Appendix

1 Materials and Methods

1.1 RNA Isolation and Quantification

Total RNA isolation from mouse fibroblast cells. The TRIzol® method (Thermo Fisher Scientific) was used to extract total RNA from mouse fibroblast cells.TRIzol maintains the integrity of RNA while disrupting the cell and cellular components. After treating the mouse fibroblast cells with TRIzol, addition of chloroform followed by centrifuging separates the solution into two visually distinct phases - the aqueous phase and the organic phase. RNA is found exclusively in the aqueous phase which was pipetted out and transferred to another tube. This aqueous phase solution is then first incubated with RNase free DNase I for 20 minutes in a 37°C water bath to remove any contaminating DNA that could have been accidentally pipetted from the interphase. After 20 minutes, RNA is then recovered from the aqueous phase by precipitation with isopropanol for 10 minutes at rtp, then yielding an RNA pellet upon centrifugation for 10 minutes at 4°C. After centrifuging, the supernatant was removed and washed with 1mL of 75% ethanol via vortexing to remove residual salt from the RNA pellet. Centrifuging was performed after 5 minutes. The supernatant is then removed via pipetting and the RNA pellet is air-dried for about 10 minutes before dissolving in 20µl DEPC water for about 10 minutes at 55-60°C on a heat block.

DEPC helps to inactivate ribonucleases through covalent modification of specific amino acids (mostly histidine residues). Wearing gloves is also important to prevent RNase contamination from our fingertips.0.1% DEPC, not heat, is used to inactivate contaminating RNases, since RNases are stable due to high percentage of cysteine bonds that result in strong disulfide bonds that render RNases to be heat-stable.

RNA Quantification. The extracted RNA from above was subsequently quantified via NanoDrop. The NanoDrop was first blanked with DEPC water before taking the readings. The solutions are also maintained at acidic pH as the 2'OH group present on RNA may cause it to undergo alkaline hydrolysis, thereby degrading the mRNA and causing lesser extraction yield.

1.2 RT-PCR (Reverse Transcription - Polymerase Chain Reaction)

Reverse Transcription. 2µl of the purified RNA was extracted using a pipette into a new sterile 0.2µl reaction tube. 8µl of RT mix containing 2µl of oligo-dT primers and 6µl of DEPC-trated H20 were already present in the reaction tubes given. Incubation of mRNA with the RT mix was at a high temperature of 72 °C for 2 minutes in the reaction tube. This is to break any intra-molecular base-pairings of RNA molecules to ensure that RNA is single-stranded so RT can take place efficiently. The tubes containing oligo-dT primers and mRNA were then gradually cooled to about 42°C for 1h to allow annealing to only mRNA poly-A tail within total RNA.

These tubes were then spun briefly in a microcentrifuge to collect the contents at the base of the reaction tube. Four tubes, each containing 2µl of RNA, underwent incubation with premixed enzyme cocktails. Two tubes, A and C were incubated with the enzyme cocktail(RT+), while tubes B and D were not incubated with the enzyme cocktail, thus acting as negative controls(RT-). The total volume of the mixture of RNA and enzyme cocktail was 20µl (Table A1). The tubes were incubated with the enzyme cocktail for 1 hour at 42 °C. The reaction was then terminated by heating at 70 °C for 10

minutes. All tubes were then placed on ice followed by addition of 80µl of nuclease-free water to prepare for the next PCR step.

Table A1. Components of the enzyme cocktail used for reverse transcription.

Volume to be added to respective tube/µl		Enzyme cocktail component
RT(+)	**RT(-)**	
4	4	5x Reaction Buffer
1	1	RiboLock Ribonuclease Inhibitor (20u/µl)
2	2	10mM dNTPs (10mM each of dATP, dCTP, dGTP, dTTP)
2	0	RevertAid Reverse Transcriptase
1	3	DEPC-treated water

Polymerase Chain Reaction(PCR). cDNA from tubes A,B,C and D were re-distributed into new tubes labelled 1,2,3 and 4 respectively. 2.5µl from each original cDNA-containing tube was pipetted into the new tubes. All new tubes 1-4 were already containing a PCR mastermix (Table A2). All contents were then mixed by pipetting, then spun down before storing on ice.

Table A2. Contents of PCR Mastermix

Volume/µl	Reagent	Final Concentration
13.50	H20	
2.50	10x PCR buffer (KOD)	1x
2.50	dNTPs, 2mM of dATP, dCTP, dGTP, dTTP each	0.2mM each
1.00	NcoI-MmLDHA-F, 10µM, forward primer for LDHA	0.4µM
1.00	EcoRI-MmLDHA-R, 10µM, reverse primer for LDHA	0.4µM
1.50	25 mM MgSO4	1.5mM
0.50	KOD DNA Polymerase, 1u/µl	0.5 units/25µl
22.50µl total volume (excluding 2.5µl DNA)		

PCR was performed using NDeI-H6-MmLDHA forward primer and EcoRI-MmLDHA as the reverse primer. KOD DNA polymerase is used as the polymerase enzyme to help with the elongation stage of PCR. KOD DNA Polymerase has a speed of 0.5min/kb at 68°C as opposed to Taq DNA polymerase which has a speed of 1min/kb at 72°C. KOD DNA polymerase also has a lower error rate of 1 mutation per 80kb as opposed to Taq DNA polymerase which has an error rate of 1 mutation per kb. Thus, KOD polymerase is chosen as the preferred polymerase for PCR in this experiment due to its

higher speed at a lower temperature and a lower mutation rate as opposed to the commonly used Taq polymerase.
Before 25 cycles of PCR was performed, the cDNA+PCR mix was incubated for 2 minutes at 94°C for DNA strand separation. After which, PCR cycling conditions for the 25 PCR cycles were as follows: denaturation at 94°C for 2 minutes to separate DNA strands of cDNA, annealing at 60°C for 1 minute for primers to anneal to the single stranded cDNA, and DNA synthesis at 68°C for 1 minute. After 25 cycles, the mixture is then incubated for 10 minutes at 68°C to ensure that all DNA is completely synthesised. Then, the RT-PCR product was stored at 4°C to preserve it.

