CHAPTER 7

Conclusions
Complex-I is a multi faceted enzyme protein complex with several functions. It is not only involved in transfer of electrons from NADH to ubiquinone but it also generates a proton motive force by pumping protons across the inner mitochondrial membrane. It also has provisions for reduction of reactive oxygen species (ROS). Hence, a large number of iron sulphur clusters are present one after the other at suitable distances to facilitate desired transfer of electrons. For several years the mitochondrial Complex-I was considered to be an L-shaped black box. This was not only because of the lack of structural information but also due to its mutation prone nature. Several mutations have been reported over the years in the 42 mammalian mitochondrial subunits resulting in different neuromuscular disorders [MITOMAP 2014]. Leigh syndrome is one such sub-acute necrotizing encephalomyelopathy with a characteristic neuropathology occurring due to mutations of Complex-I subunits. It is an energy deficit disorder where clinical symptoms depend on the areas of central nervous system that are involved. Due to the dual genetic origin of the Complex-I subunits, n-DNA or mtDNA, the disease could be inherited in an autosomal recessive or maternal fashion. In the present thesis, the human Q module has been extensively studied using computational and biophysical approaches. The aim was to evaluate the structural and functional effects of the Leigh syndrome mutations at molecular level to enhance the understanding of consequences of mutations on Complex-I. Before assembling in the mitochondria, the subunits are individually expressed either within the mitochondrial matrix or transported into it. Thus these proteins exist as individual entities before coming together to form functional assembly. So, any defect in the folding at the initial stage may be carried over to subunit assembly or directly affects assembly formation itself. Thus it is worth studying the subunits individually in isolation and with respect to each other in the Q module environment.

Among the three modules of Complex-I the Q module is the intermediate domain which links the N and P module. In addition to its structural role in Complex-I Q module is also involved in ubiquinone reduction. The four core subunits of the Q module: \textit{NDUFS2}, 3, 7 and 8 are highly conserved across species. \textit{In silico} studies of the four subunits and the Q module reflect that point/ double mutations affect the structural integrity of the individual subunits. The mutations are at evolutionarily highly conserved positions in the protein sequence. By identifying the location of the mutation its consequences to some extent can be judged. Breaking of essential
hydrogen bonds and changes in solvent accessibility observed in the mutant subunits have made them behave differently than the w-t. The changes were observed in terms of changes in fluctuation of residues during dynamic simulation, the observed changes in compactness of the structure etc. The energetic instability of subunits by mutation also affected their affinity towards the other subunits, iron-sulphur (Fe-S) cluster and n-DBQ. It would be interesting to note that in \textit{NDUFS2, 3 and 8} the mutations occurred in pairs. Also it was noted that in the case of \textit{NDUFS3} and \textit{8} the single mutations were not causing a lethal phenotype, as the individual mutations were inherited from healthy heterozygous parents [Benit et al, 2004; Loeffen et al, 1998]. Thus, the disease causing phenotype was observed only when the mutations occur together. Thus, the instability of each mutation calculated by the \textit{in silico} studies would have a cumulative effect on the subunit leading to disastrous consequences.

