

# Optimization of *Lama1* Gene Amplification and Transfection Conditions for Luciferase Assay in Dystonia

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## Abstract

LAMA1 gene is located on chromosome 18 and it encodes alpha subunit of laminin1 a heterotrimeric glycoprotein. It is expressed in different tissues including the muscles and is a crucial component of the basement membrane. Polymerase chain reaction (PCR) of LAMA1 gene identified an annealing temperature of 57°C, magnesium chloride concentration of 2.5 mM and the use of dimethyl sulfoxide and betaine to be the optimum parameters for its amplification. It was also observed that for efficient transformation of pCpGL *Escherichia coli* plasmid zeocin concentrations between 33 µg -100 µg/ml was required. Transfection on *caco-2* cells with Lipofectamine 2000 revealed increasing fluorescence intensity with increasing volumes while slight relative increase in fluorescence intensity compared to the control was detected using 1 mg/ml polyethylenimine.

The aim of the research was to optimise the amplification and transfection conditions of LAMA1 gene from a dystonic patient to enable investigations for luciferase assay. This was achieved by carrying out genomic deoxyribonucleic acid (DNA) extraction from whole blood, plasmid DNA miniprep, polymerase chain reaction (PCR) primer design, PCR, cell culture, molecular cloning in *Escherichia coli* and colorectal adenocarcinoma cells (*caco-2*) using transfecting agents (Lipofectamine 2000 and polyethylenimine) and promoter analysis using luciferase assay.

**Keywords:** Deoxyribonucleic acid (DNA), *Escherichia coli*, LAMA1 gene, Polymerase chain reaction (PCR), and Transfection.

## INTRODUCTION

Dystonia was first described as a neurological movement disorder by Oppenheim (1911) based on his observation of four children with muscle spasms of the trunks and limbs. He noticed twisted postures especially lordosis and scoliosis were aggravated by movement and they gradually progressed to postural deformities with no significant psychological abnormalities, weaknesses, sphincter disturbances or atrophy. Over the years with increasing knowledge on the disease the definition has further been modified to a neurological movement disorder as well as a multifunctional disorder with different clinical manifestations (Defazio & Gigante, 2013) but characterised similarly by intermittent or sustained muscle contractions resulting in abnormal and repetitive patterned, twisting or tremulous movements and postures (Xiao *et al.*, 2014). These muscle contractions are mostly initiated or worsened by voluntary action however, severe forms of dystonia can be initiated by involuntary movements (Albanese *et al.*, 2013; Berardelli *et al.*, 1998).

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The onset of many forms of dystonia is still partially understood. However, there are evidence of its linkage with genetic mutations and about 23 genetic loci have been implicated with the disease (Hersheson *et al.*, 2013; Charlesworth *et al.*, 2013). There has been significant progress in understanding the mechanism of dystonia as candidate genes between the dystonic loci such as *LAMA1* and *TOR1A* have been revealed by the physical map of the national centre for biotechnology information (NCBI). *LAMA1* is expressed in different tissues including the muscles and is a crucial component of the basement membrane (Hohenester and Yurchenco, 2013). *LAMA1* is located on chromosome 18 that encodes the alpha subunit of laminin1, these laminins are glycoproteins with heterotrimeric structures consisting of five alpha( $\alpha$ ) chains, three beta( $\beta$ ) chains and three gamma( $\gamma$ ) chains. Laminins have been shown to interact with the cell surfaces through certain receptors such as integrin, alpha/beta dystroglycan and syndecan to mediate their functions (Gawlik and Durbeej, 2011). Its role in the dystroglycan complex is essential in the maintenance of the structural integrity of the muscle tissues (Grewal and Hewitt, 2003). The mechanism for the alteration in gene expression has been suggested to be due to transcriptional silencing by hyper-methylation of *LAMA1* gene promoter (Kim *et al.*, 2016). DNA methylation has been implicated in several cellular processes as such it plays an important role in development. However, this mechanism also arises stochastically with age (Meng *et al.*, 2015). Identification of the proteins involved in mediating these effects and their distribution provides insights on various disease processes as well as the complexity of the process. The overall aim of the study was to optimize the amplification of *LAMA1* gene and its cloning into a CpG-free plasmid to enable investigation of the effect of methylation and deletion polymorphism.

## **MATERIALS AND METHOD**

### **Experimental design**

The study was conducted using Caco-2 cells purchased from Sigma Aldrich, UK.

### **Mammalian cell culture**

Caco-2 cells were cultured in minimum essential media (MEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 1% Non-essential amino acid (NEAA). The cells were grown in 175 cm<sup>2</sup> surface area flasks at 37°C in an incubator with a humid atmosphere and 5% CO<sub>2</sub>. Cells were grown to attain a 90% confluence after which they were detached using 0.25% trypsin/EDTA and phosphate buffer saline (PBS) solutions and sub-cultured at a sub-culturing ratio of 1:2 weekly.

Pir1 *Escherichia coli* plasmid (pCpGL) a CpG free plasmid was used to clone the target DNA sequence and it was purified using the QIAprep spin miniprep kit according to the manufacturer's protocol.

