“Expression of Human Recombinant Wild Type Isocitrate Dehydrogenase (IDH) 2”

PROJECT REPORT

Under the supervision of

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TO WHOMSOEVER IT MAY CONCERN

This is to certify that **Ms. Sai Lakshanya Rangoon**, B.S. Biomedical Engineering, University of Tennessee, Knoxville, United States has successfully completed training from **28th December 2022 to 18th January 2023** in my laboratory at School of Life Sciences, University of Hyderabad. During the training, she was involved in the project on "**Expression of Human Recombinant Isocitrate Dehydrogenase (IDH) 2**".

She was regular to laboratory and learned various Microbiological, Biochemical and Analytical techniques, and we are extremely satisfied with her performance in this tenure.

We wish her all the best for her future endeavors.

(**Dr. Raja Ram Mohan Roy**)
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ACKNOWLEDGEMENT

I would like to take this opportunity to express my gratitude to The School of Life Sciences, University of Hyderabad for giving me this earnest learning opportunity to work in one of their prestigious labs. I devote my sincere appreciation to Dr.Roy Karnati for giving me the wings to soar higher yet reminding of my responsibilities during my tenure in his lab. I deem it to be my fortune for having this wonderful opportunity to work in his lab.

Mr. Mayank Bajaj, my mentor for my project without whose support, patience and dedication, my project would not have been possible. It is my honor to have a teacher like him. I would also like to thank Vaishnavi who has played an instrumental role through every stage of my project. I would like to extend my thanks to my lab mates: Ravi Ranjan Sharma, Jitesh Gupta, Neel, Poonam, Bhageeratha for being my support system and trusting my competence during the project.

The warmest of my gratitude is dedicated to my mother, Suchitha Maddhikunta for being the most formidable pillar contributing to my success till date. Her love, dedication, courage, tolerance, and patience are few of the biggest driving forces in any pursuit of my life.

Sai Lakshanya Rangoon

ISOCITRATE DEHYDROGENASE – AN ENZYME OF ENORMOUS SIGNIFICANCE IN HUMAN METABOLISM

Isocitrate Dehydrogenase, commonly known as IDH, is an enzyme primarily involved in the Krebs Cycle or the Citric Acid Cycle (the metabolic pathway occurring in the metabolism of fats, carbohydrates, and proteins during cellular respiration). IDH is the enzyme that catalyzes the third step of the cycle for the oxidative decarboxylation of Isocitrate (ICT) to produce α-ketoglutarate, also called 2-ketoglutarate (2KG) and CO_2 and reducing NAD(P)+ to NAD(P)H.

In humans, IDH exists in three isoforms: IDH1, IDH2 and IDH3. IDH1, occurring in cytosol and peroxisomes and IDH2 occurring in mitochondria function as homodimers and catalyze the oxidative decarboxylation of ICT to 2KG simultaneously generating NAD(P)H from NAD(P)+ and the reverse reaction involving reductive carboxylation of 2KG to ICT that oxidizes NADPH to NAD(P)+. They are also known to play important roles in their forward oxidative decarboxylation reaction, in cellular defense against oxidative damage, in reductive synthesis by producing NAD(P)H and regulating dioxygenase enzyme whose co-substrate is 2KG. IDH3 is NAD-Dependent and catalyze the irreversible conversion of ICT to 2KG while reducing NAD+ to NADH.