Agarose Gel Electrophoresis. Performed on a 1% gel with EtBr/SYBR safe in 1x TAE buffer at 100V for 45 minutes to visualise 5µl of RT-PCR products. 1µl of loading dye was added to 5µl of each sample. Bands were observed under UV-light using a gel documentation system. 1kb of 1µl DNA ladder was mixed with 4µl H2O and 1µl of loading dye and was loaded into the leftmost lane.

RT-PCR Fragment Cleaning. RT-PCR products from tubes 1 and 3 (both RT+) are purified using binding and washing buffers followed by centrifugation, then quantified using NanoDrop. This is first done by mixing the remaining 20µl of solution in tubes 1 and 3 with 100µl of binding buffer, then both tubes are spun in a centrifuge at 13,000g and r.t.p. for 1 minute. The flow through is then removed and 0.7ml of washing buffer is added to each tube before centrifuging at 13,000g and r.t.p. for 1 minute. The removing of flow through and centrifuging is repeated again. Flow-through is removed and empty spin column spun again in the centrifuged under the same setting. Each spin column is then placed into a new 1.5ml tube whereby 50µl of nuclease-free H20 is added directly to the membrane of each spin column and incubated at 37°C for 5 minutes. DNA is then eluted by centrifuging at 13,000g and r.t.p. for 1 minute before taking out the spin columns and capping the tubes. This cleaned cDNA RT-PCR product is then quantified using NanoDrop.

Restriction Endonuclease (RE) Digestion. The RT-PCR product was subjected to double-digestion: 20 µl purified RT-PCR product was added to 15µl H2O, 4µl RE-Buffer 3.1 (or RE-Buffer 3 + BSA), 0.5µl NcoI, and 0.5µl EcoRI in a 1.5 ml tube. Then, the mixtures were pulsed and incubated at 37°C overnight.

To a separate 1.5ml tube, the vector plasmid was subjected to triple-digestion: 20µl of pET11-H6α vector plasmid was added to 14.5µl H2O, 4µl RE-Buffer 3.1, 0.5µl NcoI, 0.5µl EcoRI, and 0.5µl BamHI. This tube was then pulsed down before being incubated at 37°C overnight.

The use of EcoRI, BamHI and EcoRI restriction enzymes allows for the creation of compatible cohesive ends on both the RT-PCR product and the plasmid, facilitating the subsequent ligation of the cDNA fragments into the plasmid vector. This directional cloning ensures that the cDNA fragments are inserted in the correct orientation under the control of the lac-inducible T7 promoter,

1.3 Constructing Recombinant Plasmids

Cleaning-up of cut DNA fragments. The RT-PCR fragment and pET11-H6α plasmid were subjected to double and triple restriction digestion, respectively, as previously described. The resulting products underwent purification using the same clean-up method as the RT-PCR cleaning performed previously. Both the cut vector and the RT-PCR product were then quantified using NanoDrop after clean-up.

Ligation of RT-PCR cDNA into pET11-H6α plasmid. T4 DNA ligase was added to varying volume ratios of pET11-H6α and RT-PCR products for ligation to the pET11-H6α plasmid vector (Table A3). The mixture was then incubated at room temperature for 1 hour.

Table A3. The volumes of each mixture component

Vector:Insert Ratio of Reaction Tube	Volume of Mixture Constituent/µl			
	Cut pET11-H6α Vector	RT-PCR Fragment	Ligation Buffer	T4 DNA Ligase
1:1	4.5	4.5	10.0	1.0
1:2	3.0	6.0	10.0	1.0

Bacterial Cell Transformation via uptake of Recombinant Plasmid. Ligated recombinant pET11a plasmids were transformed into DHSα competent E. coli cells (30µl) using the heat shock method: incubating tubes on ice for 30 minutes, followed by heating at 42°C for 90 seconds. 150µl of LB medium was added to each tube, then incubated at 37°C with shaking for 1 hour. The cells were subsequently resuspended by gently pipetting it. 200µl of the suspension was then plated on separate LB plates containing: 100µg/ml ampicillin, 40µg/ml X-Gal and 0.1Mm IPTG and incubated upside-down at 37°C overnight.

DHSα competent E. coli cells are specifically chosen for their ability to efficiently take up foreign DNA when treated with a solution containing a divalent cation (calcium chloride) to increase the permeability of the bacterial cell walls, allowing foreign DNA to be taken up. The use of DHSα competent E. coli cells is essential for the successful introduction of the recombinant plasmids, allowing for the subsequent expression of proteins from the T7 promoter.

Small-scale Preparation of Recombinant Plasmid DNA. 4 colonies from the LB plate containing the highest percentage of white colonies is chosen. Each single colony is then transferred into a 15ml tube with 4ml of LB medium containing 50µg/ml ampicillin. Each culture was then incubated overnight on a shaking incubator at 37°C. The cultures are labelled as C1,C2,C3 and C4 respectively to indicate that they originate from different colonies (Table 4).