### **Genomic DNA extraction from whole blood**

DNA from blood sample A and B obtained from dystonic patient containing the information of gene expression was extracted using QIAamp DNA mini kit according to the manufacturer's protocol. The quality and quantity of the extracted DNA was assessed using UV spectrophotometer (ThermoFisher, UK) at an absorbance ratio of 260/280 nm and gel electrophoresis.

### **LAMA1 amplification Polymerase chain reaction (PCR) primer design**

Polymerase chain reaction (PCR) primers were designed using the Primer3 software tool, the software was set up using the following parameters; 20-22 nucleotides long, 63-65°C annealing temperature, 40-60% Guanocine-Cytocine (GC) content and a resulting amplification size of 500-1000 nucleotides. Restriction enzyme sites were added to the

resulting primers with *Pst*II sequence and *Bgl*II sequence on the forward and reverse primers respectively.

**Table 1: Summary of oligonucleotides designed from Primer3 software**

Oligo name	Sequence (5'-3')	Tm (°C)	GCcontent (%)
Primer2 forward	AACTGCAGCGTCCTCTCTCTCCAGCAGT	69.5	57.1
Primer3 forward	AACTGCAGTCCAAGTGACCTTGCCCTTCTT	66.7	48.3
Primer2&3 reverse	GAAGATCTGCCACTCGGIGGGTCTG	67.9	60

### Polymerase chain reaction (PCR)

Reaction master mix was prepared prior to addition of template DNA but some of the parameters varied due to optimization. The cycling protocol for the reactions was as follows: Taq polymerase was made inactive above 72°C, Initial denaturation at 95°C for 7 minutes, denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, extension 72°C, final extension 72°C and storage 4°C.

An excised Agarose gel fragment was weighed and 1:1 volume of binding buffer was added to it. The gel was allowed to dissolve for 10 minutes at 50°C. The solution was then transferred to a spin column (ThermoFisher, UK) and centrifuged for 1 minute. Wash buffer diluted in ethanol was added into the column and then centrifuged for 1 minute. DNA was eluted using 50 µl of elution buffer, 100 ng DNA (target *LAMA1* sequence and plasmid) was mixed with 10X reaction buffer and 10 U of restriction enzymes (*Pst*I and *Bgl*II) respectively. Reaction was incubated for 1 hour at 37°C.

Digested *LAMA1* promoter insert and digested pCpGL vector were combined with 10X ligation buffer and T4 DNA ligase in a total volume of 20 µl. The reaction was allowed and checked for at intervals of 10 minutes, 20 minutes, 30 minutes and overnight at room temperature after which reaction was applied for transformation.

### Transformation of heat shock competent *E. coli* strains

Heat shock competent *E. coli* was generated for admission of plasmid DNA into cells following a specific heat and chill cycle. A 17 hours *E. coli* colony was transferred into 100 ml L-broth and was further incubated in the shaking incubator (Hausser, UK) at 37°C for 3 hours. Bacterial cells were transferred on ice for 10 minutes, centrifuged at 4100 rpm at 4°C for 10 minutes and re-suspended in 30 ml of ice cold MgCl<sub>2</sub>-CaCl<sub>2</sub> solution. Cells were centrifuged at 4100 rpm at 4°C for 10 minutes, re-suspended in 2 ml of ice cold CaCl<sub>2</sub> and then stored at 80°C until needed.

### Transformation of *E. coli* cells

10 µl of ligated DNA was added to 200 µl of competent cells on chilled ice, reaction was then heated at 42°C for 90 seconds and rapidly transferred on ice to cool for 1 minute. 800 µl of L-broth was added to the reaction which was further allowed to incubate at 37°C for 45 minutes. Reaction was plated on L-broth agar containing zeocin antibiotic (25 µg/ml, 33 µg/ml, 50 µg/ml and 100 µg/ml) concentrations and incubated at 37°C overnight.

### Transfection of plasmid DNA using Lipofectamine 2000 transfection reagent

Caco-2 cells were seeded in 24 well plates with 4x10<sup>4</sup> cells per well and were further cultured in complete MEM media (MEM, 2 mM glutamine, 1% Non-essential amino acid (NEAA), 1% penicillin/ streptomycin and 10% FCS) for 24 hours. Plasmid DNA was diluted in 100 µl media (containing no serum or antibiotic) and Lipofectamine 2000 at varying concentrations (0.75, 0.95, 1.15, 1.35, 1.55 and 1.75) µl by vortexing and then reaction was incubated at room temperature for 25 minutes. Growth media from cells was removed and

replaced with 0.5 ml fresh media and 100  $\mu$ l DNA-Lipofectamine complex after which it was gently mixed using the plate shaker (Bio Tex, UK). Cells were incubated at 37°C for 24 hours following treatment.

#### **Transfection of plasmid DNA using Polyethyleneimine (PEI) transfection reagent**

Caco-2 cells were seeded in 24 well plates with  $5.71 \times 10^4$  cells per well and were further cultured in complete MEM media for 24 hours. Plasmid DNA was diluted in 1 ml media (containing no serum or antibiotic) and 105  $\mu$ l of 1 mg/ml PEI by vortexing and then reaction was incubated at room temperature for 10 minutes. Growth media from cells was removed and replaced with fresh media and DNA-PEI complex in a drop wise fashion after which it was gently mixed using the plate shaker. Cells were incubated at 37°C for 24 hours following treatment.