Although the four subunits of the Q module were cloned and detailed studies were planned, in the case of three of them, \textit{NDUFS2, 8} and ND1, there were difficulties during protein expression stage itself or faced with solubility problems of expressed proteins, hence further studies on them could not be completed. The subunits amenable to cloning and expression, \textit{NDUFS3} and \textit{NDUFS7}, were studied using biochemical and biophysical techniques. These are amongst the reported top 10 proteins most prone to mutations leading to Complex-I deficiency. \textit{In vitro} studies of \textit{NDUFS3} and \textit{7} highlight the common features of their mutants causing Leigh syndrome. Mutants of both subunits shown to be energetically unstable \textit{in silico} had higher aggregation propensities with spectroscopic features of amyloid like fibrils. The T145I+R199W mutant of \textit{NDUFS3} showed a different native structure compared to the w-t although the stabilities under different pH conditions, temperature dependence and Gdn-HCl concentrations were found to be somewhat similar as that of w-t. The higher aggregation propensity, differences in secondary and tertiary structure, tryptophan microenvironment and the higher fluctuation of residues in mutant during molecular dynamics simulation indicate the unstable nature of the mutant. \textit{NDUFS3} is involved early in the assembly of the peripheral arm. Hence, mutation in the subunits disrupting its correct folding would result in hindrance during the early stages of assembly. Hence, even though the expression levels of mutant \textit{NDUFS3} may remain normal in the cell, mutations affect the assembly process of Complex-I thereby resulting in the accumulation of assembly intermediates as observed in patient cell lines [Benit et al, 2004].
NDUFS7 plays a functional role in the Q module. It harbours partial binding site for ubiquinone. It is expressed in all tissues similar to housekeeping genes and is the fourth most mutated protein resulting in Complex-I deficiency. Hence, mutations in this gene cause a spectrum of disorders depending on its expression levels in the tissues. The protein possesses the iron-sulphur cluster N2 which plays a vital role in maintaining the structural integrity and functionality of the subunit. Molecular dynamics studies indicate that the absence of cluster N2 has a detrimental effect on the binding of n-DBQ revealing the involvement of cluster N2 in binding. The mutants V122M and R145H showed the absence of signature peak of N2 cluster in UV-visible spectra. The mutants destabilize the cluster N2 which would have an effect on their binding ability. Thus, although the mutation sites are away from the n-DBQ binding site in the structure they can influence the binding of n-DBQ. This was actually proved when reduced binding affinity for n-DBQ was shown in the fluorescence based assay of mutants V122M and R145H of NDUFS7. The MD simulation studies also showed the early breakage of one critical hydrogen bond formed with the substrate in the mutants. Thus, even when the mutants showed a fairly similar secondary and tertiary structure with similar stabilities in comparison with the w-t; differences were seen in the aggregation propensities and n-DBQ binding ability as observed in both biophysical and computational studies.

Thus, the mutations affected both the subunits structurally as well as functionally. The subunits NDUFS3 and 7 have been shown to assemble early during the Complex-I assembly process. NDUFS3 forms the early peripheral arm while NDUFS7 acts as an anchor joining the assembled Q module to ND1 in the membrane. Thus, mutations lead to the lack of a fully assembled Complex-I and consequently leads to energy deficit. This may be due to the structurally modified mutant proteins or availability of less number of molecules for assembly pre-empted by aggregation. In the absence of a fully and correctly assembled complex large amount of ROS generation results and that further leads to cellular damage. Muscles and nerves are the most energy dependant tissues and hence are the worst hit by the deleterious mutations. The wide spectrum of symptoms observed in the cases of such mutations are the result of different regions affected by the Complex-I deficiency and resultant ROS damage.
The points to highlight on the observations recorded and conclusions drawn from this study are:

(1) Modelling the subunits structures has helped in identifying the location of mutations in the structure, which in turn has exemplified mutational effects on function based on structure.

(2) Molecular dynamics simulation has demonstrated the effects of mutations on the local and global stability of subunits.

(3) Modelling the Q module has brought out the varied influence of mutations on the stability of assembly, iron-sulphur cluster and ubiquinone binding.

(4) Cloning the subunits and biophysical studies has helped to assess the folding and stability of individual subunits.

(5) Preparation of selected mutants using site-directed mutagenesis and further experimentation has helped to compare the stability of mutants with that of the wild-type.

(6) Results from both computational and experimental approaches have helped us to conclude that the deleterious mutations affect folding and stability of individual subunits as well as assembly, affect stability of iron-sulphur cluster and reduce ubiquinone binding affinity.

(7) Experimental studies have specifically demonstrated the increased propensity of mutant subunits for aggregation in solution compared to their corresponding wild-type protein.

(8) Investigations on subunit NDUFS7 and mutants showed that the signature peak of UV-visible spectrum for iron-sulphur cluster N2 was absent in mutants.

These studies, thus, take us closer towards understanding the molecular effects of pathological mutations in subunits of Complex-I leading to genetic disorders. The effects have been studied via both computational and biophysical approaches. Crystal structure of the human subunits or the entire eukaryotic complex will of course give further insights into understanding the nature of the structural changes resulting in disease phenotype. However, present studies highlight the important roles played by some of the core subunits making up the Q module.
APPENDIX

Cloning of two nuclear encoded and one mitochondrial encoded subunits of human mitochondrial Complex-I

Introduction

NDUFS2 and 8 are two of the core subunits of Complex-I Q module. Mt-ND1 (mitochondrially encoded NADH dehydrogenase 1) gene is encoded by the mt-DNA. It is a 955 bp gene containing no introns. The predicted polypeptide has a molecular weight of 35.6 kDa. This protein is part of the membrane domain of Complex-I and links the membrane domain to the hydrophilic domain.