1.5ml of the bacterial culture is carefully decanted into a 1.5ml microcentrifuge, then centrifuged at 14,000g for 1 minute. The bacterial pellet needs to be firmly attached to the side of the tube after centrifugation. After which, the supernatant is discarded via aspiration and repeat the centrifugation with another 1.5ml of the bacterial culture. The supernatant was then removed via aspiration and the bacterial cell pellet is left to dry by inverting the tube upside-down onto a paper towel for a few minutes until all the residual supernatant has drained out of the tube.

Table A4. Constituents and purpose of Solutions A,B,C and D.

Solution	Purpose	Constituents
A	Cell Resuspension	100µg/ml RNaseA + 10mM ETDA + 50mM Tris (pH7.5)
B	Cell Lysis	0.2M NaOH + 1% SDS solution

C	Neutralisation	4.08M guanidine hydrochloride + 0.759M potassium acetate + 2.12M glacial acetic acid
D	Column Wash	60mM potassium acetate + 8.3 mM Tris-HCl + 0.04mM ETDA + 60% ethanol

The bacterial cells were then resuspended using vortex mixing in 250µl of Solution A, followed by gentle mixing (by inverting the tube 5x) with 250µl of Solution B at r.t.p. Vortexing was not done upon addition of Solution B as the chromosomal DNA would be sheared. After which, 10µl of alkaline protease solution was added and the tube is gently mixed. The tube is then incubated for <5 minutes at r.t.p (if incubated for >5 minutes, the plasmid DNA would be nicked). Then, 350µl of Solution C is added to the tube and gently mixed by inverting the tube 5x. The resultant bacterial lysate is then centrifuged for 10 minutes in a microcentrifuge at 14,000g.

While the centrifuge was running, plasmid DNA purification units were prepared by insertion of one Miniprep spin column into a 2ml Collection Tube. After 10 minutes was up, all the cleared cell lysate (supernatant) was micropipetted into the spin column. The spin column containing the supernatant was then centrifuged for 1 minute at 14,000g at r.t.p. The spin column was then removed from the collection tube and the flowthrough was discarded. After discarding flowthrough, the spin column was placed back in to the Collection Tube. Then, 750µl of Solution D was added and the Collection Tube was centrifuged for 1 minute at 14,000g at r.t.p. Flow-through was then discarded and 250µl of Solution D was added into the spin column and the Collection Tube was centrifuged for 14,000g for 2 minutes at r.t.p.

Each spin column is then transferred into a sterile, new 1.5ml microcentrifuge tube. The plasmid DNA in the spin column was eluted by adding 50µl of nuclease-free water, and incubated at 2 minutes at r.t.p. The spin column was then centrifuged for 1 minute at 14,000g at r.t.p. The plasmid DNA was then quantitated via NanoDrop before storing at -20°C for long-term storage.

Plasmid DNA Digestion. From each colony taken from the Small-scale Preparation of Recombinant Plasmid DNA step, pipette 5µl of each colony sample into 3 new reaction tubes, resulting in 15 tubes in total. The last 3 tubes were positive controls containing a control plasmid. Refer to Table A5 for clearer breakdown of the reaction tubes used:

Table A5. Breakdown of the tubes that were prepared before conducting RE digest. 5µl of each colony (from the Small-scale Preparation of Recombinant Plasmid DNA step) was pipetted into each of the 3 tubes, A total of 15 tubes was thus used.

Reaction Tube Content Source	**Colony 1**	**Colony 2**	**Colony 3**	**Colony 4**	**Positive Control Plasmid**
(Quantity) x (Volume) of reaction tubes	3 tubes x 5µl each	3 tubes x 5µl each	3 tubes x 5µl each	3 tubes x 5µl each	3 tubes x 5µl each

To each set of 3 tubes per colony, each tube was subjected to reaction with a different reaction each (Table A6). Each tube was then subjected to the relevant treatment (Table A7) before pipetting the relevant amounts into each reaction tube. The reactions were then incubated at 37°C for 1 hour.

Table A6. Treatment subjected to the 3 tubes respectively.

Reaction Tube (out of the 3 tubes)	Treatment
1st	Uncut (i.e. no treatment)
2nd	Eco-RI digested plasmid
3rd	Eco-RI/NcoI double-digested plasmid

Table A7. Constituents for each treatment per tube.

Treatment	Constituent (µl)					
	Plasmid	H20	Buffer	EcoRI	NCoI	Total
Uncut plasmid	5.0	4.0	1.0	-	-	10.0
EcoRI-digested plasmid	5.0	3.5	1.0	0.5	-	10.0
EcoRI/NcoI double-digested plasmid	5.0	3.5	1.0	0.5	0.5	10.0

Agarose Gel Electrophoresis. Performed on a 1% agarose gel with EtBr Safe in 1x TAE buffer at 100V for 45 minutes. 2µl of loading dye was added to 10µl mixture present in each tube. Each 12µl of each tube was then loaded into individual wells. 1µl of 1kb DNA ladder was mixed with 2µl of loading dye and loaded into an individual well. The resulting bands were visualised under UV light and photographed. Based on this gel electrophoresis results, the plasmid that was most cleanly digested was selected for sequencing.

1.4 Sequencing of DNA

Mastermix Preparation. 5µl big dye + 5µl sequencing buffer + 15µl template is mixed together to form 5 portions of the mastermix, whereby one portion is to account for pipetting error. To 4 separate MicroAmp™ PCR tubes, add 5µl of the mastermix. Then to each of the 4 tubes, 5µl of each primer (pET11aF, LDHA 430-450, pET11aR, and LDHA 570-550) was added into a separate PCR tube respectively and mixed i.e. one primer per tube.

PCR. PCR was conducted with the following parameters: 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 2 minutes, repeated for 25 cycles. The PCR machine was then set to hold at 4°C.