#### **Luciferase reporter assay**

Growth media from transfected cells was removed and cells washed with PBS, lysis reagent was dispensed into each well and allowed to incubate at room temperature for 15 minutes. Afterwards 20  $\mu$ l of cell lysate was transferred into 24 well plates with clear flat bottom and the assay performed with 100  $\mu$ l of luciferase assay reagent. The emitted light was detected using a microplate reader programmed with 30 seconds delay. Results were represented in percentage relative increase.

#### **Statistical analysis of data**

Luminescence intensities of the luciferase reporter assay using transfecting agents PEI and Lipofectamine 2000 were both expressed in percentage increase relative to the control as determined by the equation:

$$\% \text{ increase in luminescence intensity} = [(L_p - L_{\text{basal}}) / L_{\text{basal}}] \times 100$$

Where  $L_p$  is the luminescence intensity in the presence of the plasmid DNA and  $L_{\text{basal}}$  is the luminescence intensity in the absence of any plasmid DNA.

## **RESULTS**

#### **Determining the quality and quantity of genomic DNA (gDNA) extract.**

The level of purity and concentration of the extracted DNA was assessed by measuring the absorbance at ( $OD_{260}:OD_{280}$ ) using a spectrophotometer and visualising them under ultra-violet (UV) light following agarose electrophoresis. The results obtained were 1 and 3 for blood samples A and B respectively.

The absorbance value of blood sample B was greater than that of blood sample A. With reference to the standard DNA absorbance purity at  $OD_{260}:OD_{280}$  ( $\geq 1.8$ ), sample B is of pure quality whereas sample A has some impurities. The presence of impurities such as phenol and proteins which absorb near 280 nm are suspected to be the cause of the decrease.

The quality and quantity of the genomic DNA extract was further verified using agarose gel electrophoresis, the gel was stained with ethidium bromide and visualised under UV illumination.



Plate I: agarose gel electrophoresis depicting gDNA extracts of blood samples A and B.

Lane (1) molecular weight marker in base pairs, lanes (2-3) sample A, lane (4) sterile water and lanes (5-6) sample B.

In both cases, samples showed neat specific bands on the gel, however thicker bands were detected with blood sample B. These indicates that there is more DNA in blood sample B than in blood sample A and the absence of smears indicates that both DNA samples are of good quality as there is no degraded DNA present.

#### **Optimisation of PCR reaction for *LAMA1* gene amplification.**

To optimise the amplification of *LAMA1* gene, different PCR assay parameters such as template DNA concentration, magnesium concentration, primer and annealing temperature were optimised for a series of reactions and the resulting amplicons were detected under UV illumination following agarose gel electrophoresis and ethidium bromide staining of gel.

#### **Conventional PCR assays using primer1 pair**

A series of PCR assays were adapted and optimised using primer1 pair and different reaction parameters including magnesium concentration, annealing temperature and DNA concentration.