Mt-ND1 is considered a mutational hotspot in the case of mitochondrial disorders. The initial diagnosis of any suspected mitochondrial disorder always involves the screening of Mt-ND1 gene sequence for mutations. Mutations in ND1 are known to cause disorders like LHON, MELAS, dystonia etc.

Materials and Methods

1. RNA isolation, cDNA preparation, primer design and PCR amplification

Total RNA was isolated from the human colorectal adenocarcinoma cell line HT29 (1x10^6 cells) using Trizol® Reagent (Life Technologies, Cat#10296-010) as per the manufacturer’s instructions. Purified RNA samples were analyzed by denaturing agarose gel electrophoresis and concentration was spectrophotometrically determined using Nanodrop (Thermo Scientific, USA). One μg of purified RNA was used for the preparation of cDNA using the SuperScript™ III First Strand Synthesis System (Life Technologies, Cat#18080-051).

1.1 NDUFS2

Suitable primers (NDUFS2F: 5’ AAC ATA TGG CGG CGC TGA GGG 3’ & NDUFS3R: 5’ ATA AGC TTC ACC GAT CTA CTT CTC CAA ATA CAA TAT C 3’) were designed based on the RNA sequences downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) for amplification of the full length NDUFS3 ORF. The PCR reactions were set in a 50 μl volume containing 1X Pfu buffer (20 mM Tris-HCl pH 8.8 at 25°C, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-
100, & 0.1 mg ml⁻¹ nuclease free BSA), 1 unit of Pfu polymerase, 200 µM each of
dNTP and forward and reverse primers and 100 ng of the amplified cDNA. Cycling
conditions were 95°C for three minutes, followed by 30 cycles of 95°C for ten
seconds, 45°C for forty-five seconds, and 72°C for one minute thirty seconds. The
amplicon of desired size (808 bp) was gel extracted using the QIAQuick Gel
Purification kit (Qiagen, Cat#28704), which was used for TA cloning in pGEM-T
vector (Promega).

1.2 NDUFS8

Suitable primers (NDUFS8F: 5’CCG AAT TCA TGC GCT GCC TGA CCA 3’ &
NDUFS8R: 5’ TGC TCG AGT CAC CGA TA C AAG TAG TCA 3’)) were designed
based on the RNA sequences downloaded from NCBI (http://www.ncbi.nlm.nih.gov/)
for amplification of the full length NDUFS7 ORF. The PCR reactions were set in a 50
µl volume containing 1X Pfu buffer (20 mM Tris-HCl pH 8.8 at 25°C, 10 mM KCl,
10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, & 0.1 mg ml⁻¹ nuclease free
BSA), 1 unit of Pfu polymerase, 200 µM each of dNTP and forward and reverse
primers and 100 ng of the amplified cDNA. Cycling conditions were 95°C for 3 min,
followed by 30 cycles of 95°C for 10 s, 50°C for 45 s, 72°C for 1 min and final
extension of 72°C for 20 min with hold of 25°C forever. The amplicon of desired size
(656 bp) was gel extracted using the QIAQuick Gel Purification kit (Qiagen,
Cat#28704), which was used for TA cloning in pGEM-T vector (Promega).

2. TA cloning and sub-cloning in the bacterial expression vector

The purified amplicon was A-tailed using Taq polymerase in 1X Thermopol buffer
(NEB) containing 200 µM dATP and 5 units of Taq DNA polymerase (NEB) at 72°C
for 20 minutes. The A-tailed amplicons were then cloned into pGEM-T vector
(Promega), followed by chemical transformation into E. coli DH5α (Invitrogen). The
plasmids from the positive colonies, screened through colony PCR, were purified by
the standard alkaline lysis method. Full length NDUFS2 cDNA ORF or NDUFS8
cDNA ORF from pGEM-T vector was further subcloned into pET 28b(+) vector
(Novagen) between Nde I and Xho I sites. Sequences were confirmed by DNA
sequencing using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v3.1
(ABI, Cat#4337457) in an automated 3730 DNA analyzer (ABI). Appropriate plasmids were transformed into \textit{E. coli} BL21(DE3) for protein expression.

