3		9				
NDK_{log}		100		100		100		100				
		3.50877		2.69541		0.37523		1.46198				
100/ndk_{log}		2		8		5		8				
relative error		49.3095		14.8686		12.4801		57.8026				
		3		5		2		3				
		431.929		50.1347		122.851		102.046				
NDK_{stat}		8		7		8		8				

NDK_{stat (cf)}	296.703	83.7163	162.663	99.9149
relative	2	1	5	3
error	127.241	40.7307	19.5192	23.0911
	8	7	3	3

11.5 Appendix 5. Knockout Production Primer Design

E. coli ndk inactivation primers

>ndk (*Escherichia coli* str. K-12 substr. MG1655) plus 200 bp. upstream
ggtgatggatgacgtggggcaaactcgcgacagtgaaatgtcatgcaatagtctgtttt
gtccctgattggttgctaaaactcattttattttaaaaaaatggttaccttcatcaatagtc
aacggccctggttgcctcattataatccgcgcatctcgtagcgtggtacagacaacaacag
aacaatttacagaggtaaaaATGGCTATTGAACGTACTTTTTCCATCATCAAACCGAACG
CGGTAGCAAAAACGTCATTGGTAATATCTTTGCGCGCTTTGAAGCTGCAGGGTTCAAAA
TTGTTGGCACAAAATGCTGCACCTGACCGTTGAACAGGCACGTGGCTTTTATGCTGAAC
ACGATGGAAAACCGTTCTTTGATGGTCTGGTTGAATTCATGACCTCTGGCCCGATCGTGG
TTTCCGTGCTGGAAGGTGAAAACGCCGTTACAGCGTCACCGCATCTGCTGGGCGCGACCA
ATCCGGCAAACGCACTGGCTGGTACTCTGCGCGCTGATTACGCTGACAGCCTGACCGAAA
ACGGTACCCACGGTTCGATTCCGTCGAATCTGCCGCTCGCGAAATCGCTTATTTCTTTG
GCGAAGGCGAAGTGTGCCCGCGACCCGTTAAtaatttcgtaaatgccgcgtgcaaacgt
ggcatccgtgcgccagaatgtgtacaatgcagcgccccggacgagcagccgctcaccgg
ggcgtttcttttttcaaccctccagggggccataacgtgtaataacgaggccggaatagca
tatgtctgaacaattagtcacacctgaaaacgtcaccacgaaagatggaaaa

Forward homology

GCTATTGAACGTACTTTTTCCATCATCAAACCGAACGTGTAGGCTGGAGCTGCT
IC

Reverse homology

ATCAAAGAACGGTTTTCCATCGTGTTCAGCATAAAAAGGGAATTAGCCATGGTCC
AT

Check primers

F: cattataatccgcgcatct

R: cggcctcgttattacaggtt

11.5 Appendix 5. Site Directed Mutagenesis Primer Design

SDM primers for ndk (*E. coli*)

ATGGCTATTGAACGTACTTTTTCCATCATCAAACCGAACGCGGTAGCAAAAAACGTCATTGGTAATATCTTTGCGCGCTTTGAAGCTGCAGGGTTC
 AAAATTGTTGGCACCAAAATGCTGCACCTGACCGTTGAACAGGCACGTGGCTTTTATGCTGAACACGATGGAAAACCGTTCTTTGATGGTCTGGTT
 GAATTCATGACCTCTGGCCCCGATCGTGGTTTTCCGTGCTGGAAGGTGAAAACGCCGTTTCAGCGTCACCCGCGATCTGCTGGGCGCGACCAATCCGGCA
 AACGCACTGGCTGGTACTCTGCGCGCTGATTACGCTGACAGCCTGACCGAAAACGGTACCCACGGTTCTGATTCCGTCTGAATCTGCCGCTCGCGAA
 ATCGCTTATTTCTTTGGCGAAGGCGAAGTGTGCCCGCGCACCCGTTAA

MAIERTFSIIKPNNAVAKNVIGNIFARFEAAGFKIVGTKMLHLTVEQARGFYAEHDGKPFDFGLVEFMTSGPIVVSVLEGENAVQRHRDLLGATNPA
 NALAGTLRADYADSLTENGTHGSDSVESAAREIAYFFGEGEVCPTR