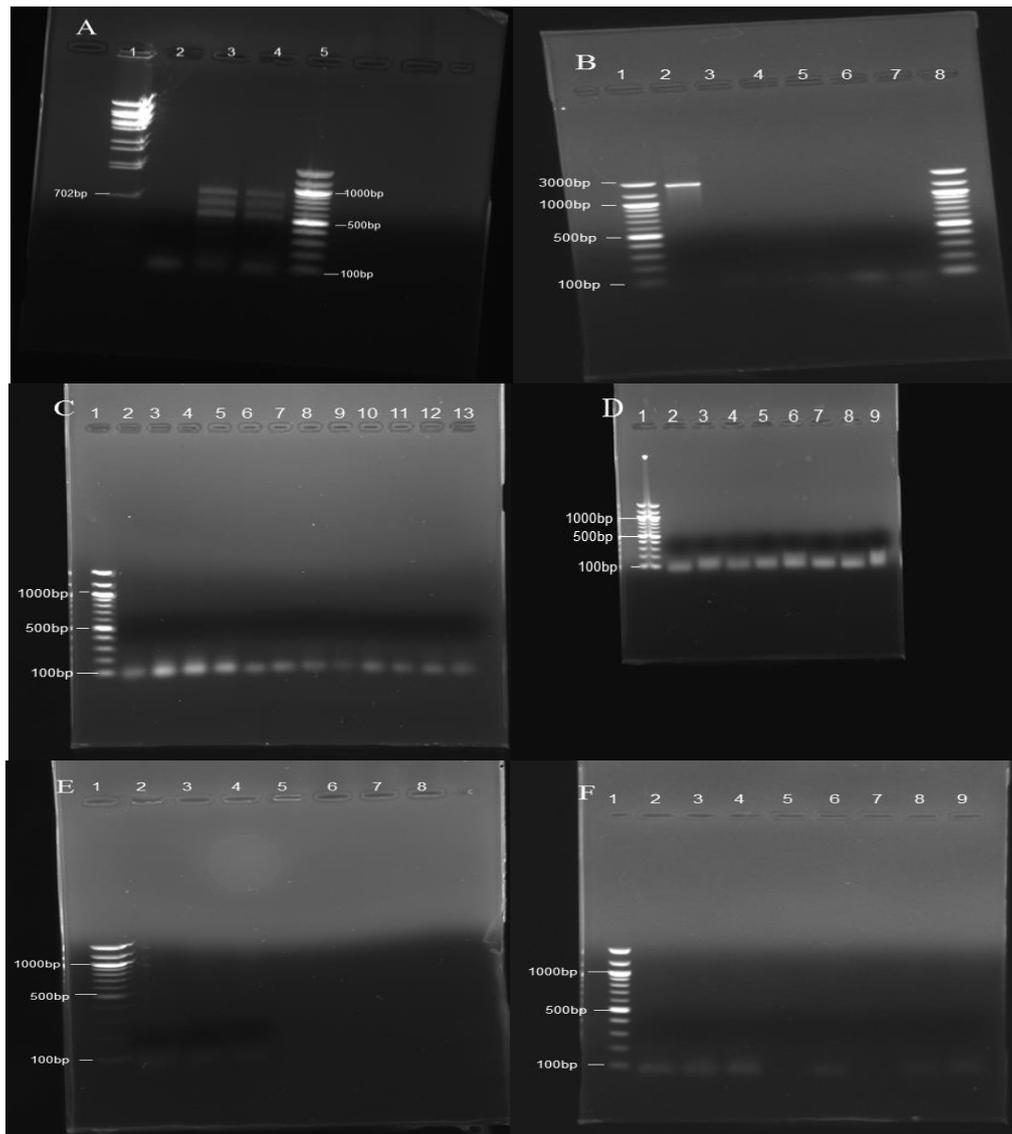


Plate II: agarose gel electrophoresis of PCR amplicons using primer pair 1.

(A) PCR assay at 57°C annealing temperature with lanes (1,5) molecular weight marker in base pairs, lane (2) negative control, lanes (3-4) samples with 2.5 mM MgCl<sub>2</sub>. (B) PCR assay at 57°C annealing temperature with lane (1,8) molecular weight marker, lane (2) positive control, lanes (3-7) samples with gDNA concentrations 50 ng, 80 ng, 100 ng, 200 ng and 300 ng respectively. (C) PCR assay at 57°C annealing temperature with lane (1) molecular weight marker, lane (2-3) negative control, lanes (4-13) samples with two lanes each of 1.5 mM, 2 mM, 2.5 mM, 3 mM and 3.5 mM MgCl<sub>2</sub> respectively. (D) PCR assay at 57°C annealing temperature with lane (1) molecular weight marker, lane (2-3) positive control DNA, lanes (4-9) primers flanking alpha-synuclein housekeeping gene with two lanes each of 1.5 mM, 2 mM and 2.5 mM MgCl<sub>2</sub> respectively. (E) PCR assay at 60°C annealing temperature with lane (1) molecular weight marker, lane (2) negative control and lanes (3-4) samples with 2.5 mM MgCl<sub>2</sub>. (F) PCR assay at 55°C annealing temperature with lane (1) molecular weight marker, lane (2) negative control and lanes (3-4) samples with 2.5 mM MgCl<sub>2</sub>.

PCR amplicons at annealing temperature 57°C as in (A) showed faint non-specific bands between 1000 bp to 500 bp and below 100 bp, the faint non-specific bands could be as a result of various factors including low template DNA concentration, contamination with exogenous DNA and inappropriate MgCl<sub>2</sub> concentration. The bands below 100 bp on the gel

could be due to primer dimer hybridization or hairpin structures that prevent primer annealing; these are suspected because of the high GC content of the primer pair and template sequence. Repeats of the reaction at the same annealing temperature but different template DNA concentrations (**B**), MgCl<sub>2</sub> concentrations (**C**) and primers flanking alpha-synuclein housekeeping gene (**D**) revealed no band on the gel except for faint bands below 100 bp. These results indicate that no amplification occurred except for primer dimer hybridisation or hairpin structures. Change in the annealing temperature to 55°C (**F**) also depicted primer dimer hybridisation or hairpin structures. However, at a higher annealing temperature of 60°C (**E**) very faint almost invisible band below 100 bp was seen. Having adjusted the annealing temperature, magnesium concentration and template DNA concentration other factors that can affect the specificity and sensitivity of the PCR assay includes poor primer specificity, DNA and primer degradation and polymerase enzyme inactivation. In order to check the validity of Taq polymerase and the reaction mix, hot start PCR assay was run with positive control and a master mix.

### PCR assay optimisation using hot start PCR.

Samples were further assayed using hot start PCR as there was no amplification with the conventional PCR assay. This is to eliminate any inadequacies associated with the polymerase enzyme as well as reduce possible self-annealing and formation of secondary structures associated with high GC content primer and template. Reaction master mix containing betaine and dimethyl sulphoxide (DMSO) to enhance annealing of primers to template and hence amplification was utilised.



Plate III: agarose gel electrophoresis of hot start PCR amplicons using primer pair 1 and reaction master mix containing betaine.

Hot start PCR at 57°C annealing temperature lanes (1) molecular weight marker, lane (2) negative control, lane (3) positive control, lanes (4-5) samples with 1.5 mM MgCl<sub>2</sub> and lanes (6-7) samples with 2.5 mM MgCl<sub>2</sub>.

