CHAPTER 4: OPTIMISATION OF TRANSFECTION CONDITIONS FOR MA104, COS-7, BSR AND HEK293H CULTURED CELLS USING eGFP

4

4.1 Introduction

Eukaryotic cells are able to take up and express foreign, external DNA with very low efficiency under normal physiological conditions (Rauth and Kucherlapatki, 1984). This inefficiency can be attributed to the cell membrane's lipid bi-layer of most mammalian cells. In order to introduce foreign DNA in the mammalian cell of interest, this barrier must first be overcome. Numerous transfection methods and reagents have been developed and commercialised to transport charged molecules across this eukaryotic cell membrane. Transfection techniques vary greatly and may include chemical and lipid transfections or physical procedures such as electroporation. Unfortunately, not all transfection methods can be applied to the entire spectrum of cellular systems. A successful transfection depends on 3 factors, namely: acceptable transfection efficiency, low cytotoxicity and reproducibility (Karra and Dahm, 2010).

Due to the fact that a large number of rotavirus strains tend to grow well in the African green monkey kidney cell line, MA104, transfection experiments with this cell system would be ideal. However, MA104 cells are notoriously difficult to transfect (Richards et al., 2013, Taraporewala et al., 2006). The broad aim of the study is to establish a reverse genetics system for rotavirus. Successful and efficient transfection of rotavirus-derived nucleic acids into specific mammalian cell lines will be imperative to the success of both transcript- and plasmid only based systems. Four different transfection reagents, FuGENE® 6 (Roche), X-tremeGENE 9 (Roche), X-tremeGENE HP (Roche), and Lipofectamine ® 2000 (Life Technologies) were considered.

The FuGENE® 6 transfection reagent (Roche) is widely used to transfect a variety of mammalian cells with a wide range of xenobiotics (Chen et al., 2007, MacCorkle et al., 1998). FuGENE® 6 is a multi-factor, lipid-based delivery system which is able to form a complex with DNA and transport it over the lipid bi-layer into mammalian cells. This transfection reagent has a high transfection efficiency in HeLa, COS-1, COS-7 and CHO cells

with a low cytotoxic effect (Roche, 2006). X-tremeGENE 9 is part of a new transfection reagent range from Roche. This transfection reagent is a non-liposomal, multi-component reagent with a low cytotoxicity and possesses the capability to provide high transfection efficiency in a wide range cell lines (Roche, 2011a). The X-tremeGENE HP transfection reagent is a mixture of lipids and other components provided in 80% ethanol. X-tremeGENE HP is the high performance equivalent of X-tremeGENE 9 and is designed to transfect a wide range of mammalian cells with very low cytotoxicity (Roche, 2011b). Lipofectamine ® 2000 reagent is often used for transfecting DNA and RNA into eukaryotic cells, and is a multi-component liposome formulation consisting of polycationic lipid 2, 3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N, N-dimethyl-1- propanaminium trifluoroacetate and the neutral lipid dioleoyl phosphatidylethanolamine. This transfection reagent is popular for transfecting shRNA and siRNA used in gene knockdown studies (InvitroGen, 2002).

In this chapter, the transfection efficiency of four different transfection reagents, in a variety of mammalian cell lines, is described. This is done to identify the most effective transfection agent for the development of a rotavirus reverse genetics system. Four mammalian cell lines, which supports rotavirus replication (MA104, COS-7, BSR and HEK 293H), where chosen to be transfected with four distinct transfection reagents, FuGENE® 6 (Roche), X-tremeGENE 9 (Roche), X-tremeGENE HP (Roche), and Lipofectamine ® 2000 (Life Technologies). The aim of this chapter is to evaluate the four transfection reagents based on their (1) transfection efficiency, (2) cytotoxicity and (3) reproducibility in order to determine which transfection reagent, and cell line, are best suited for rotavirus transcript- and plasmid transfections.

The transfections were performed in four mammalian cell lines, MA104, COS-7, BSR and HEK 293H. The MA104, COS-7 and HEK 293H lines were chosen because of their availability and the fact that all are susceptible to rotavirus SA11/Wa infection and can sustain rotavirus replication (Ji et al., 2002, Londrigan et al., 2000, Troupin et al., 2010). Rotaviruses Wa and SA11 were adapted to BSR cells. MA104 cells are ideal for rotavirus propagation, but have very poor transfection efficiency. On the other hand, COS-7, BSR and HEK 293H cells have very high transfection efficiency, but rotavirus proliferation is poor and not nearly comparable to that in MA104 cells (Ciarlet et al., 2001, Ward et al., 1984).