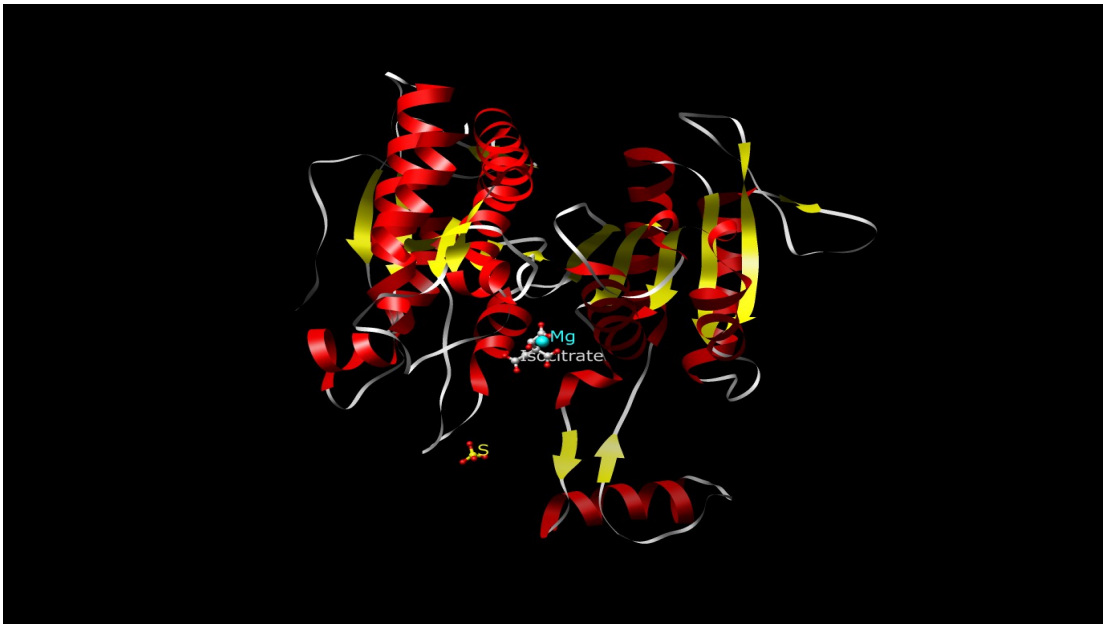

Figure 1: Crystallographic structure of E. coli isocitrate dehydrogenase

MUTATIONS OF IDH

Studies have shown that IDH1/2 mutations occur in nearly 80% of grade II/III oligodendrogliomas, astrocytomas, and oligoastrocytomas. Several malignancies and genetic diseases, such as gliomas, myeloid neoplasms, Ollier disease and Maffucci syndrome were characterised by mutations found in IDH 1 and IDH 2. The mutant proteins of IDH1 and IDH2 display neomorphic enzymatic function by catalysing NAD(P)H dependent reduction of α-ketoglutarate producing an oncometabolite D-2-Hydroxyglutarate (D-2HG). This impacts the epigenetic cell activity by blocking the cellular differentiation through competitively inhibiting the α- ketoglutarate dependent dioxygenases which are involved in histone and DNA demethylation, hypoxia, angiogenesis, and maturation of collagens of the extracellular matrix. The mutation also affects the cellular metabolism, redox state, and DNA repair. Evidence suggests that IDH mutations drive other oncogenic mutations in several hematologic and solid tumors.

D-2HG that is released in the serum or urine by cancer cells with IDH1 or IDH2 mutations is an important biomarker reflecting the altered enzymatic activity of the mutant enzymes. D-2HG levels are of particular interest for both the diagnosis and monitoring of patients with IDH1 or IDH-2 mutant malignancies. But efforts for utilizing the mutations for targeted therapy is still underway. Relevant preclinical models and results of early Phase I trials in adults with hematologic malignancies demonstrate that targeting IDH1/2 mutant is a valid strategy to combat oncogeneic disorders.

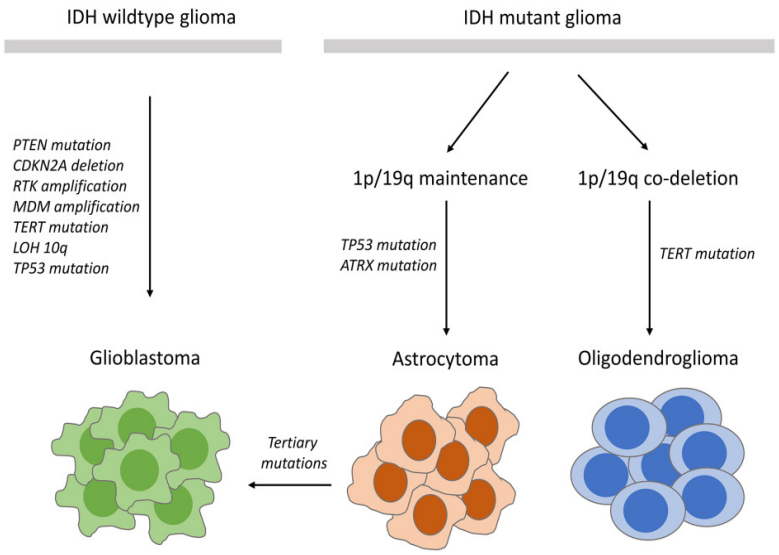

Figure 2: Schematic demonstrating the different genetic alterations noted between isocitrate dehydrogenase (IDH) wild-type and IDH-mutant glioma.