The hot start PCR assay illustrated no amplification band except for the positive control. Having adjusted all other parameters except the primers it is possible that the failure of the reaction may be due to primer denaturation therefore, new primer sets were used with sequences a little farther from the initial primer.

### PCR assay using primers 2 and 3.

Due to amplification failure obtained from the PCR assays, new primers (2 and 3) were designed to amplify *LAMA1* gene. Reaction master mix containing DMSO and betaine was utilised in the reaction to enhance the activity of the primer.

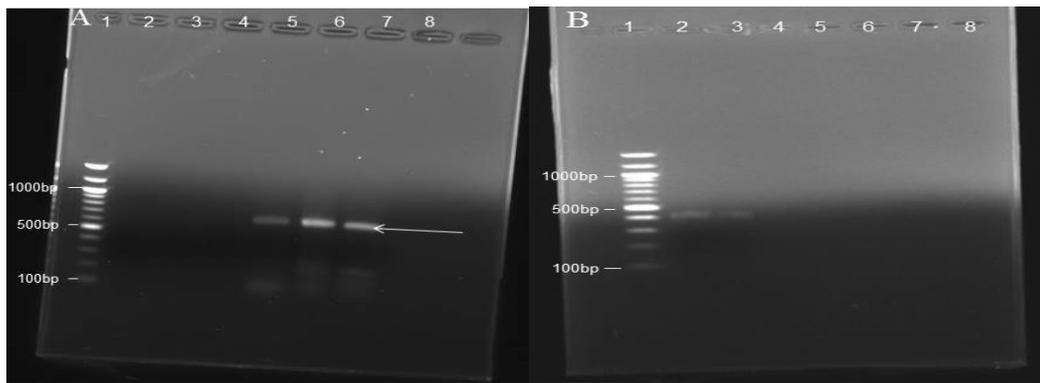


Plate IV: agarose gel electrophoresis of PCR amplicons using primer pair 2 and 3 and reaction master mix containing betaine but with additional 1 mM MgCl<sub>2</sub> in (B). 8 µl PCR amplicons was mixed with 2 µl 5x loading buffer.

(A) PCR assay at 57°C annealing temperature with lane (1) molecular weight marker, lanes (2-4) samples with primer 2, lanes (5-7) samples with primer 3 and lane (8) negative control. (B) PCR assay at 57°C annealing temperature with lane (1) molecular weight marker and lanes (2, 3) samples with primer 3.

Reaction (A) showed no amplification with primer 2 as there were no visible bands. However, clear bands as shown by arrow illustrating amplicons at 570 bp and other faint non-specific bands of lower sizes were seen with primer 3. Primer 3 was further verified in a second reaction (B) which showed corresponding weak bands. Primer 3 based on these results have been able to amplify the gene of interest.

### Molecular cloning of *LAMA1* gene using pCpGL plasmid

The expression vector was prepared and purified using the QIAprep spin miniprep kit and then digested using restriction enzymes *Pst*I and *Bgl*III. The size of pCpGL plasmid was verified using agarose gel electrophoresis.



Plate V: agarose gel electrophoresis of *Pst*I and *Bgl*III restriction enzyme digest of pCpGL plasmid.

Lanes (1, 5) molecular weight markers in base pairs, lanes (2-4) digested pCpGL plasmid. Samples illustrated specific bands with no smears on the gel affirming the presence and quality of pCpGL plasmid.

### Molecular cloning of digested pCpGL and digested *LAMA1* gene.

The pCpGL digest and digested *LAMA1* gene were ligated using DNA T4 ligase and samples were taken after 10 minutes, 20 minutes, 30 minutes and overnight. These samples were transformed on L-agar/zeocin (25 µg/ml) plates and left to culture overnight.