4.2 Materials and Methods

4.2.1 Evaluation of chemical based transfection reagents

4.2.1.1 Cultured cells and eGFP encoding plasmids

MA104 (passage 25 or #25), BSR (#10) and HEK 293H (#9) cell lines were used for transfection optimisation and were kindly provided by Prof. A. C. Potgieter, Deltamune, South Africa. COS-7 (#30) cells were also utilised and were a gift from Dr. S. Prince, Department of Human Biology, University of Cape Town, South Africa. Cells were cultured in Dulbecco's modified essential medium (D-MEM; Hyclone) containing 10% FBS (Hyclone), 1% penicillin/streptomycin/amphotericin B (Gibco) and 1% non-essential amino acids (Lonza). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. The peGFP-N1 vector (Clonetech) was used for expressing eGFP (see ***Appendix C*** for plasmid map). JM109 competent cells (Clontech) were used to amplify the peGFP-N1 plasmids. Cultures were grown to a cell density of approximately 4 x 10^9 cells per milliiter, which corresponds to a cell pellet weight of approximately 3 g/L LB medium. The QIAGEN plasmid purification kit was used for plasmid extraction according to the manufacturer's instructions.

4.2.1.2 Transfection procedures of the different chemical transfection reagents

Initial transfections were performed as described by the specific manufacturer. The expression of eGFP was used to determine the efficacy of transfection reagents to successfully transfect cells. This was perfomed by estimating the total amount of cells expressing eGFP. After extensive optimization the following transfection procedures were utilised for each transfection reagent:

Optimized FuGENE® 6 transfection procedure

Cultured cells were propagated in D-MEM medium containing 10% foetal bovine serum and nonessential amino acids (Lonza) with antibiotics to reach a confluency of between 70 – 90%. The FuGENE® 6 transfection mixture consisted of 97 µl serum free D-MEM, 2 µl FuGENE® 6 transfection reagents and 1 µg eGFP containing plasmid DNA (2:1 Transfection reagent to DNA concentration ratio). This mixture was then incubated for 30-60 minutes at room temperature, as prescribed by the manufacturer. Cells were thoroughly washed with phosphate-buffered saline (PBS, SIGMA) to remove excess foetal bovine serum (FBS)

containing media. Failure to remove most of the FBS containing media impacted negatively in subsequent plasmid derived eGFP expression. The transfection mixture (100 µl) was applied in a drop wise manner to the appropriate wells (24 well plate; Nunc™). The wells were gently swirled to ensure even distribution over the plate surface. Plates were centrifuged at 300 rpm for 1 minute to assist with the absorption. Plates were incubated for 10 minutes followed by the addition of 100 µl serum free media to ensure that the cells were completely covered and did not dry out during incubation. At least a total of 180 µl of media was needed to cover all cells in a single well of a Nunc™ 24 well plate. Cells were incubated for 5 hours at 37°C to allow time for absorption. According to the manufacturer's instructions, the FuGENE® 6 reaction mixture had to be removed after 5 hours of incubation and replaced with 1 ml of DMEM (containing 10% FBS) and these directions were followed.

Optimised X-tremeGENE 9 and X-tremeGENE HP transfection procedure

Cultured cells were propagated in D-MEM medium containing 10% foetal bovine serum and nonessential amino acids (Lonza) with antibiotics to reach a confluency of between 70 – 90%. The transfection protocol for X-tremeGENE 9 and X-tremeGENE HP is identical and will be discussed simultaneously. The optimal transfection mixture for a 24-well plate consisted of 97 µl serum free media, 2 µl X-tremeGENE transfection reagent and 1 µg of eGFP containing plasmid DNA. This mixture was incubated for 15-30 minutes at room temperature as prescribed by the manufacturer. Prolonging the incubation time to 1 hour, considerably increased plasmid based eGFP expression. Cells were washed with PBS to remove excess foetal bovine serum containing media. According to the manufacturer, removal of all the serum containing media is not necessary. Even though the impact of serum on the transfection efficiency was much less than when compared to the FuGENE 6 reagent, it still influenced the success of eGFP expression. It is advised to wash the cells at least once with PBS to ensure optimal plasmid uptake. The transfection mixture was applied to the appropriate wells in a drop wise manor. The wells were gently swirled to ensure even distribution over the entire plate surface. Plates were incubated for 10 minutes followed by the addition of 100 µl serum free media to assure that the cells were completely covered and did not dry out during incubation. The removal of the XtremeGENE reaction mixtures are not necessary and were left on the cells for the duration of the experiments. Not removing the transfection cocktail greatly improved plasmid derived eGFP expression.