Ethanol Precipitation. To each MicroAmp™ PCR tube, add 80µl of prepared ETOH/sodium acetate (consisting 3.0µl 3M sodium acetate, pH4.6, 62.2µl non-denatured 95% ethanol and 14.5µl deionised water) and mix. Then, transfer each mixture to a separate 1.5ml Eppendorf tube and briefly vortex. The tubes were then left on ice for 15minutes for the ethanol products to precipitate. The Eppendorf tubes were subsequently spun for 20 minutes at maximum speed at r.t.p. After which, the supernatant was carefully aspirated with a pipette and discarded. The resulting pellet was barely visible to the eye.

To rinse the pellet, 500µl of 75% ethanol was added to the pellet and was subsequently vortexed. The ethanol-pellet mixture was then microcentrifuged at maximum speed before aspirating and discarding the supernatant. As much as possible of the ethanol was removed (as ethanol will impact sequencing) before the pellet was dried at 50°C on a heat block for 10 minutes. The pellet was monitored to ensure it did not over-dry. Subsequent steps were performed by an external lab to be sequenced by professionals who used the ABI PRISM 3100 to sequence the pellets.

Using BLAST to perform Sequence Analysis. Sequencing results were conducted by an external laboratory. These results were then aligned using BLAST on the NCBI website to generate a comprehensive plasmid sequence, aiding in identifying discrepancies and distinguishing between sequencing errors and mutations.

2 Tables of Migration Distances and Standard Curves

Table A8. Calculated migration distance for each bp.

Molecular Size (bp)	Log(Molecular Size)	Distance Migrated by DNA (cm)
10000	4	1.2
8000	3.903089987	1.45
6000	3.77815125	1.7
5000	3.698970004	2
4000	3.602059991	2.3
3500	3.544068044	2.5
3000	3.477121255	2.8
2500	3.397940009	3.1
2000	3.301029996	3.5
1500	3.176091259	4.1
1000	3	5
750	2.875061263	5.7
500	2.698970004	6.4
250	2.397940009	7.4

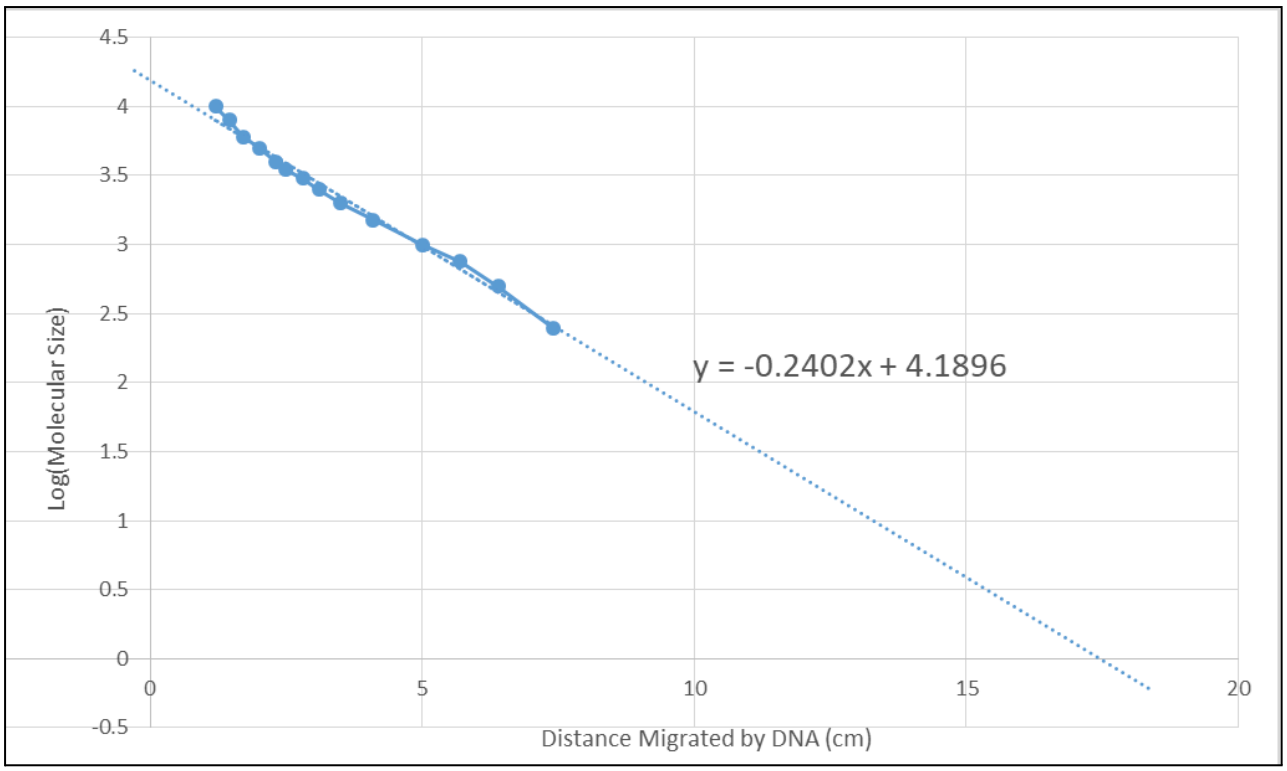

Fig. A1 Graph plot of Log(molecular size) against distance migrated by DNA(cm). Standard Curve Equation is: log (mol. size) = k * migration +c