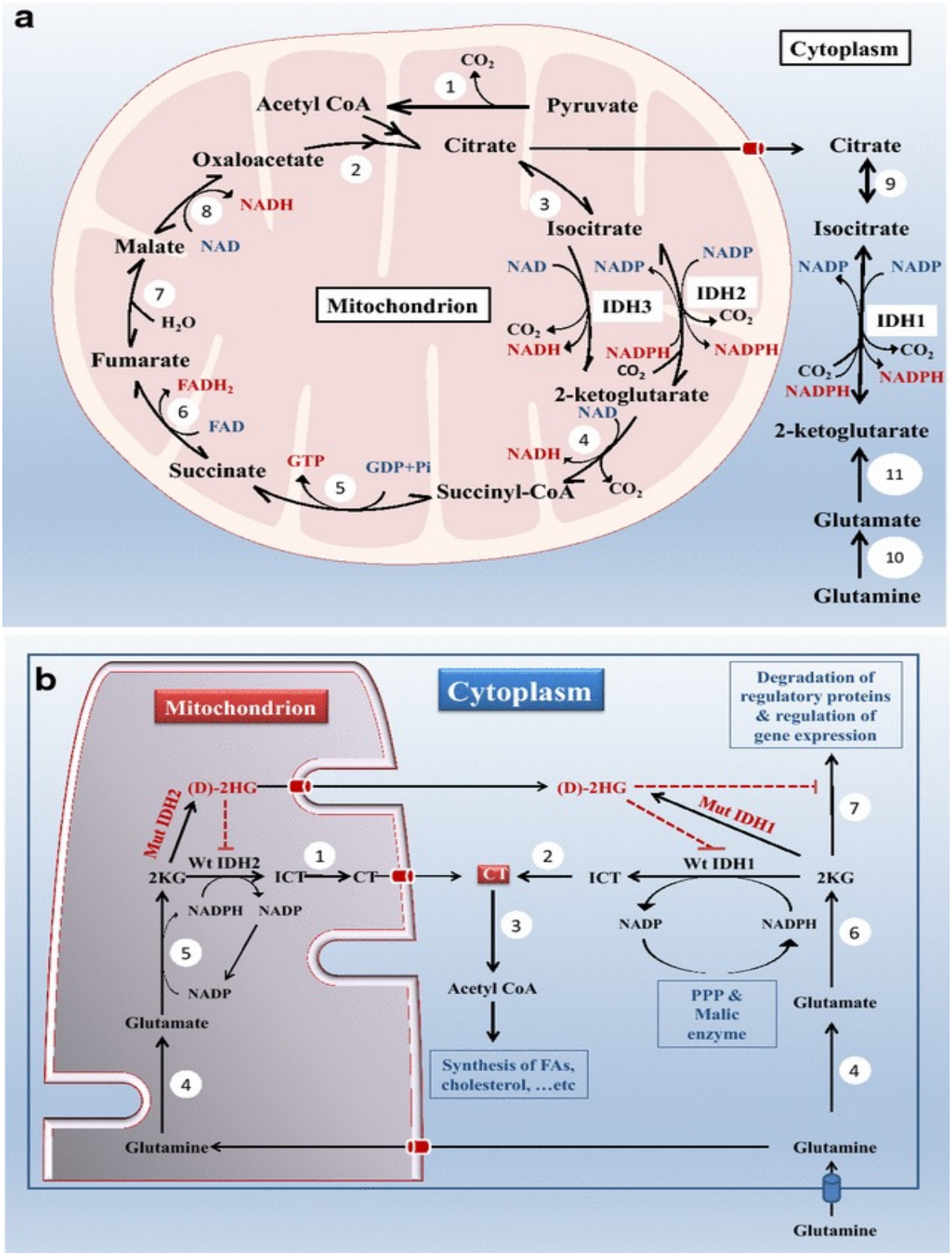

Figure 2: The subcellular localization of the three isocitrate dehydrogenase isoforms and their roles in normal cellular metabolism. *IDH* isocitrate dehydrogenase, *1* pyruvate dehydrogenase, *2* citrate synthase, *3* mitochondrial aconitase, *4* 2-ketoglutarate dehydrogenase, *5* succinyl CoA synthetase, *6* succinate dehydrogenase, *7* fumarate hydratase, *8* malate dehydrogenase, *9* cytosolic aconitase, *10* glutaminase, *11* glutamate transaminase.

