2.3.7.2 Transformation of Competent DH5α

An aliquot of competent DH5α in glycerol was removed from storage at -80 ºC and thawed on ice. pGL3 luc plasmid (~10 ng) was added to the eppendorf (in less than 10 µl) and mixed gently with the pipette tip to avoid shearing the pDNA. This aliquot was incubated on ice for 30 min, then at 42 ºC for 1 min before returning to ice for 2 min (heat shock). LB medium (800 µl) was added and the eppendorf incubated for 45 min at 37 ºC with shaking at 150 rpm. This culture was then pour plated onto an LB agar plate containing ampicillin (100 µg/ml).

2.3.7.3 Amplification of Transformed DH5α

Amplification of DH5α was performed according to the QIAGEN® plasmid purification method (Qiagen, 2000) and all buffers used are included in the Megaprep plasmid purification kit. A single colony from the ampicillin selection plate was picked with an inoculation loop and inoculated into 10 ml of LB medium containing ampicillin (100 µg/ml) and incubated for 8 h at 37 ºC under shaking at 220 rpm. All of this starter culture was inoculated into 500 ml of LB medium in a 1L aerated flask and grown overnight (16 h) at 37 ºC under shaking at 220 rpm.

2.3.7.4 Isolation and Purification of the pGL3 luc Plasmid

Transformed DH5α (prepared as described above) were pelleted by centrifugation at 6000 RCF for 15 min at 4 ºC. A QIAfilter Mega-Giga cartridge was attached to a 1 L Pyrex bottle. The bacterial pellet was re-suspended thoroughly using a pipette in a total volume of 50 ml of the buffer P1 (50 mM Tris.Cl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and transferred to a 500 ml bottle. To this, 50 ml of the buffer P2 (200 mM NaOH, 1 % SDS (w/v)) was added and mixed thoroughly by inversion of the bottle 4-6 times. This solution was incubated at room temp for 5 min. Neutralisation of pH was achieved by addition of 50 ml of chilled buffer P3 (3.0 M potassium acetate pH 5.5) and the solution was mixed by inversion 4-6 times. A white precipitate formed.

The lysate was transferred to the QIAfilter Mega-Giga cartridge prepared at the start and incubated at room temperature for 10 min to allow the protein precipitate to float on top of the rest of the solution. The precipitate was removed by vacuum filtration and the solution collected. The vacuum was stopped and 50 ml of buffer FWB (750 mM NaCl, 50 mM 4-Morpholinepropanesulfonic acid (MOPS) pH 7.0, 15 % isopropanol (v/v))

was added with gentle stirring of the white precipitate. Then the vacuum reapplied and the solution was combined with the previous filtrate. A sample (120 µl) of the cleared lysate was removed and stored for analysis.

The buffer ER (12.5 ml) was then added to the filtered lysate and mixed by inversion 10 times before a 30 min incubation on ice. A QIAGEN-tip 2500 was equilibrated with 35 ml of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15 % isopropanol (v/v), 0.15 % triton X-100) with flow being gravity driven and flow stopping when the meniscus reached the top of the column. The filtered lysate was then added to the column. The QIAGEN-tip was washed with 200 ml of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15 % isopropanol (v/v)) and a 160 µl sample of the eluent was taken for analysis.

DNA was then eluted from the column with 35 ml of buffer QN (1.6 M NaCl, 50 mM MOPS pH 7.0, 15 % isopropanol). A sample of the eluent (22 µl) was taken for analysis. The DNA was precipitated from the eluent solution by addition of isopropanol (26 ml). The centrifuge tube was marked on the back to indicate the expected position of the pellet and the solution centrifuged at 15000 RCF for 30 min at 4 ºC. The supernatant was carefully decanted to avoid loss of the DNA pellet. Ethanol (70 % v/v) was used to wash the pellet and then re-centrifuged at 15000 RCF for 10 min at 4 ºC. The ethanol was decanted and the pellet air dried for 10 min. The dried pellet was re-dissolved in 2 ml of buffer TE (10 mM Tris.Cl pH 8.0, 1 mM EDTA).

2.3.7.5 Quantification of pGL3 luc

Quantification of plasmid concentration was made using both UV absorbance and quantification of band intensity in agarose gel. The UV absorbance (280 nm) of a 50 µg/ml solution of pDNA has an absorbance of 1. Therefore, by measuring the UV absorbance (280 nm) of the isolated pGL3 luc (as described above), it was possible to calculate the concentration in a sample of plasmid.

For the agarose gel characterisation a known concentration of pDNA was loaded onto a lane and the band intensity of the sample compared, intensity was measured with ImageQuant (Amersham, UK). Agarose gel electrophoresis also confirms the identity of the plasmid through the comparison of restriction digests of the stock and plasmid preparation. As can be seen in Fig. 2.6 the retention factor (rf) of the complete plasmid and EcoR1/BamH1 restriction digest fragments are the same in the stock (lanes 3 and 4) and plasmid preparation (lanes 5 and 6) confirming the plasmid production.