Optimised Lipofectamine ® 2000 transfection procedure

Cultured cells were propagated in D-MEM medium containing 10% foetal bovine serum and nonessential amino acids (Lonza) without antibiotics to reach 70% confluence. Transfection complexes were prepared by diluting 1 µg DNA in 50 µl serum free D-MEM. Another complex was prepared by mixing 2 µl Lipofectamine ® 2000 reagent in 50 µl serum free D-MEM. The two mixtures were combined to a total volume 100 µl and incubated at room temperature for 30 minutes. 200 µl of this complex was then added to each well and incubated for 6 hours at 37°C in a CO_2 incubator. After 6 hours, 200 µl of serum containing media (10% FBS) was added to the mixture and incubated further. Failure to add foetal bovine serum containing media lead to mass cell death.

4.2.1.3 Preparation of transcriptionally active rotavirus SA11 double-layered particles

Rotavirus SA11 was propagated in bulk by incubating rotavirus-infected MA104 cells in D-MEM containing 1 µg/ml porcine trypsin IX (Sigma) and supplemented with 1% non-essential amino acids (Gibco) and 1% penicillin/streptomycin/amphotericin (Lonza). Infected cultures were harvested when the cytopathic effect (CPE) reached ~80%. Cells were harvested by freeze-thawing and the cellular component was separated from the culture medium by centrifugation at 3000 rpm for 5 minutes at 4 °C. The remaining supernatant was ultra-centrifuged at 30 000 rpm, using a TH641 rotor in a Sorvall ultracentrifuge at 4 °C, for 1 hour. The two pellets were pooled and resuspended in 8 ml of 10 mM Tris/HCl buffered saline (TBS), pH 7.5. After resuspension, 3.2 ml Vertrel® (DuPont) was added and the mixture homogenised. The homogenates were centrifuged at 2000 rpm at 4 °C for 5 minutes and pooled. The rotavirus outer capsid (VP4 and VP7) was removed with 10 mM EDTA and incubated at 37 °C for 1 hour (Estes et al., 1979) followed by pelleting the double-layered particles (DLPs) by ultra-centrifugation in a TH641 rotor at 31 200 rpm for 1.5 hours. The resulting pellet was resuspended in TBS and purified by CsCl (Sigma) gradient ultra-centrifugation at 35 000 rpm for 16 hours at 15 °C with a TH641 rotor. The band containing the DLPs was extracted using a syringe and the remaining CsCl removed by dialysis in TBS overnight. An aliquot of the purified DLPs was analysed with a 10% SDS-PAGE gel and staining with Coomasie Brilliant Blue (Merck) to confirm that DLPs had been obtained.

4.2.1.4 In vitro transcription of the rotavirus SA11 genome segments using double-layered particles

For the *in vitro* transcription of rotavirus SA11 genome segments, a transcription mixture was prepared containing 100 mM Tris/HCl (pH 8.0), 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT (Roche), 5 mM rATP (Promega), rCTP, rGTP, rUTP, at 2.5 mM each, 1 mM *S*-Adenosyl methionine (SAM) (Sigma), 6% PEG6000 and 0.4 U/µl RNasin® Plus (Promega) (Mason et al., 1980). The transcription reaction was performed at 40 °C for 6 h. Total RNA was extracted from the transcription reaction using TRI-reagent LS (Molecular Research Centre), following a standard RNA extraction protocol (Jere et al., 2011). Genomic dsRNA was removed by precipitating ssRNA with 2 M LiCl at 4 °C for 16 hours followed by centrifugation at 13 100 rpm for 30 minutes at 4 °C. The mRNA pellet was washed with 70% ethanol and dissolved in nuclease-free water (Promega) containing 0.5 U/µl RNasin® Plus. A ND-1000 spectrophotometer (NanoDrop Products, Wilmington, USA) reading was taken to access the purity of the transcripts.