b The roles of wild type and mutant isocitrate dehydrogenases 1 and 2 in cellular metabolism. *CT* citrate, *(D)-2HG* (D) 2-hydroxy glutarate, *FAs* fatty acids, *ICT* isocitrate, *IDH* isocitrate dehydrogenase, *Mut* mutant, *PPP* pentose phosphate pathway, *Wt* wild type, *2KG* 2-keto glutarate, *1* mitochondrial aconitase, *2* cytosolic aconitase, *3* citrate lyase, *4* glutaminase, *5* glutamate dehydrogenase, *6* glutamate transaminase, *7* 2-ketoglutarate dependent dioxygenases. The *dotted red line* indicates inhibition (Picture Credit: Al-Khallaf, H. Isocitrate dehydrogenases in physiology and cancer: biochemical and molecular insight. Cell Biosci 7, 37 (2017). https://doi.org/10.1186/s13578-017-0165-3

EXPRESSION OF HUMAN IDH-2 IN *E. coli* BL21DE3

I. Preparation of Competent Cells

1) The primary(5 ml) and secondary(25 ml) bacterial cultures for *E.coli* strains BL21DE3(used for expression) and DH5α(used for cloning and plasmid confirmation) were prepared in falcon tubes.
2) The secondary cultures obtained were incubated on ice for 20 minutes.
3) The cultures were subjected to centrifugation at 4000 rpm for 10 minutes and the supernatant was discarded.
4) The bacterial pellets(of E.coli BL21DE3 and DH5α thus obtained) was treated with 0.1M $CaCl_2$ solution and incubated in ice for 30 mins
5) The solutions were subjected to centrifugation at 4000 rpm for 10 mins
6) The bacterial pellets thus obtained contained competent E.coli cells of strains BL21DE3 and DH5α.
7) The pellets obtained were aliquoted in 0.1ml tubes and stored in 15% glycerol and 0.1M $CaCl_2$ solution at -80°C.

II. Transformation of IDH2 WT

1) Plasmid(extrachromosomal DNA used as a vector) stocks containing pET-41a genetically engineered with IDH2 WT gene exhibiting antibiotic resistance to Kanamycin was added to the aliquots containing competent cells of *E. coli* BL21DE3 and DH5α.
2) The mixture was incubated on ice for 30 minutes.
3) The mixture was then subjected to heat shock of 42°C for 90s.
4) The mixture was then incubated in ice for 10 mins.
5) The aliquots were incubated in 1 ml L.B Broth at 37°C for 1 hour and then centrifuged at 6000 rpm for 5 minutes and the supernatant was discarded.
6) The bacterial pellets obtained contained transformed cells of *E. coli* BL21DE3 and DH5α. The pellets were then plated on Kanamycin induced L.B Agar plates with 0.05 ml L.B broth and incubated at 37°C for 16 hours.
7) Transformed colonies of *E. coli* DH5α and BL21DE3 were obtained.