The plasmids from the transformed colonies were isolated by the alkaline lysis method. \textit{NDUFS2} was sub-cloned in pET 28b(+) vector (Promega) between Hind III and Xho I. \textit{NDUFS8} was sub-cloned in pGEX-4T1 vector (Promega) between EcoR I and Xho I. The plasmids were transformed chemically into \textit{E. coli} DH5α (Invitrogen). The plasmids were then isolated from transformed colonies by the alkaline lysis method and further transformed in \textit{E. coli} BL21(DE3) expression cells. Colony PCR and Sequencing was done using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v3.1 (ABI, Cat#4337457) in an automated 3730 DNA analyzer (ABI) to confirm positive clones.

3. **Synthesis of the ND1 gene**

The sequence of the ND1 gene was downloaded from NCBI. 24 overlapping oligonucleotides of 60 base pairs each were designed using the software DNAWorks which on annealing would form the ND1 construct. The primers were annealed and ND1 was amplified using the protocol described by Hoover DM and Lubkowski J, 2002 [Hoover et al 2002]. In short it involves 2 PCR steps. All the 24 oligonucleotides were synthesized at 50 nmol scale. They were mixed and diluted to a final concentration of 1 ng/µl. The PCR reaction 1X Pfu buffer, 200 µM dNTP’s and the primer mix was subjected to the following cycling conditions 95°C for five minutes after which the reaction is kept on hold at 80°C for 1 min and Pfu polymerase is added. This is followed by 10 cycles of 95°C for 30 seconds, 62°C for 30 seconds, 72°C for 20 seconds with an increase of 5 seconds per cycle. One µl of this reaction mixture is mixed with 1X Pfu buffer, 200 µl dNTP’s, outermost oligonucleotides used as forward and reverse primers and Pfu polymerase and subjected to following reaction conditions 95°C for 5 min, 95°C for 20 seconds, 65°C for 30 seconds, 72°C for 1 minute 30 seconds for 30 cycles. The amplified product was checked by Agarose gel electrophoresis and the amplicon of desired size were purified using the QiaQuick Gel Purification kit (Qiagen Cat.#28704).
Table 1: 24 oligonucleotides designed for in-vitro synthesis of ND1 gene.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>ND1-1</td>
<td>ATGCCAATGGCGAATCTGCTCCTGCTGATCGT</td>
</tr>
<tr>
<td>ND1-2</td>
<td>GTTCGGTCAGCATCAGAAACGCCATCGCAATCAGGATCGGAACGATCAGCAGGAGCAGAT</td>
</tr>
<tr>
<td>ND1-3</td>
<td>GTTTCTGATGCTGACCGAAGAGTTAAATTCTGGTTTACATGAGCTCCGTGAAAGGCTCAA</td>
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<tr>
<td>ND1-4</td>
<td>GTCGCGGAACCGCTGGAGGAGACCATACGGACCAACGACGTTTGGACCTTTACGGAGCTG</td>
</tr>
<tr>
<td>ND1-5</td>
<td>CAGCGCGAATGGGTCGGGAGGTGATGTACAGGGTGATGGTGCTAGTCGCCGGTTTCAG</td>
</tr>
<tr>
<td>ND1-6</td>
<td>CGTCAGCGCCAGGGTAGGCGCGGTGATGTACAGGGTGATGGTGCTAGTCGCCGGTTTCAG</td>
</tr>
<tr>
<td>ND1-7</td>
<td>TACCGTGACCGCTGACAGTGCATCTCCTCTCTGAGACCTCGTGCTGCTAAACCCGCT</td>
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<td>ND1-8</td>
<td>AGAGGTCGCTACAGCAGGCTAGAAGAGGAGGACATCAGGAGGAATGATAGCGAGC</td>
</tr>
<tr>
<td>ND1-9</td>
<td>TCTTTATCCCTGCCGACCTCTTCTGCAAGTTTATAGCATTCTGCTGCTGGGGGCTG</td>
</tr>
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<td>ND1-10</td>
<td>GCAACCGCAGCCACGGCACCAGATAGCGCTGATTAGTTAGTTAGGTAGACGCCCACACGAGAC</td>
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<td>ND1-11</td>
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<td>ND1-12</td>
<td>AGGTTGGAGAGGGTAAAAGGGGAGGACATCAGGAGGCTAGACAGGAGAATGATAGCGAGC</td>
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<td>ND1-13</td>
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<td>ND1-14</td>
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<td>ND1-20</td>
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<td>ND1-21</td>
<td>GCTGACTGCGCTTTTCTCTGCTGATCCGACTCCCGCTCTCCCGTTATGATCAGGCT</td>
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<td>ND1-22</td>
<td>CAGAGTCCAGAGGCGAGAAGTTTTTCCACAGGGAGGTGCAATGCTGATCATAACGGAAGGCG</td>
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<td>ND1-23</td>
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<td>ND1-24</td>
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</tr>
<tr>
<td>MTND1R</td>
<td>TACTCGAGTACAGGCTGAGGTGGAAATG</td>
</tr>
</tbody>
</table>