4.2.1.5 Transfection and expression determination of rotavirus SA11 transcripts

Roughly 16 hours before transfection, BSR, COS-7, HEK 293H and MA104 cells were seeded in 24-well plates (Nunc™). At approximately 70–90% confluence, the cells were transfected using the XtremeGENE HP transfection reagent (Roche) as described in ***section 4.2.1.2***. After 24 hours the transfection with rotavirus SA11 transcripts (from DLPs), cells were fixed with 300 µl, 4% paraformaldehyde (Merck). Cells were then permeabilized by 300 µl, 0.25% Triton X-100 (Merck). The HRP/DAB detection kit (Abcam) was used to prepare cells for rotavirus VP6 immunostaining. Hydrogen peroxide (40 µl) was added to each well and incubated for 30 min at room temperature, rocking gently, to block endogene peroxidase. An Abcam protein blocker (40 µl) was added to the cells and incubated for 30 min at room temperature, rocking gently. The primary antibody (goat polyclonal to rotavirus NCDV (Biotin) - Abcam) was diluted 1:200 in PBS and 300 µl added to each well and incubate at room temperature for 2 hours. After incubation, cells were thoroughly washed with PBS. The secondary antibody (Donkey polyclonal Secondary Antibody to Goat IgG - H&L (HRP) - Abcam) was diluted 1:400 and 400 µl added to each well and incubated for 1 hour followed

by a PBS wash. To visualize the secondary antibody, 20µl of DAB chromagen solution (Abcam) was mixed with 1 ml of the DAB substrate and 300 µl added to each well. eGFP was used as an expression control. The immunostained cells were then visualised with an Eclipse TE2000-S microscope (Nikon) and images were captured using the NIS-Elements (2.30) software (Nikon).

4.2.2 Evaluation of the effectiveness of electroporation in four different mammalian cell lines using eGFP

The potential use of electroporation in the development of a rotavirus reverse genetics system was evaluated in four mammalian cell lines (MA104, COS-7, BSR and HEK 293H). Electroporation is a widely utilized method of cell membrane permeabilization in order to deliver specific DNA, siRNA, proteins or drugs into a variety of cells.

Cells were electroporated in the presence of 1 µg eGFP plasmid using the GenePulser Xcell (Biorad). Cells were grown to confluence in a 25 cm^2 flask (Nunc^{TM}). Cultured cells were harvested with 10µg/ml trypsin-EDTA (Sigma). The trypsin was neutralised by 10% FBS containing D-MEM, pelleted by centrifugation and thoroughly washed with PBS to remove all traces of FBS. Cells were resuspended in PBS and ~ 1 x 10^6 cells were placed into a 0.1 cm GenePulser cuvette (Biorad). Pre-programmed protocols with cell specific parameters were used for each mammalian cell line (***Table 4.1***).

Table 4.1: Electroporation parameters for the different mammalian cell lines

	MA104	**COS-7**	**BSR**	**HEK 293H**
Voltage (V)	110	110	140	110
Pulse length (ms)	20	20	25	25
Number of pulses	1	1	1	1

After the pulse, cells were seeded in a 6 well plate (Nunc^{TM}) in D-MEM containing 10% FBS. eGFP expression was noted every 6 hours using a fluorescent light microscope and cell viability was determined after 24 hours by trypan blue (Fluka) staining 48 hours after transfection. A total of 200 cells were counted of each cell line using a haemocytometer and the cell viability calculated. All experiments were at least performed in triplicate.

4.3 Results and discussion

The efficient transfection of both rotavirus transcript and rotavirus expression plasmids will be crucial to the success of a reverse genetics system. In this chapter, different cell lines and transfection reagents are examined to determine the best transfection conditions.

4.3.1 Determination of the best transfection reagent to DNA ratio

In order to determine the most efficient transfection reagent: DNA ratio of FuGENE® 6 (Roche), X-tremeGENE 9 (Roche), X-tremeGENE HP (Roche), and Lipofectamine ® 2000 (Life Technologies), cells were seeded in a 24 (2 cm^2) well plate. Four different cell lines (MA104, COS-7, BSR and HEK 293H) were transfected with each transfection reagent as indicated in ***section 4.2.1***. Different transfection reagent to [DNA] ratios were tested with the intention of determining the ratio best suited for our needs. Cell lines were transfected with 1 µg of eGFP and incubated for 24 hours. In general, it is clear from these experiments that a higher transfection reagent to DNA concentration ratio was more effective in expressing larger amounts of the eGFP plasmids in cells (***Figure 4.1***).

The FuGENE® 6 transfection reagent (Roche) is a multi-component, lipid-based delivery systems. It is evident that this transfection reagent is relatively ineffective at low transfection reagent to DNA concentration ratio (***Figure 4.1A***). The 6:1 ratio is effective in most cell types tested, but due to high transfection reagent concentration, this transfection method is very expensive.