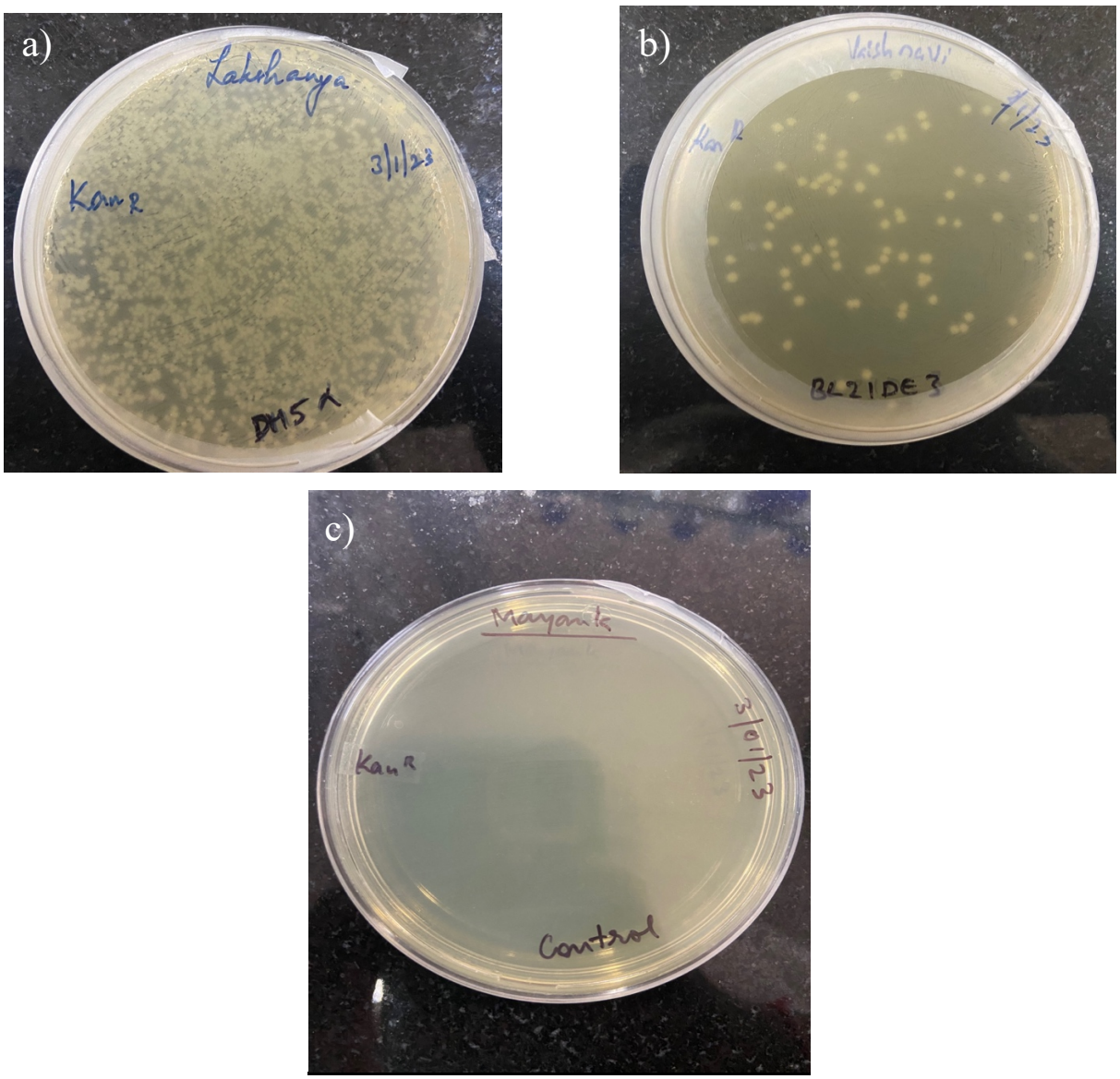

Figure 3: (a) Transformed *E.coli* DH5α colonies (b) Transformed *E.coli* BL21DE3 colonies (c) Control

III. Plasmid Isolation

1) Primary(10 ml) culture of the transformed colony of *E.coli* DH5α was prepared and pelleted into an aliquot using centrifugation at 11000g.
2) The bacterial pellet thus obtained was treated with 0.25ml of A1Buffer and resuspended by vortexing. (Resuspension)
3) The solution was then treated with 0.25ml of A2 Buffer and mixed gently by inverting the tube 6-8 times. (Lysis)
4) The solution was incubated at room temperature for 5 minutes.
5) The solution was then treated with 0.3ml of A3 Buffer and mixed gently by inverting the tube 6-8 times until the blue coloured solution turned colourless. (Neutralization)

6) The solution was incubated at room temperature for 5 minutes and then subjected to centrifugation at 11000g for 7 minutes.
7) A nucleospin column was inserted in the collection tube and the supernatant(0.7ml) was decanted into the column
8) The nucleospin along with the collection tube was subjected to centrifugation at 11000g for 1 minute twice and the flow-through was discarded
9) The pellet was treated with 0.5ml AW Buffer and then subjected to centrifugation at 11000g for 1 minute at room temperature.(Washing)
10) The pellet was treated with 0.6ml A4 Buffer and then subjected to centrifugation at 11000g for 1 minute and the flow-through was discarded and the empty collection tubes were subjected to centrifugation at 11000g for 4 minutes.
11) An autoclaved aliquot was placed under the nucleospin column and treated with 0.05ml of AE Buffer and incubated at room temperature for 1 minute. The aliquot-nucleospin set up was then subjected to centrifugation at 11000g for 1 minute.(Elution)
12) The aliquot containing the isolated plasmids was stored at -20°C.