4. **TA cloning and sub-cloning of the synthesized ND1 gene**

The synthesized and purified ND1 gene was A tailed and cloned into pGEM-T vector (Promega) as already described. This was then sub-cloned into pGEX-4T1 (Promega) expression vector.

All the constructs were confirmed by colony PCR and sequencing using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v3.1 (ABI, Cat#4337457) in an automated 3730 DNA analyzer (ABI).
5. Expression studies

5.1 IPTG induction

Single colony of *E. coli* BL21(DE3) clone was inoculated in 5 ml LB containing a suitable antibiotic (Ampicillin 100 µg ml⁻¹ or Kanamycin 60 µg ml⁻¹) and incubated overnight at 37°C (200 rpm). The overnight grown culture was spun at 3500 rpm for 10 min at 25 °C. The cell pellet was resuspended in 5 ml LB containing suitable antibiotic and transferred to 100 ml LB with suitable antibiotic. The cells were grown till OD read 0.3; at 37 °C and shaking at 150 rpm. The cells were then aseptically induced with 0.1-0.5 mM IPTG and were further grown at 18 °C, 28 °C and 37 °C with a shaking at 200 rpm till O.D₆₀₀ = 0.6-0.7. 1ml of the above sample was used to check the expression profile. Cells were harvested by spinning at 4000 rpm for 15 minutes at 4°C. The pellet was redissolved in 20 ml Lysis Buffer (100 mM Tris, 200 mM NaCl, 0.01 % IGEPAL® CA630 and 1 mg ml⁻¹ lysozyme) and was kept on ice for 30 minutes with intermittent shaking. Cells were lysed by sonication at 60% Power in Esquire Biotech Ultrasonic homogenizer with a pulse of 6 s followed by pause of 8 s for 10 minutes. The sonicated solution was centrifuged at 10,000 rpm for 45 minutes at 4°C. The pellet and supernatant were separated. The expression profile was checked on 12% SDS-PAGE gel followed by Coomassie Brilliant Blue R-250 staining.

5.2 Autoinduction

The autoinduction protocol was similar to the protocol described in Materials and methods section 11. In brief, the primary culture grown overnight in LB containing suitable antibiotic was transferred to fresh LB containing suitable antibiotic. The autoinduction medium was prepared as described in section 11. The primary culture was inoculated in the autoinduction medium (1 % inoculum). The flasks were incubated at 37 °C for 2, 4 and 6 hours (standardized for soluble expression) followed by a prolonged incubation at either 16 °C, 18 °C and 20 °C. 1ml of the above sample was used to check the expression profile. Cells were harvested by spinning at 4000 rpm for 15 minutes at 4°C. The pellet was redissolved in 20 ml Lysis Buffer (100 mM Tris, 200 mM NaCl, 0.01 % IGEPAL® CA-630 and 1 mg ml⁻¹ lysozyme) and was kept on ice for 30 minutes with intermittent shaking. Cells were lysed by sonication at
60% Power in Esquire Biotech Ultrasonic homogenizer with a pulse of 6 s followed by pause of 8 s for 10 minutes. The sonicated solution was centrifuged at 10,000 rpm for 45 minutes at 4°C. The pellet and supernatant were separated. The expression profile was checked on 12% SDS-PAGE gel followed by Coomassie Brilliant Blue R-250 staining.

**Results**

1. RNA was isolated from both healthy human blood and HT29 cell line. The concentration of RNA isolated from HT 29 cell line was estimated to be 415 ng/µl. This RNA was further used for cDNA preparation.

![Fig 1: Formaldehyde Agarose gel for identifying RNA extracted, Lane 1: from blood and Lane 2: from HT29 cell line.](image1)

CDNA was prepared and used for further amplification of the genes with suitable primers. NDUFS2 and 8 amplified and were identified depending upon their appropriate sizes. NDUFS2 was ~1.4 kb and NDUFS8 was 646 bp.