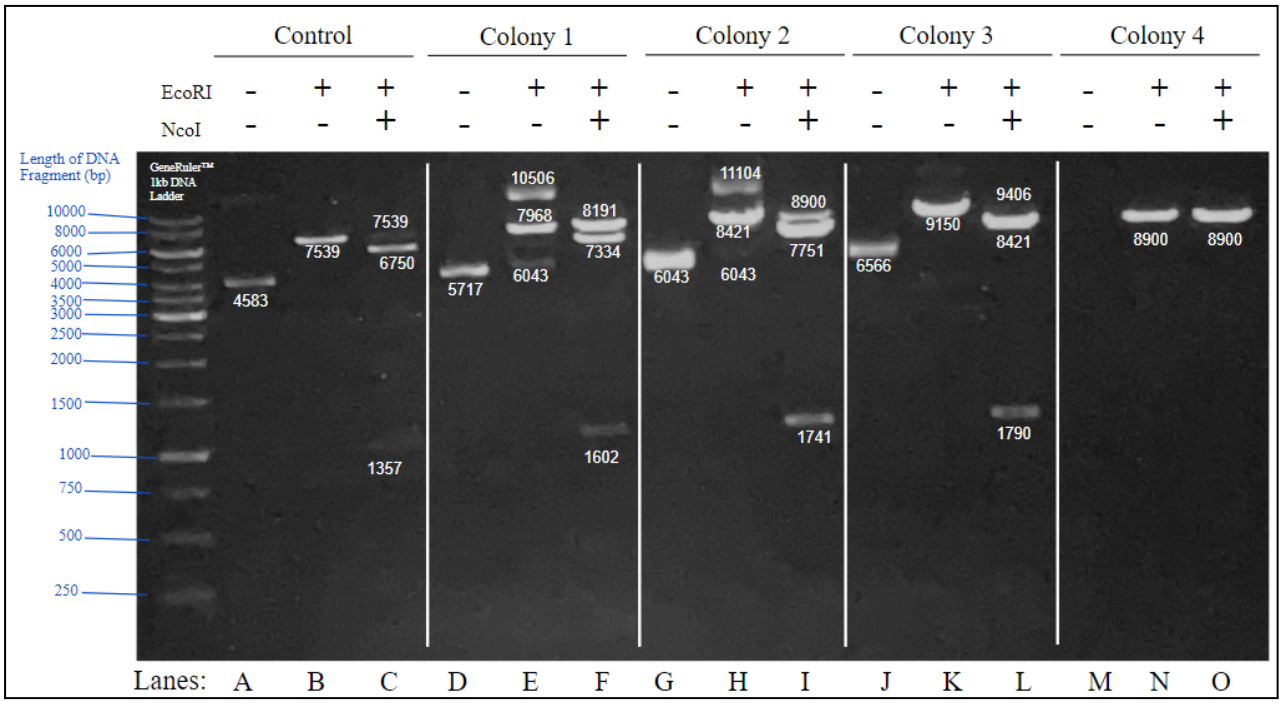

Fig A2. Estimated sizes of the sample bands based on the standard curve equation in Fig. A1.