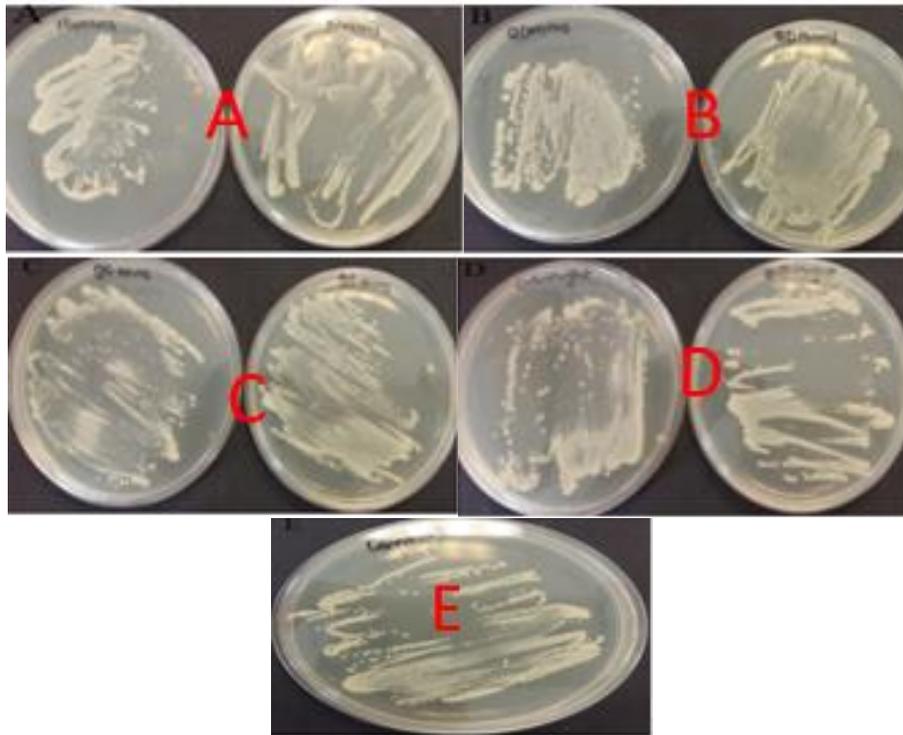


Plate VI: transformation on *E. coli* with varying ligation times.

(A) Transformation with 10 minutes ligation (B) Transformation with 20 minutes ligation (C) Transformation with 30 minutes ligation (D) transformation with overnight ligation and (E) Transformation with competent cells only.

Plates A, B, C and D showed visible colonies and smears alongside the control (E). All cases showed similar culture growth, these could be attributed to either the effectiveness of the antibiotic used or the efficiency of the competent cells. Higher doses of zeocin (33  $\mu\text{g}/\text{ml}$ , 50  $\mu\text{g}/\text{ml}$  and 100  $\mu\text{g}/\text{ml}$ ) were then used to verify the effectiveness of the antibiotic.

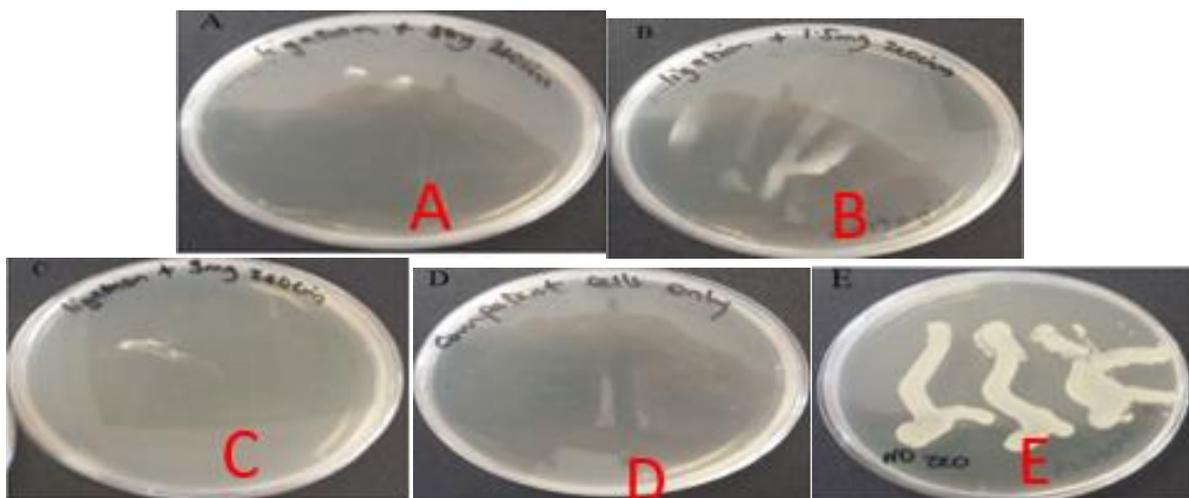


Plate VII: transformation on *E. coli* with varying concentrations of zeocin antibiotic.

(A) Transformation with L-agar/zeocin 33  $\mu\text{g/ml}$  (B) Transformation with L-agar/zeocin 50  $\mu\text{g/ml}$  (C) Transformation with L-agar/zeocin 100  $\mu\text{g/ml}$  (D) Transformation using competent cells only on L-agar/zeocin 100  $\mu\text{g/ml}$  (E) Transformation with no zeocin on L-agar plate.

There were lesser smears with higher concentrations of zeocin as compared to the lower concentration (25  $\mu\text{g/ml}$ ) and the control (E), these concentrations also proved to be more effective as the control (D) showed no visible colony or smear.

Using colonies from the transformed plates, a colony PCR assay was set-up to amplify cells with the plasmid. Bacterial cultures using L-broth/zeocin (33  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$ ) was also incubated for 16-20 hours at 37°C to further grow cells with plasmids. PCR amplicons and miniprep of cultured cells were run on agarose gel to verify the presence of plasmids.

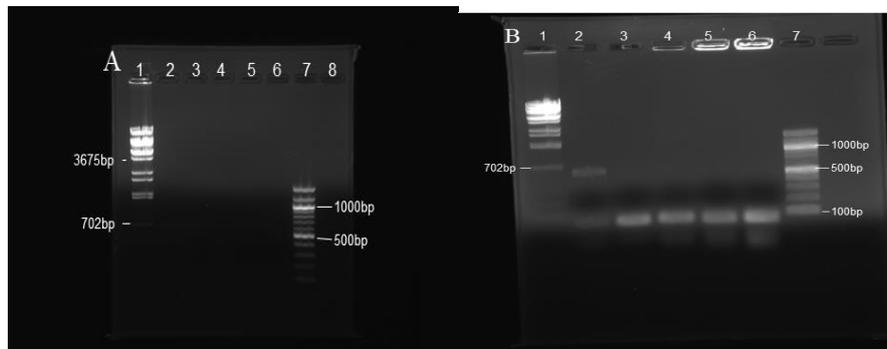


Plate VIII: agarose gel electrophoresis of transformed cells.