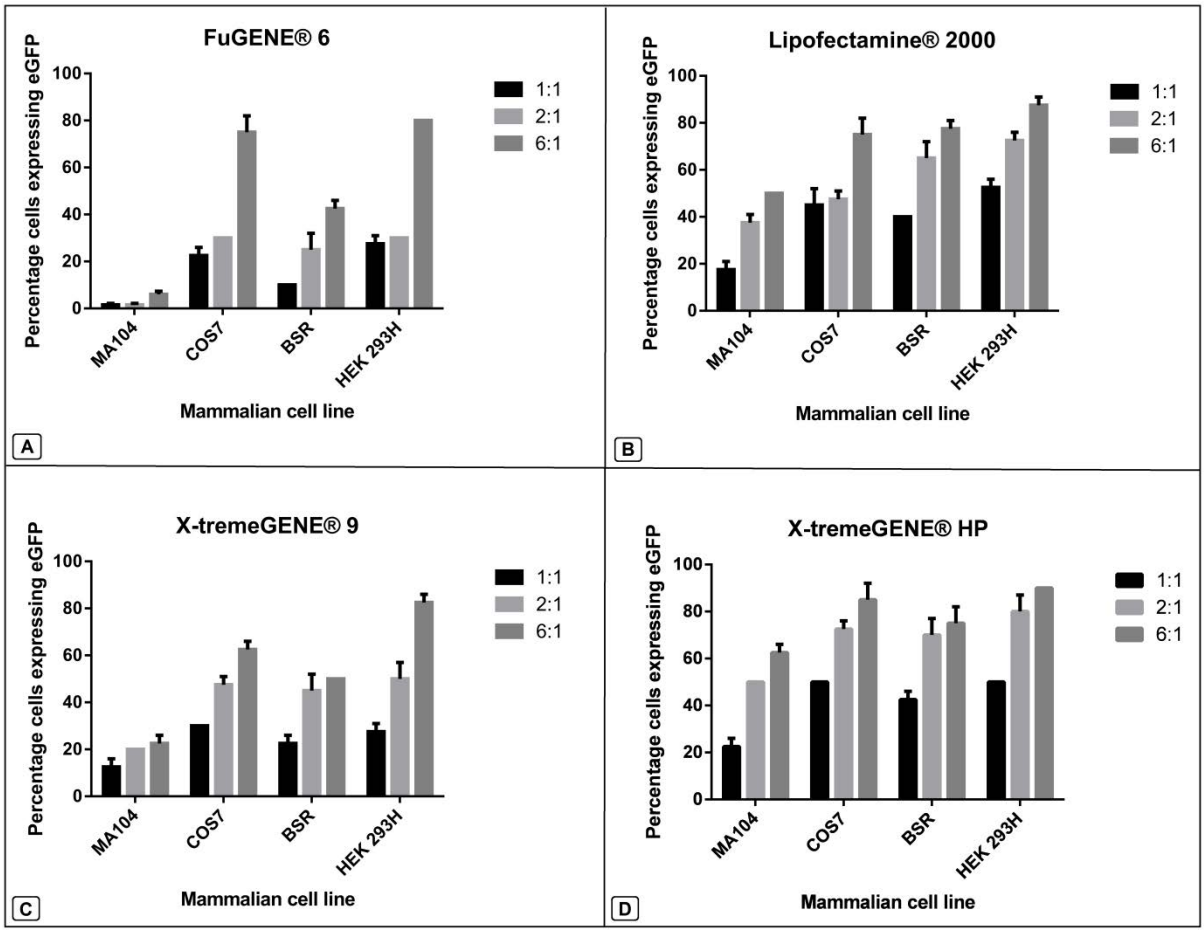

Figure 4.1: ***Comparison of eGFP expression in cultured cells transfected with different ratios of transfection reagents (µl) to eGFP DNA (µg).*** *MA104, COS-7, BSR and HEK 293H cells were transfected with 1 µg of eGFP gene containg plasmid DNA using the transfection reagents (A) FuGENE® 6, (B) Lipofectamine ® 2000, (C) XtremeGENE 9 and (D) XtremeGENE HP. All experiments were at least performed in triplicate.*