3 BLAST Sequence Alignments

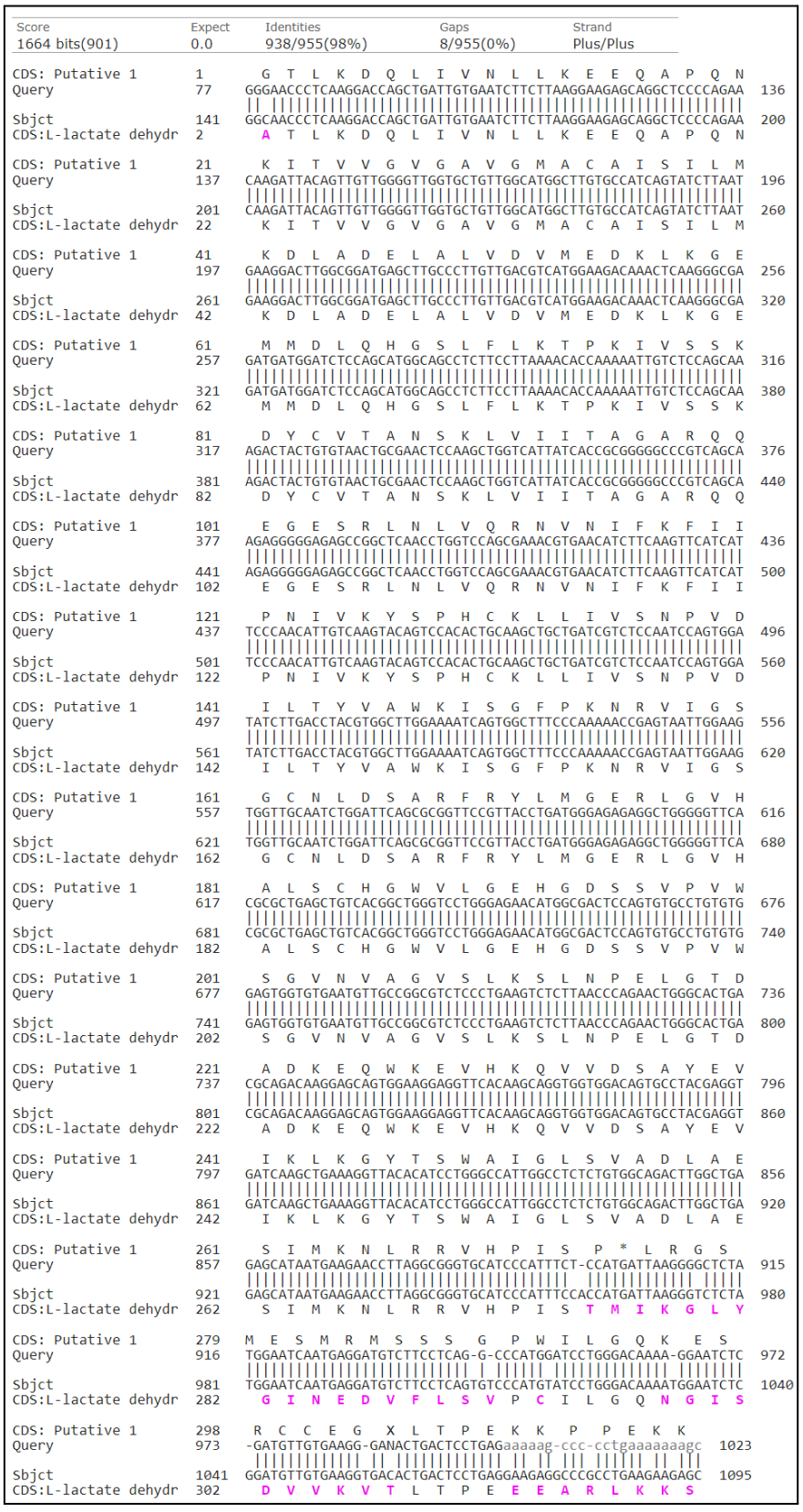

Fig A3. Pairwise alignment of pET11aF against NM_010699.2. pET11aF had a 2% amino acid mismatch as seen by the significantly larger number of pink alphabets (which indicate amino acid mismatches)from position 895 onwards. (nucleotide on left, CDS on the right)

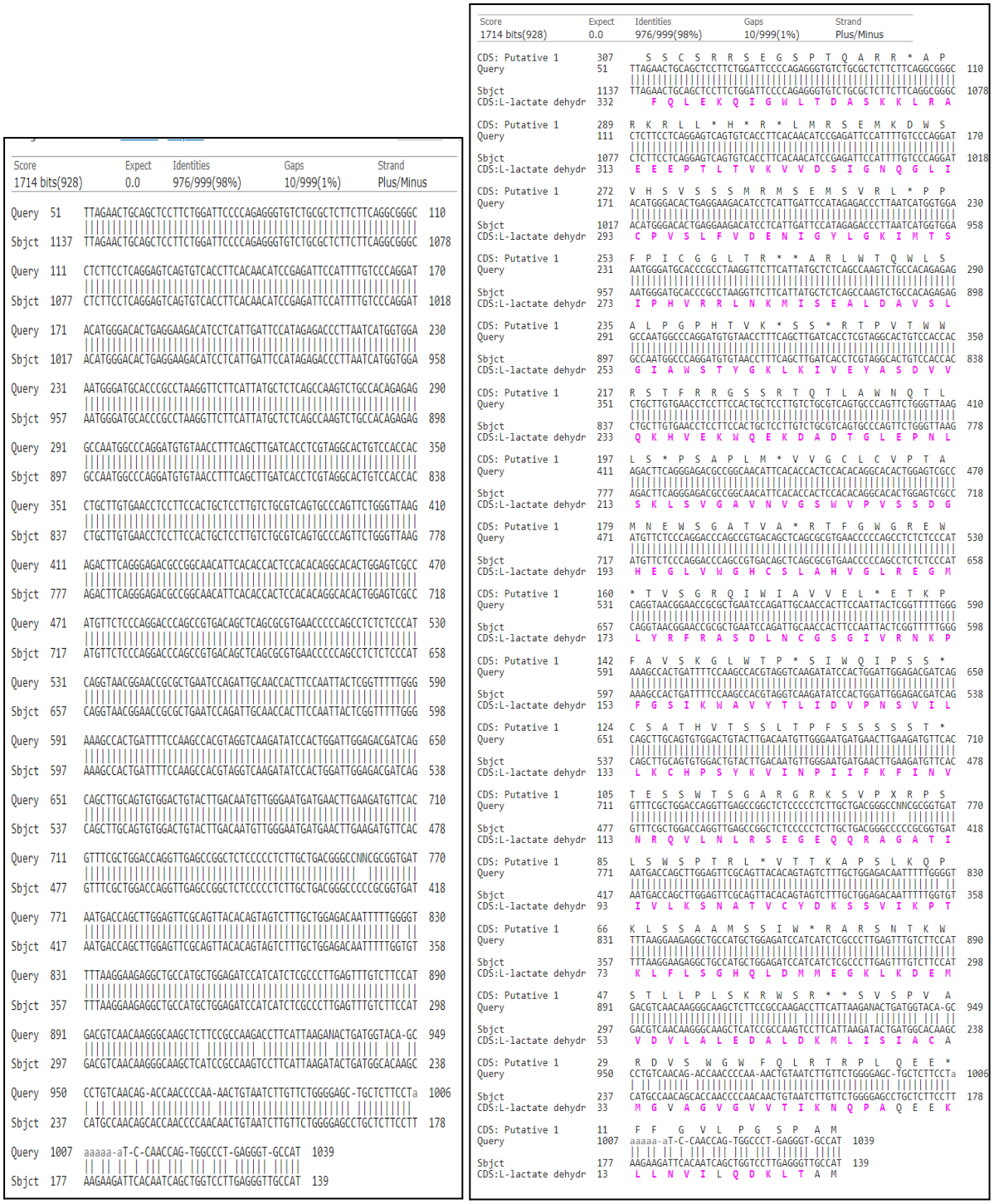

Fig A4. Pairwise alignment of pET11aR sequence
against NM_010699.2 (nucleotide on left, CDS on the right)