![Fig 2: Agarose gel showing amplification of the gene constructs (A) Lane 1: 1 kb plus DNA ladder, Lane 2: PCR product amplified with NDUFS2 primers and (B) Lane 1: 1 kb plus DNA ladder, Lane 2: PCR product amplified with NDUFS8 primers from cDNA prepared from RNA isolated from HT29 cell line.](image2)
2. Cloning in pGEMT vector and colony PCR to confirm positive clones.

Several colonies were obtained after cloning the amplified PCR product into pGEMT vector. Of these a few colonies were subjected to colony PCR and appropriate clone was chosen for further plasmid isolation.

Figure 3.1: Agarose gel electrophoresis Lane 1-15: Colony PCR products for colony number 1-15 for NDUFS2 gene cloned in pGEMT vector and transformed in *E.coli* DH5α cells. Lane 16: 1 kb NEB ladder. Star indicates: Amplified NDUFS2 colony PCR product.

Colony number 7, 11 and 13 show amplified constructs of ~1.4 kb which is the size of the NDUFS2 gene. (Figure 3.1)

Colony number 2, 3, 4 and 7 showed amplified bands at ~850 bp. The size of the NDUFS8 gene is 655 bp and including the extra bases incorporated by the primers this is the appropriate size (Figure 3.2).

Figure 3.2: Agarose gel electrophoresis Lane 1: 1 kb plus DNA ladder, Lane 2-9: Colony PCR products for colony number 1-8 for NDUFS8 gene cloned in pGEMT vector and transformed in *E. coli* DH5α cells. Star indicates the presence of NDUFS8 Colony PCR product.
The potential positive clones were used for plasmid isolation and the plasmids were used for sub-cloning in expression vector and were sequenced to confirm the presence of the gene.


Colony PCR was performed to confirm positive clones followed by sequencing.

NDUFS2 was sub-cloned in pET 28b(+) between Nde I and Hind III.

![Figure 4.1: Agarose gel electrophoresis Lane 1-15: Colony PCR products for colony number 1-15 for NDUFS2 gene cloned in pET 28b(+) vector and transformed in E coli DH5α cells, Lane 16: 1 kb NEB Ladder. Star indicates the presence of NDUFS2 Colony PCR product. Colony number 3, 8, 11, 13 and 14 show bands of appropriate sizes and are positive clones (Figure 4.1). NDUFS8 was sub-cloned in pGEX 4T1 between Eco RI and Xho I.](image1)

NDUFS8 was sub-cloned in pGEX 4T1 between Eco RI and Xho I.

![Figure 4.2: Agarose gel electrophoresis Lane 1: 1 kb NEB Ladder, Lane 2-16 Colony PCR products for colony number 1-15 for NDUFS8 gene cloned in pET 28b(+) vector and transformed in E coli DH5α cells. Star indicates the presence of NDUFS8 Colony PCR product.](image2)
Colony number 2, 3, 5, 6, 10 and 12 showed bands of appropriate size and are positive clones (Figure 4.2)

The plasmids from the positive clones were sequenced to confirm the presence of desired gene and transformed into *E coli* BL21(DE3) expression cells. The clones were then checked for expression.

4. **Soluble protein expression**

Recombinant *NDUFS2* and 8 proteins were expressed only as inclusion bodies in all the conditions of expression.

5. **In vitro synthesis of ND1 gene.**

![Image](A.png)

*Figure 5: A: PCR product after 1st PCR reaction. B: PCR product after 2nd PCR reaction. Arrow indicates the presence of amplified ND1 gene.*

ND1 is 956 bp. In the first PCR reaction a general assembly of the oligonucleotides takes place. ND1 got synthesized in the 2nd PCR reaction where the outermost oligonucleotides were used for amplification (Figure 5). The amplified PCR product was cloned into pGEM-T vector. 96 colonies were screened and sequenced to get an appropriate clone. The construct was further sub-cloned in pET 26b(+) (Nde I – Xho I), 28b(+) (Nde I-Xho I), pET 30b(+) (EcoRI – Xho I), pET 44b(+) (EcoR I – Hind III) and pGEX4T1. Expression was tried by inducing the cells at different IPTG
concentrations (0.1-1 mM) at different temperatures (16°C, 18°C, 21°C, 28°C, 37°C) in different expression hosts (Rosetta, pLysS, pLysE); however the clones didn’t show overexpression of the ND1 gene. Autoinduction was also tried, however, it also didn’t improvise the expression profile for carrying out further research on it.