IV.Agarose Gel Electrophoresis

1) 50ml of 0.8% Agarose gel was prepared using 1X TAE Buffer (pH=8.0)
2) 0.002ml of Ethylene Bromide(EtBr) was added to the gel and poured into the casting tray with a well-comb.
3) The gel was allowed to cool down and solidify for 15 minutes at 4°C
4) After polymerization, the gel tray was set in the Electrophoresis unit and the electrophoresis tank was filled with 1X TAE Buffer.
5) The well comb was gently removed.
6) The wells were individually filled with 0.003ml Commercial DNA Marker, 0.012 ml of the isolated Plasmid and the control. (dyed with 0.005ml of loading dye B7024S)
7) The gel was run for 100V for 1 hour.(until the dye reached 70-80% of the gel)
8) After electrophoresis, the power was disconnected and the gel was visualized in UV transilluminator to observe the bands.
9) The observations were recorded and the gel was discarded.

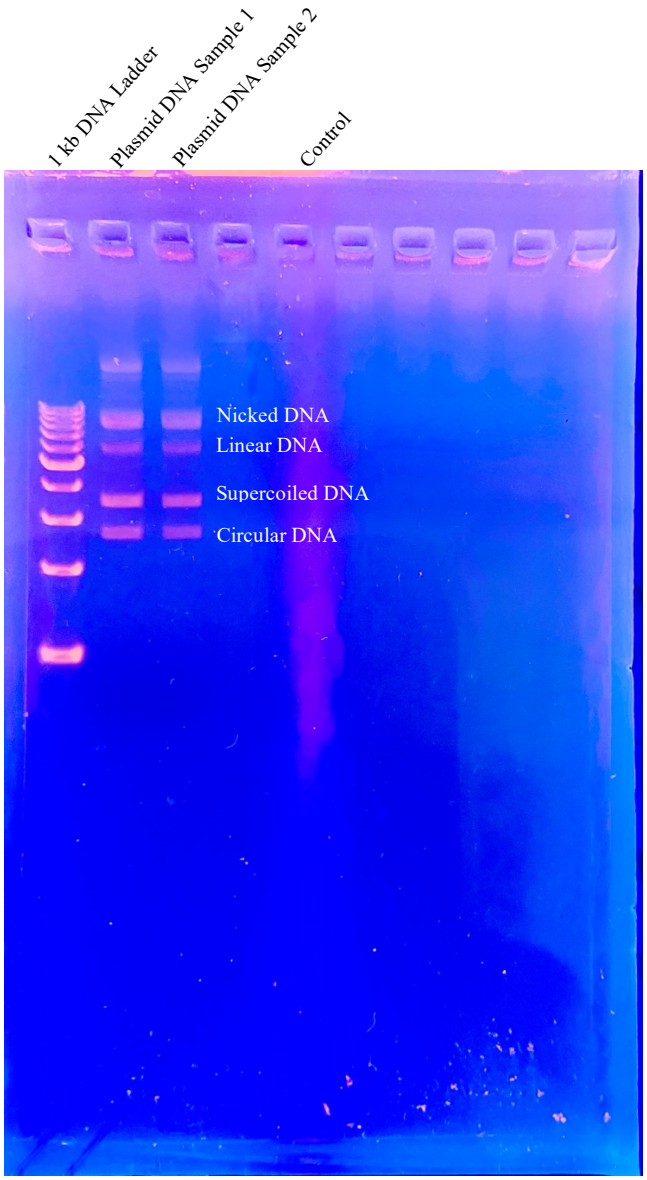

Figure 4: Results obtained from the Agarose gel Electrophoresis

V. Expression of Recombinant IDH2 WT using IPTG

1) Primary culture(10 ml) followed by Secondary culture(250 ml) of the transformed colony of *E.coli* BL21DE3 was prepared. The 2° culture was incubated for 4 hours at 37°C until the O.D reaches 0.5-0.6
2) 5ml of the 2° culture was harvested in a falcon tube as uninduced sample.
3) The rest of the 2° culture was treated with 1mM Isopropyl β-D-1-thiogalactopyranoside(IPTG-the reagent that induces transcription and expression of the protein IDH in the transformed cells) and incubated at 37°C for 4 hours.
4) The induced and the uninduced culture was subjected to centrifugation at 4500rpm for 10 minutes at room temperature.
5) The supernatant was discarded in both the cultures and the pellets were resuspended in 20mM Tris Chloride Buffer(pH =8.0).