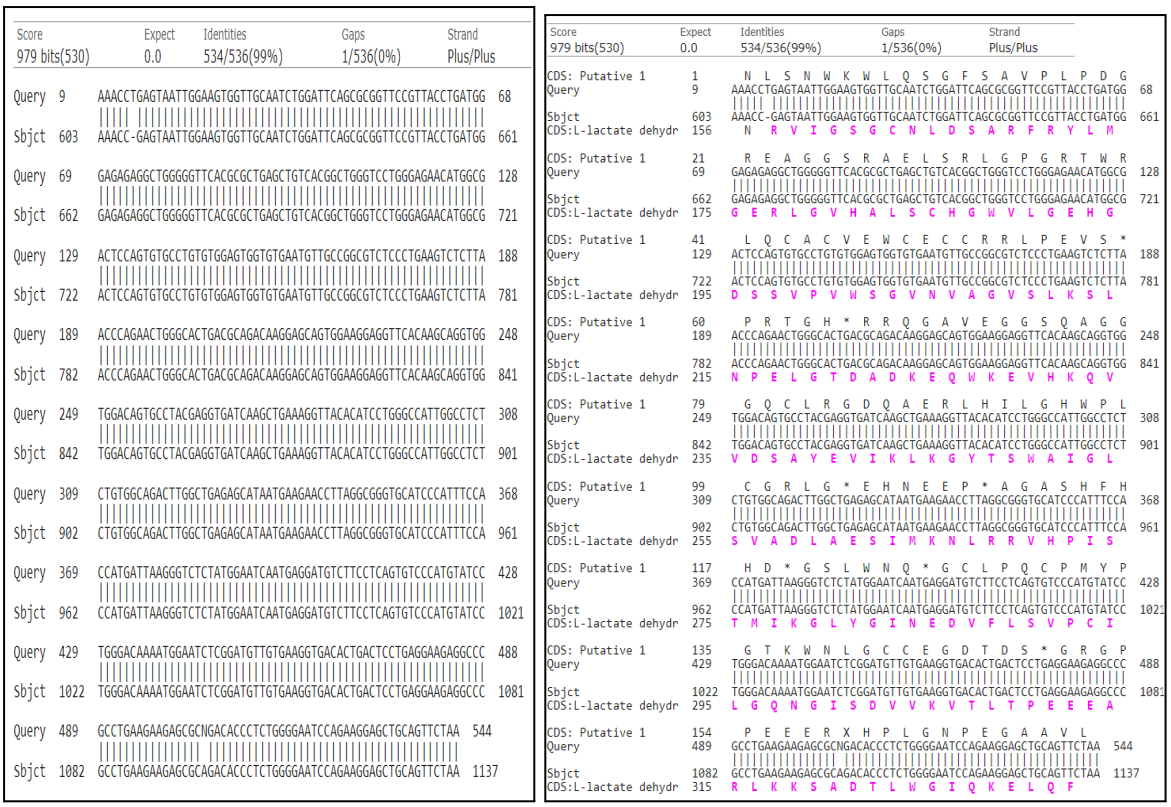

Fig A5. Pairwise alignment of LDHA430-450 cut fragment against NM_010699.2. Only one mismatch was found at query position 15. (nucleotide on left, CDS on the right)

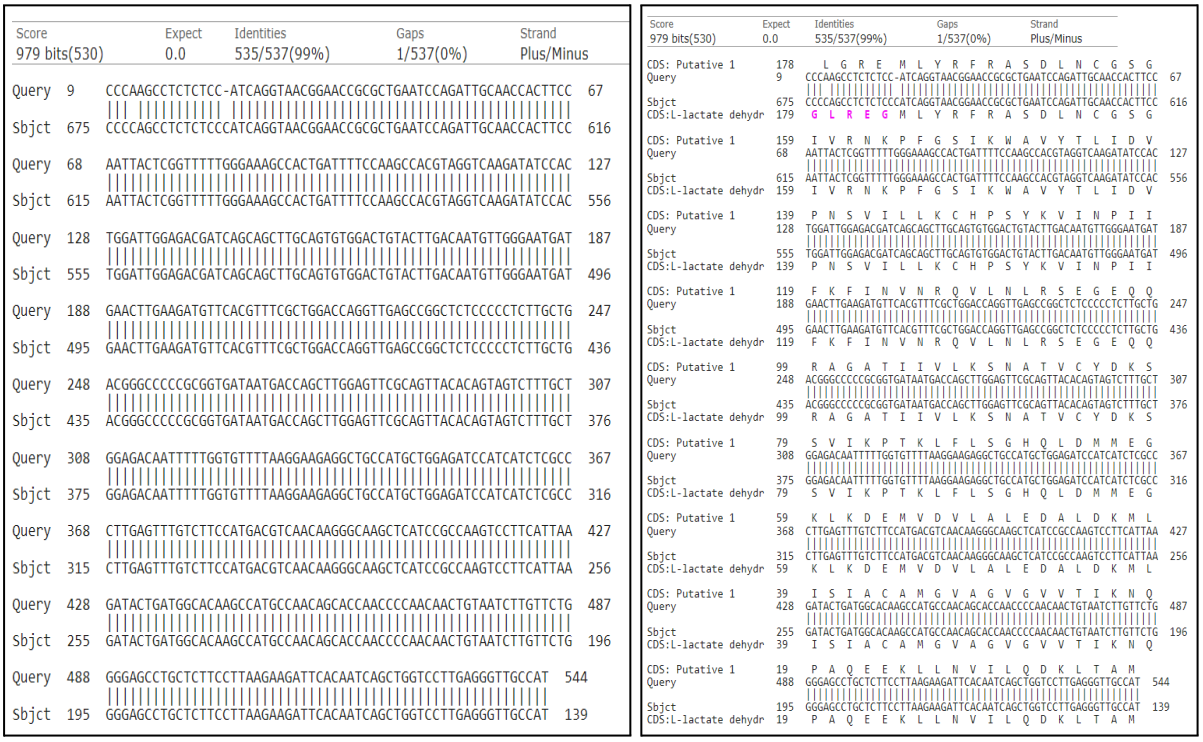

Fig A6. Pairwise alignment of LDHA570-550 cut fragment against NM_010699.2 Only one mismatch was found at query position 25.(nucleotide on left, CDS on the right)