(A) Miniprep cells following incubation showing lanes (1, 7) molecular weight markers in base pairs, lane (2) negative control, lanes (3-6) transformed *E. coli* cells. (B) Colony PCR amplicons of transformed *E. coli* cells showing lanes (1,7) molecular weight markers in base pairs, lane (2) positive *LAMA1* control and lanes (3-6) colony PCR amplicons.

There were no visible bands on both images except for the positive control and non-specific bands below 100 bp on (B) which may be due to primer dimer hybridisation. These images indicate that there were no plasmids in the transformed cells.

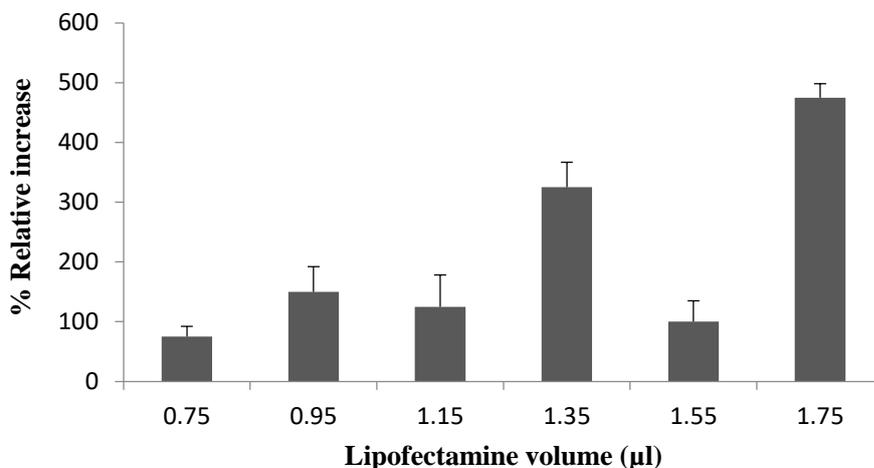


Figure 1: Transfection of caco-2 cells with varying volumes of Lipofectamine 2000.

There was a pattern of increasing transfection efficacy with increasing volume of Lipofectamine 2000. However, there were deviations with Lipofectamine volumes of 1.15  $\mu$ l and 1.55  $\mu$ l. At the lowest volume used 70% luminescence increase relative to the control was attained indicating that Lipofectamine 2000 even at that concentration is highly effective in mediating the transfer of DNA on caco-2 cells.

The concentration of PEI used had 6.7% increased effect, relative to the control (no plasmid DNA) indicating that the volume of PEI used only slightly mediated the transfer of pCpGL plasmid in caco-2 cells.

## DISCUSSION

Determination of the purity and concentration of the extracted nucleic acid is the foremost practise of studies in good gene analysis as not only does it enable reliable and reproducible results by decreasing the chances for erroneous data, it also determines the working volume of sample and confirms the suitability of the sample for further analysis. Low DNA concentration in samples can result to significantly low amplification yields in PCR while very high yields can affect polymerase enzyme activity (Schade, 2014). The purity and concentration of nucleic acids are determined by absorbance values of 260 nm / 280 nm ratio  $\geq 1.8$  for DNA and  $\geq 2$  for RNA using UV spectrophotometry. Other methods include agarose gel electrophoresis and fluorescent DNA binding dyes (Wahlberg *et al.*, 2012). In this study, the extracted DNA samples A and B yielded absorbance values 1 and 3 respectively, the lesser value obtained from sample A indicates presence of some impurities. These impurities could be as a result of the presence of proteins, chaotropic salts or phenol amongst other contaminants that absorb light at about 280 nm (Boesenberg-Smith *et al.*, 2012). The pH of a sample is also known to interfere with the absorbance values as acids and bases are reported to lower and increase UV absorbance values respectively (Haque *et al.*, 2003).

Agarose gel electrophoresis of both samples revealed specific bands but sample B had a stronger DNA intensity. This indicates that sample B has a higher concentration of DNA than A. However, both samples yielded specific bands with no lower molecular weight smears which often results from DNA degradation suggesting that the extracted DNA is of good quality (Desjardin and Conklin, 2010).

To obtain amplicons with high specificity and precision, it is imperative to optimise PCR parameters as genes respond differently to the reaction parameters. PCR amplification of genes with high GC content is an established problem as it has been associated with formation of secondary structures, self-annealing, mismatch and high annealing temperatures (Frey *et al.*, 2008). To amplify *LAMA1* gene promoter region which has the aforementioned characteristics, it was imperative to optimise the PCR assay parameters for high specificity and efficiency. Using the genomic DNA, different PCR parameters including primers, annealing temperatures, magnesium concentrations and template concentrations were optimised to obtain *LAMA1* amplicons with high yield, specificity and precision for further downstream analysis.