6) The uninduced sample was subjected to Sonication(Stimulation of the cell membranes to lyse using Ultrasonic Vibrations)(Amplitude:37%, Pulse:30s ON and 30s OFF, Temperature: 4°C) for 10 minutes produce uninduced whole cell lysate.
7) The induced sample was also subjected to sonication(Amplitude:37%, Pulse:30s ON and 30s OFF, Temperature: 4°C) for 30 minutes. The induced sample with cell lysate was subjected to centrifugation at 15000rpm for 30 minutes at room temperature. The supernatant and the pellet were collected.

VI. Sodium Dodecyl Sulphate –Polyacrylamide Gel Electrophoresis (SDS PAGE)

1) 5ml of 10% SDS-PAGE Resolving gel and SDS-PAGE Stacking gel was prepared using the following concentrations:

Components	**SDS PAGE RESOLVING GEL**	**Components**	**SDS PAGE STACKING GEL**
Water	1.900 ml	Water	3.400 ml
30% Acrylamide	1.700 ml	30% Acrylamide	0.850 ml
1.5M Tris Buffer(pH= 8.8)	1.300 ml	1 M Tris Buffer(pH= 8.8)	0.625 ml
Sodium Dodecyl Sulphate(SDS)	0.050 ml	Sodium Dodecyl Sulphate(SDS)	0.050 ml
10% Ammonium Persulphate(APS)	0.050 ml	10% Ammonium Persulphate(APS)	0.050 ml
Thermo-Scientific Tetramethyl ethylenediamine (TEMED).	0.002 ml	Thermo-Scientific Tetramethyl ethylenediamine (TEMED).	0.005 ml

2) The gel prepared was set up between two stacked vertical glass plates and wells were set up using wide comb giving a loading capacity of about 0.03ml.
3) 0.06ml of the supernatant, pellet and whole cell lysate from the induced sample, and 0.06ml of the uninduced whole cell lysate were taken in separate aliquots and mixed with 0.012ml 5X Loading Dye.

4) After polymerization of the gel occurred, The comb was gently removed.
5) The wells were loaded with 0.005ml of the Commercial Protein Marker and 0.025ml of the samples in each well.
6) The Gel was then subjected to 2 hours of electrophoresis at 80V in 1X SDS PAGE running buffer.

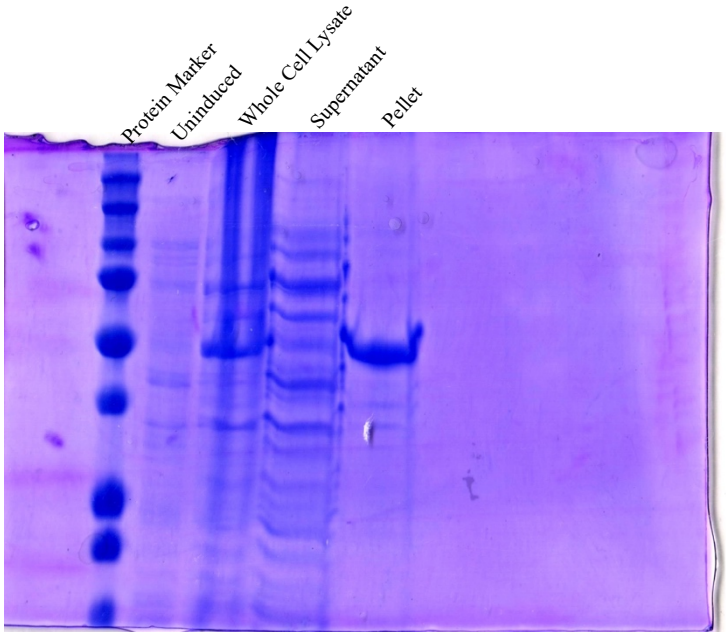

Figure 5: Results obtained from the SDS Page

CONCLUSION

The agarose gel electrophoresis confirmed the presence of plasmid in the transformed colonies. The human recombinant isocitrate dehydrogenase 2 enzyme was expressed at 1mM IPTG at 37^{o}C for 4 hrs. The expressed protein was observed in 10% SDS PAGE and protein was obtained in the pellet from the centrifugation of induced whole cell lysate.