'E' 4 to 'A' 4

a78c	5'-ggcagccatatggctattgcacgtactttttccatcatc-3'			
a78c_antisense	5'-gatgatggaaaaagtacgtgcaatagccatatggctgcc-3'			
a78c	39	79.50°C	-45.65 kcal/mole	1.5%
a78c_antisense	39	79.50°C	-48.58 kcal/mole	2.8%

'KP' 11-12 to 'AA' 11-12:

a31g_a32c_c34g	5'-gaacgtacttttccatcatcgcagcgaacgcggtagcaaaaaacg-3'			
a31g_a32c_c34g_antisense	5'-cgtttttgctaccgcgttcgctgcgatgatggaaaaagtacgttc-3'			
a31g_a32c_c34g	46	78.13°C	-51.84 kcal/mole	8.0%
a31g_a32c_c34g_antisense	46	78.13°C	-49.20 kcal/mole	4.1%

'G' 79 to 'A'79

g236c			5'-gtttccgtgctggaagctgaaaacgccgttcag-3'		
g236c_antisense			5'-ctgaacggcgttttcagcttccagcacggaaac-3'		
g236c	33	80.38°C	-42.72 kcal/mole		7.0%
g236c_antisense	33	80.38°C	-43.43 kcal/mole		9.4%

Kpn Loop deletion

Delete AA 103-105

del307-315			5'-cgcaactggctggtactgattacgctgacagcc-3'		
del307-315-antisense			5'-ggctgtcagcgtaatcagtagccagtcg-3'		
del307-315	32	81.63°C	-43.92 kcal/mole		36.3%
del307-315-antisense	32	81.63°C	-42.62 kcal/mole		37.9%

'H' 117 to 'A' 117

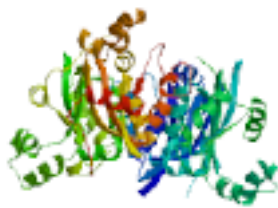
Primer Name	Primer Sequence (5' to 3')				
c349g_a350c			5'-gaccgaaaacggtaccgccggttctgattccgtc-3'		
c349g_a350c_antisense			5'-gacggaatcagaaccggcggtagccgttttcggtc-3'		
c349g_a350c	34	78.68°C	-45.47 kcal/mole		9.6%
c349g_a350c_antisense	34	78.68°C	-47.67 kcal/mole		5.6%

11.6 Appendix 6. Swiss Predicted Model Data

Workunit: P000006 Title:



Model Summary:



Model Information:

Modelled residue range: 2 to 143
 Based on template: 2hurC (1.62 Å)
 Sequence Identity [%]: 98.592
 Evaluate: 3.59502e-72

Quaternary structure Information:

Template (2hur): TETRAMER
 Model: TETRAMER

Ligand Information:

Ligands in the template: SO4: 4.
 Ligands in the model: none.

Quality Information:

QMEAN Z-Score: 0.481

Global Model Quality Estimation:

QMEAN4 global scores:

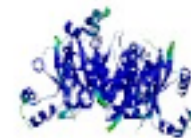
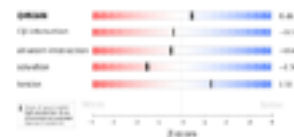
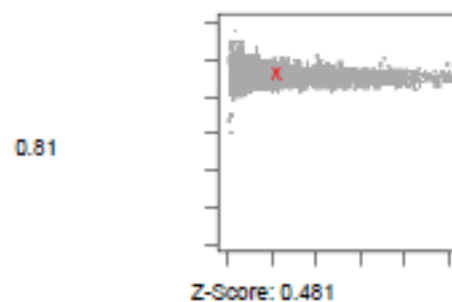
QMEANscore4: Estimated absolute model quality:

Score components:

Local scores:

Coloring by residue error:

Residue error plot:



QMEAN4 global scores:

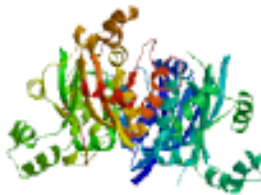
The QMEAN4 score is a composite score consisting of a linear combination of 4 statistical potential terms (estimated model reliability between 0-1). The pseudo-energies of the contributing terms are given below together with their Z-scores with respect to scores obtained for high-resolution experimental structures of similar size solved by X-ray crystallography:

Scoring function term	Raw score	Z-score
C_beta interaction energy	-179.44	-0.33
All-atom pairwise energy	-13920.48	-0.46
Solvation energy	-32.83	-1.50
Torsion angle energy	-205.04	1.33
QMEAN4 score	0.810	0.48

If you publish results from QMEAN, please cite the following paper:

Benkert P, Biasini M, Schwede T. (2011). "Toward the estimation of the absolute quality of individual protein structure models." *Bioinformatics*, 27(3):343-50.