Conventional PCR assay with different PCR parameters using primer1 pair yielded no positive outcome (see Plate II) except for non-specific bands on gel A which could not be reproduced and faint bands below 100 bp. The bands below 100 bp are either as a result of primer dimer hybridisation or formation of hairpin structures whose resulting effect is a decrease in the availability and binding capacity of the primers. Based on the non-specific band obtained at 57°C annealing temperature, a higher temperature was used as it has been reported that non-specific bands become more specific with increasing temperatures (Kim *et*

*al.*, 2008) but it yielded no amplification. Adjustments with varying concentrations of magnesium and template DNA had no positive impact on the reactions as no amplification was seen across all concentrations.

To validate the reaction, reagents as well as eliminate primer dimer or hairpin formation, hot start PCR using master mix containing DMSO and betaine known to increase amplification efficiency and increase the chances of reducing and breaking the secondary structures (Hensen and Justesen, 2006) was employed. The hot start PCR however yielded no amplification but the primer dimer or hairpin structures were eliminated, this is in concordance with reports by Roux (2009). This reaction validates the PCR protocol and the reagents used but the result questioned the integrity of the primer.

The new primers (primer 2 and 3) were designed with the following factors in consideration: GC content, self-complementarity and complementarity at the 3' termini of the primer which have been reported to promote primer dimer hybridisation (Kalender *et al.*, 2009). Specific bands of the required product size were obtained with primer 3 at 57°C annealing temperature and 2.5 mM MgCl<sub>2</sub> concentration (see Plate IV) confirming that primer 1 had been denatured. A repeat of the assay using primer 3 with a higher concentration of MgCl<sub>2</sub> showed specific weak bands this result confirms that for *LAMA1* amplification 57°C annealing temperature and 2.5 mM MgCl<sub>2</sub> concentration are the optimum parameters. The weak bands obtained could be as a result of the magnesium concentration being too high for the reaction (Innis *et al.*, 2012). Studies have shown that clear specific bands become weaker with increasing concentrations due to the ability of magnesium to complex with DNA thus altering the reaction's annealing temperature, DNA and primer dissociation temperature and stability (Owczarzy *et al.*, 2008). Changes in magnesium chloride concentration has been reported to be an important parameter not only for band specificity but also polymerase enzyme's fidelity as it plays a catalytic role and serves as a co-factor for polymerase enzyme activity (Batra *et al.*, 2006).

To increase the chances of a successful cloning a crucial factor to consider is the cloning vector, this has to be free from contaminants and fully digested to increase the efficiency of ligation. In this study, this was determined by using agarose gel electrophoresis. Clear bands of the expected size were obtained and the bands were also thin indicating that the vector DNA is linear as opposed to the circular shape of bacterial DNA due to the digestion. Longer ligation duration has been reported to yield more colonies than ligation of shorter duration, other factors that affect ligation includes addition of condensing agents such as polyethylene glycol which induces macromolecular crowding by mimicking higher DNA concentrations in the reaction (Lund *et al.*, 1996). Similar images were obtained at the different ligation durations when they were transformed on L-agar/zeocin plates, the control which had competent cells only also yielded similar image. This could be as a result of inadequate antibiotic concentration and degradation, contamination or low efficiency of competent cells (Das and Dash, 2014).

Varying concentrations of zeocin were used to rule out the possibility of inadequate antibiotic concentration and degradation (see Plate VII). Results showed less smears at higher concentrations indicating that the zeocin antibiotic concentration used initially was inadequate which is in concordance with report by Sharma *et al.* (2014). The other control (no zeocin antibiotic) had thick visible smears showing clearly that the bacteria with or without the resistance gene survived. The absence of bands on gel from colony PCR and mini-prepped yield from L-broth culturing indicates that the transformation was not successful. Possible reasons for transformation failure include inefficient ligation due to

DNA damage by UV light during gel extraction process (Grundemann and Schomig, 1996) and inefficient competent cells.

Factors that affect transfection efficiency includes frequency of passaging of cells, presence of an antibiotic, presence of serum in the media, strength of the promoter as well as the type of plasmid used (Fang and Shen, 2010). 1 mg/ml PEI at 105 µl yielded slight increase relative to the control, other investigations have shown higher yields with increasing PEI concentrations. Transfection with PEI can be affected by factors such as sample pH which has been reported to affect PEI-DNA complex formation (Utsuno and Uludag, 2010). Lower sample pH has been linked with higher intensities hence increasing complex formation. The structure and molecular mass of the PEI used also plays a crucial role in PEI transfection as higher molecular mass has been associated with increased transfection efficacy and high cytotoxicity and vice versa, this however has been optimised by crosslinking and grafting the low molecular weight PEI (Jere *et al.*, 2009).

Studies have shown up to 70%-90% higher fluorescence intensity with 1 µl of Lipofectamine 2000 which further increased with increasing volume however, a declining effect was observed with 4 µl which was reported to be as a result of cell toxicity (Karra and Dahm, 2010).

#### **CONCLUSION**

To enable specificity of *LAMA1* gene and reproducibility of results, it is essential to optimize the parameters for amplification and transfection.

#### **RECOMMENDATION**

Further studies should be conducted to investigate the mechanism of *LAMA1* gene in dystonia.

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