Lipofectamine ® 2000 proved to be the second best transfection (***Figure 4.1B***). The Lipofectamine ® 2000 reagent was very effective at the higher ratios (2:1 and 6:1). The XtremeGENE 9 is also a multi-component, lipid-based transfection reagent from Roche and proved to be more effective in lower transfection reagent to DNA ratios (1:1 and 2:1) than the FuGENE® 6 reagent (***Figure 4.1C***) and was the third best transfection reagent tested. The XtremeGENE HP transfection reagent, also in the XtremeGENE range, is formulated to transfect "hard-to-transfect" cells and proved to be the most efficient transfection reagent tested (***Figure 4.1D***). All transfection reagent to DNA ratios demonstrated more competent eGFP expression in comparison to any other transfection reagents examined. Another

advantage of the XtremeGENE transfection reagent series is the option to leave the transfection mixture on the cells for the entirety of the transfection experiment. Other transfection reagents like FuGENE® 6 and Lipofectamine® 2000 must be removed from the cells after a few hours to ensure the reduction of unwanted cell death due to cytotoxicity of the reagent. It was decided to use the 2:1 transfection reagent to DNA ratio in all subsequent studies due to its effective plasmid delivery and affordability.

4.3.2 Evaluation of the effect of different transfection reagents on cell viability

Low cytotoxicity is one of the requirements of a good transfection reagent (Karra and Dahm, 2010). During transections, prolonged exposures to transfection reagents are sometimes necessary in order to maximize uptake of the desired molecules. Due to this fact, transfection reagents, that have very little or no negative effect on cell viability, are favoured. Cells were exposed to the four transfection reagents at a 2:1 transfection:[peGFP-N1] ratio as described in ***section 4.2.1***. Cell viability was determined by trypan blue (Fluka) staining 48 hours after transfection.

The viability of cells exposed to different transfection reagents ranged from ~ 70 – 90%. X-tremeGENE 9 was the least cytotoxic transfection reagent tested and cell viability ranged between 83-91% in different cell lines (***Figure 4.2***). Lipofectamine ® 2000 proved to be the most cytotoxic transfection reagent among those tested with the number of viable cells ranging from 71-83% after 48 hours. Shorter incubation times (2-4 hours) prevented most of the cell death caused by the Lipofectamine ® 2000 reagent, but eGFP expression is negatively affected (data not shown). X-tremeGENE HP and FuGENE® 6 also displayed relatively low cytotoxic effects across the cell lines examined. The X-tremeGENE transfection reagents can be left on the cells for the entire duration of the transfection experiment. Very little negative effects on cell viability were observed in this study. This characteristic may prove useful when transfecting “hard to transfect” cell lines. When Lipofectamine ® 2000 was left on cells, mass cell death ensues within 10 hours and must thus be removed within 5 hours for a successful transfection.