Model Summary:



Model Information:

Modelled residue range: 2 to 143
 Based on template: 2hurC (1.62 Å)
 Sequence Identity [%]: 99.296
 Evaluate: 1.08086e-73

Quaternary structure Information:
 Template (2hur): TETRAMER
 Model: TETRAMER

Quality Information:

QMEAN Z-Score: -0.016

Ligand Information:

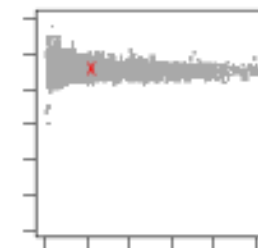
Ligands in the template: SO4: 4.
 Ligands in the model: none.

Global Model Quality Estimation:

QMEAN4 global scores:

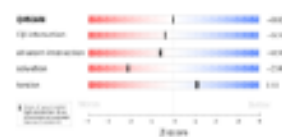
QMEANscore4: Estimated absolute model quality:

0.775



Z-Score: -0.016

Score components:



Local scores:

Coloring by residue error:



Residue error plot:

QMEAN4 global scores:

The QMEAN4 score is a composite score consisting of a linear combination of 4 statistical potential terms (estimated model reliability between 0-1). The pseudo-energies of the contributing terms are given below together with their Z-scores with respect to scores obtained for high-resolution experimental structures of similar size solved by X-ray crystallography:

Scoring function term	Raw score	Z-score
C_beta Interaction energy	-176.29	-0.39
All-atom pairwise energy	-13617.65	-0.58
Solvation energy	-21.87	-2.09
Torsion angle energy	-196.36	1.11
QMEAN4 score	0.775	-0.02

If you publish results from QMEAN, please cite the following paper:

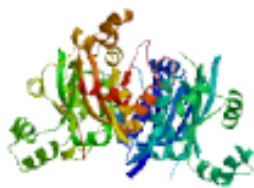
Benkert P, Biasini M, Schwede T. (2011). "Toward the estimation of the absolute quality of individual protein structure models." *Bioinformatics*, 27(3):343-50.

Mutant 2

Workunit: P000005 Title:



Model Summary:



Model Information:

Modelled residue range: 2 to 140
Based on template: 2hurC (1.62 Å)
Sequence Identity [%]: 97.887
Evalue: 3.3897e-71

Quaternary structure information:

Template (2hur): TETRAMER
Model: TETRAMER

Ligand information:

Ligands in the template: SO4: 4.
Ligands in the model: none.

Quality information:

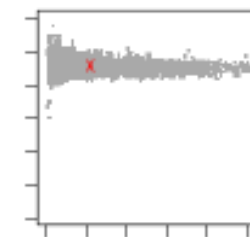
QMEAN Z-Score: 0.216

Global Model Quality Estimation:

QMEAN4 global scores:

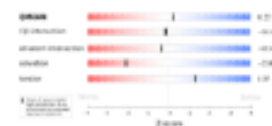
QMEANscore4: Estimated absolute model quality:

0.791



Z-Score: 0.216

Score components:



Local scores:

Coloring by residue error:



Residue error plot:

QMEAN4 global scores:

The QMEAN4 score is a composite score consisting of a linear combination of 4 statistical potential terms (estimated model reliability between 0-1). The pseudo-energies of the contributing terms are given below together with their Z-scores with respect to scores obtained for high-resolution experimental structures of similar size solved by X-ray crystallography:

Scoring function term	Raw score	Z-score
C_beta interaction energy	-186.75	-0.16
All-atom pairwise energy	-13819.11	-0.34
Solvation energy	-22.11	-2.08
Torsion angle energy	-198.28	1.27
QMEAN4 score	0.791	0.22

If you publish results from QMEAN, please cite the following paper:

Benkert P, Biasini M, Schwede T. (2011). "Toward the estimation of the absolute quality of individual protein structure models." *Bioinformatics*, 27(3):343-50.

Mutant 3

12. LIST OF REFERENCES

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