4 BLAST Analysis

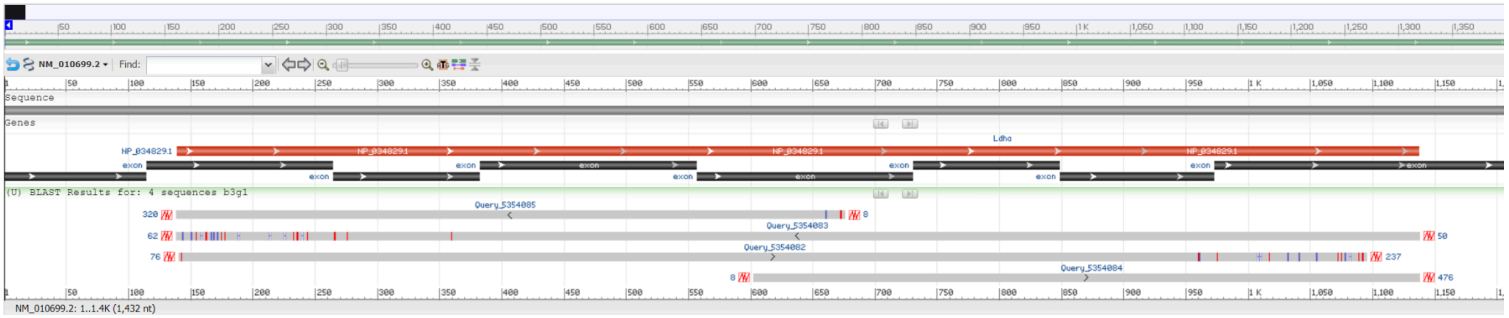

Fig A7. Graphical Representation: alignment of (from top to bottom) LDHA 570-550, pET11aR, pET11aF and LDHA 430-450 sequences against NM_010699.2. From the diagram, there are no aligned red lines common in all the sequences, showing that no mutation was present. For a mismatch to be characterised as a mutation, the mismatch needs to appear in the same location in more than one sequence.

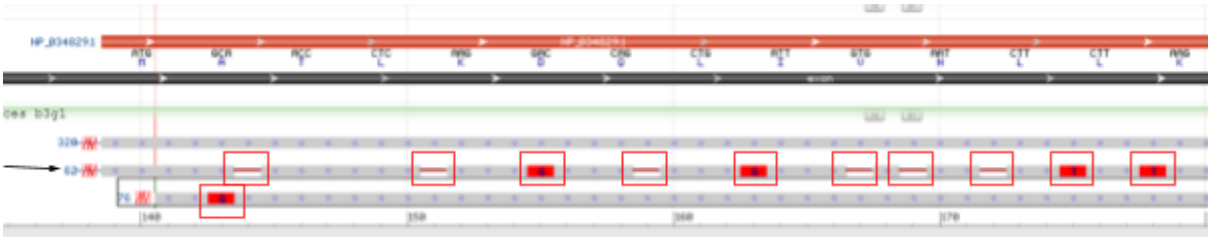

Fig A8. Close-up of mismatching. The red boxes indicate either a sequencing error or a mutation. The middle sequence as indicated by the arrow (belonging to the pET11aR sequence) had many red boxes at its end, so it is plausible that these are sequencing errors and not mutations since these mismatches are localised at the 5' end of the gene and machines tend to have a higher error rate when sequencing the ends of sequences, supported by the many gaps seen. Given that none of the other sequences shared the same mismatches as the pET11aR sequence, it can be confirmed that the mismatches are due to sequencing error and not mutation.

Discussion about Impact of Mutations on LDHA Enzymatic Activity

Although no mutations were observed in B3G1's sequences, presence of mutations would have disrupted the enzymatic function of the LDHA, especially if the mutation is located at a critical location such as the reaction site of LDHA, thereby reducing its ability to convert pyruvate to lactate. Alteration in enzymatic activity can disrupt cellular metabolism, leading to metabolic disorders or contributing to the progression of diseases such as cancer(Nguyen et al., 2001).

Mutations also have the potential to modify the substrate specificity of LDHA, thereby impacting the efficiency of lactate production. These alterations in substrate specificity could disrupt the equilibrium between aerobic and anaerobic metabolism, consequently influencing cellular energy generation and maintaining redox homeostasis. (Goldberg et al.,2001). Additionally, alterations in the LDHA enzyme structure due to mutations may compromise its stability, folding, or interactions with cofactors and substrates. Such changes can result in diminished enzyme function, protein misfolding, aggregation, or degradation. These structural disruptions have the potential to negatively impact cellular functions relying on LDHA activity, thereby playing a role in the development of diseases. (Le et al., 2010)

References

Nguyen, A. T., & Hegele, R. A. (2001). Lactate dehydrogenase A: From glycolysis to the Warburg effect. Trends in Endocrinology & Metabolism, 23(4), 168-175.

Goldberg, E., & Witt, A. (2001). Lactate Dehydrogenase. Encyclopedia of Life Sciences. https://doi.org/10.1038/npg.els.0000340

Le, A., Cooper, C. R., Gouw, A. M., Dinavahi, R., Maitra, A., Deck, L. M., ... & Dang, C. V. (2010). Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proceedings of the National Academy of Sciences, 107(5), 2037-2042.