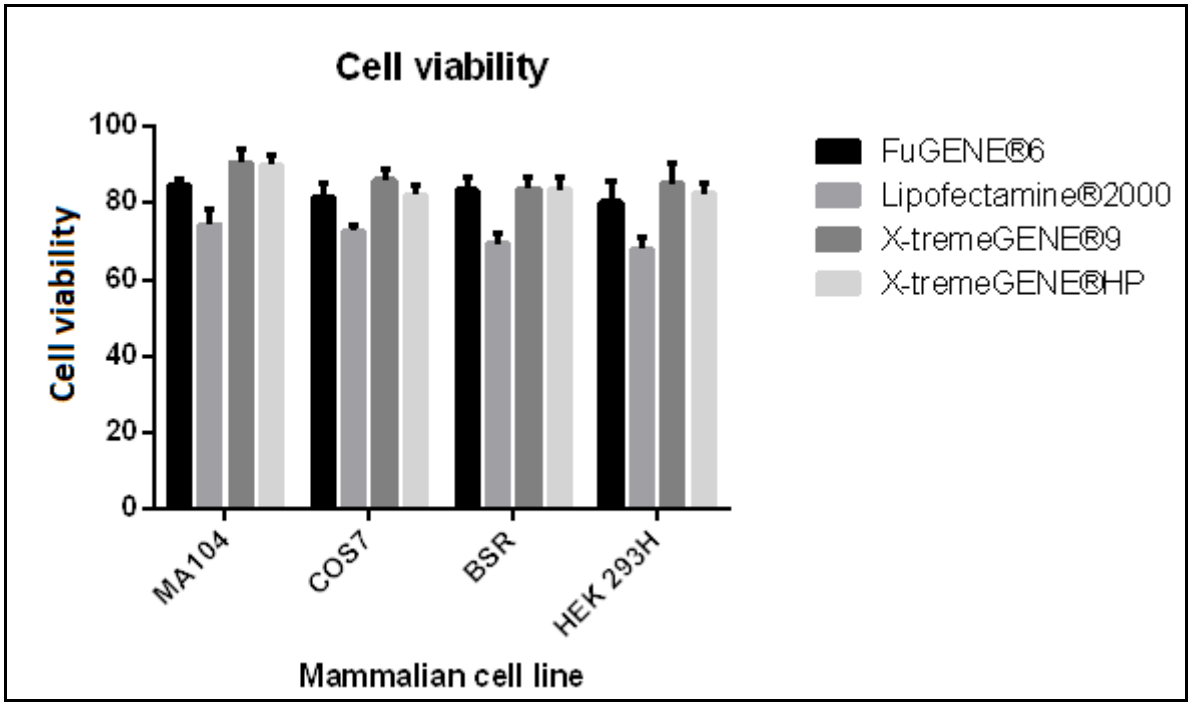

Figure 4.2: ***The influence of the four different transfection reagents on cell viability of MA104, COS-7, BSR and HEK 293H cultured cells.***

4.3.3 Optimisation of transfection methods and determination of the most effective transfection reagent for each cultured cell type

In order to determine the most effective transfection reagent for each cell type, all four cell lines (MA104, COS-7, BSR and HEK 293H) were transfected with the plasmid expressing eGFP using the four different transfection reagents. The eGFP expression was monitored with a fluorescent light microscope every 2 hours for 50 hours.

For experimental optimization, the four different cell lines were cultured to different confluences and transfected with a 2:1 ratio of transfection reagent (µl) to eGFP containing plasmid DNA (µg). The cell confluence that seems to yield the best eGFP expression ranges from 60-90% cell coverage (data not shown). Cells were propagated in D-MEM medium containing 10% foetal bovine serum, penicillin/streptomycin/amphotericin B (Lonza), and nonessential amino acids (Lonza). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. ***Figure 4.3*** summarises the transfection efficiency of each examined transfection reagent on different cell types. Significant differences, between the transfection efficiencies of the tested transfection agents on the different cell cultures, were observed. MA104 cells

proved to be the most challenging to effectively transfect and eGFP expression ranged from 10 – 65% (***Figure 4.3 (A)***). Transfections with the X-tremeGENE HP transfection reagent resulted in the highest percentage (65%) of MA104 cells expressing eGFP over a 50 hour period. Lipofectamine ® 2000 accounted for just over 50% of eGFP expression in MA104 cells but it also resulted in low cell viability (***Figure 4.2***). X-tremeGENE 9 and FuGENE® 6 transfections were ineffective in successfully transfecting MA104 cells and only accounted for roughly 30% and 10% eGFP expression, respectively. The expression of eGFP in Cos-7 cells ranged from between 70 - 90% after transfection with the different transfection reagents. Lipofectamine ® 2000 was the most effective transfection reagent with just under 90% of cells expressing eGFP after 50 hours, followed by FuGENE® 6 (~80%), X-tremeGENE HP (~80%) and X-tremeGENE 9 (~70%) (***Figure 4.3 (B)***). In BSR cells, Lipofectamine ® 2000 and X-tremeGENE HP accounted for ~80% of the cells expressing eGFP, trailed by X-tremeGENE 9 (~70%) and FuGENE® 6 (~65%) (***Figure 4.3 (C)***). The HEK 293H cells proved to be the cell line with the highest transfection efficiency. The expression of eGFP in HEK 293H cells ranged from between 80 - 95%. X-tremeGENE HP were the most effective transfection reagent with just under 95% of cells expressing eGFP after 50 hours, followed by Lipofectamine ® 2000 (~90%), FuGENE® 6 (~85%) and X-tremeGENE 9 (~80%) (***Figure 4.3 (D)***).

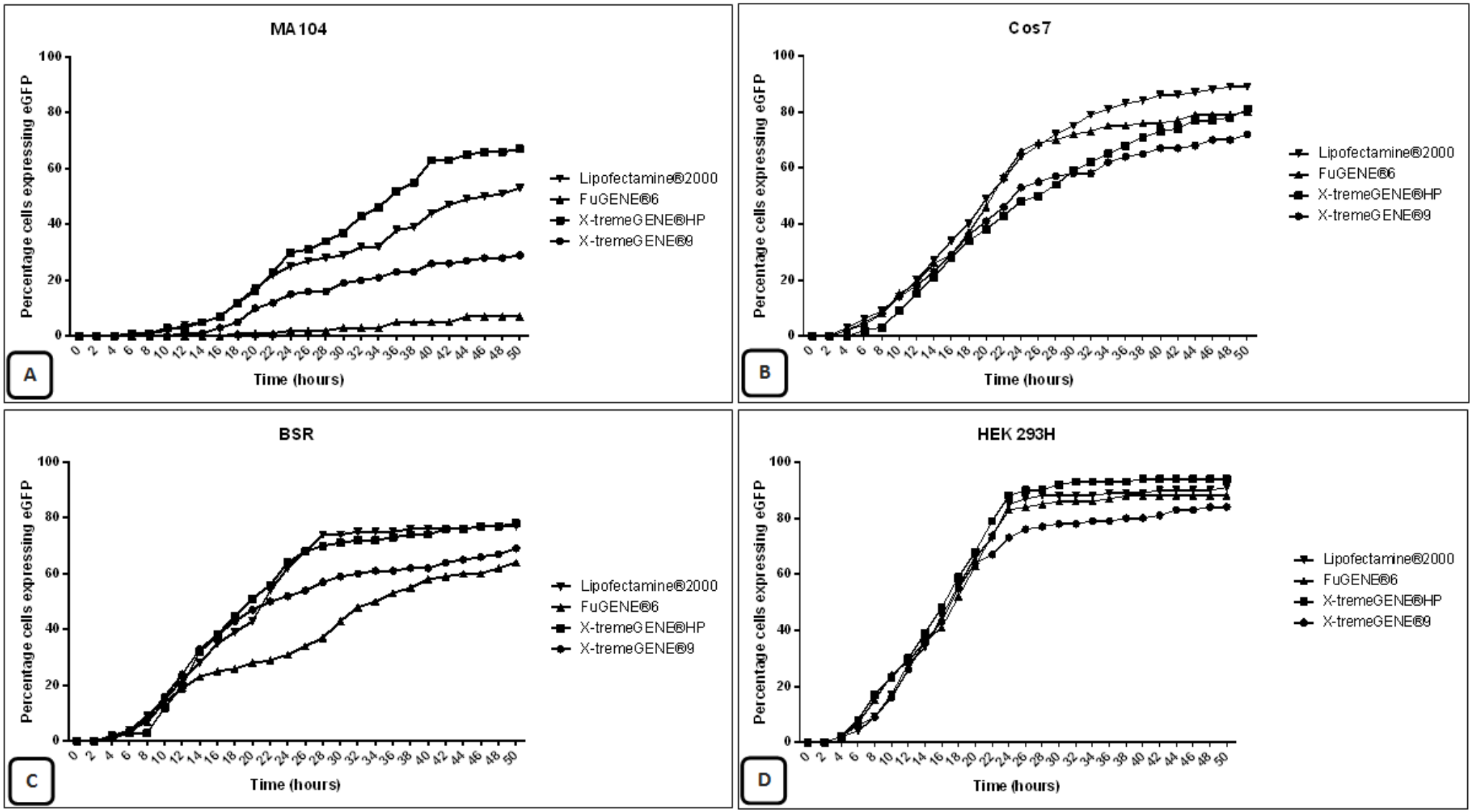

Figure: 4.3: ***Efficacy of four different transfection reagents for transfecting and expressing eGFP containing plasmids in (A) MA104, (B) COS-7, (C) BSR and (D) HEK 293H cells***

4.3.4 Determining the four different transfection reagents ability to successfully transfect rotavirus transcripts

Not only plasmid DNA, but also rotavirus transcripts will be transfected during the course of this study. The effectiveness of the different transfection reagents to transfect rotavirus transcripts was determined by transfecting MA104, COS-7, BSR and HEK 293H cultured cells. The expression of VP6 was monitored over a 24 hour period (***Figure 4.4***). MA104 cells proved to be the best suited for expressing rotavirus VP6. The expression of VP6 ranged from 2 – 80% depending on the transfection reagent and cell type. Lipofectamine ® 2000 and XtremeGENE HP proved to be the best suited to successfully transfect and express *in vitro* derived rotavirus VP6 transcripts over a broad range of cultured cells (***Figure 4.4***). FuGENE® 6 and X-tremeGENE 9 had poor transfection efficiency, accounting for less than 20% of cells expressing rotavirus VP6.

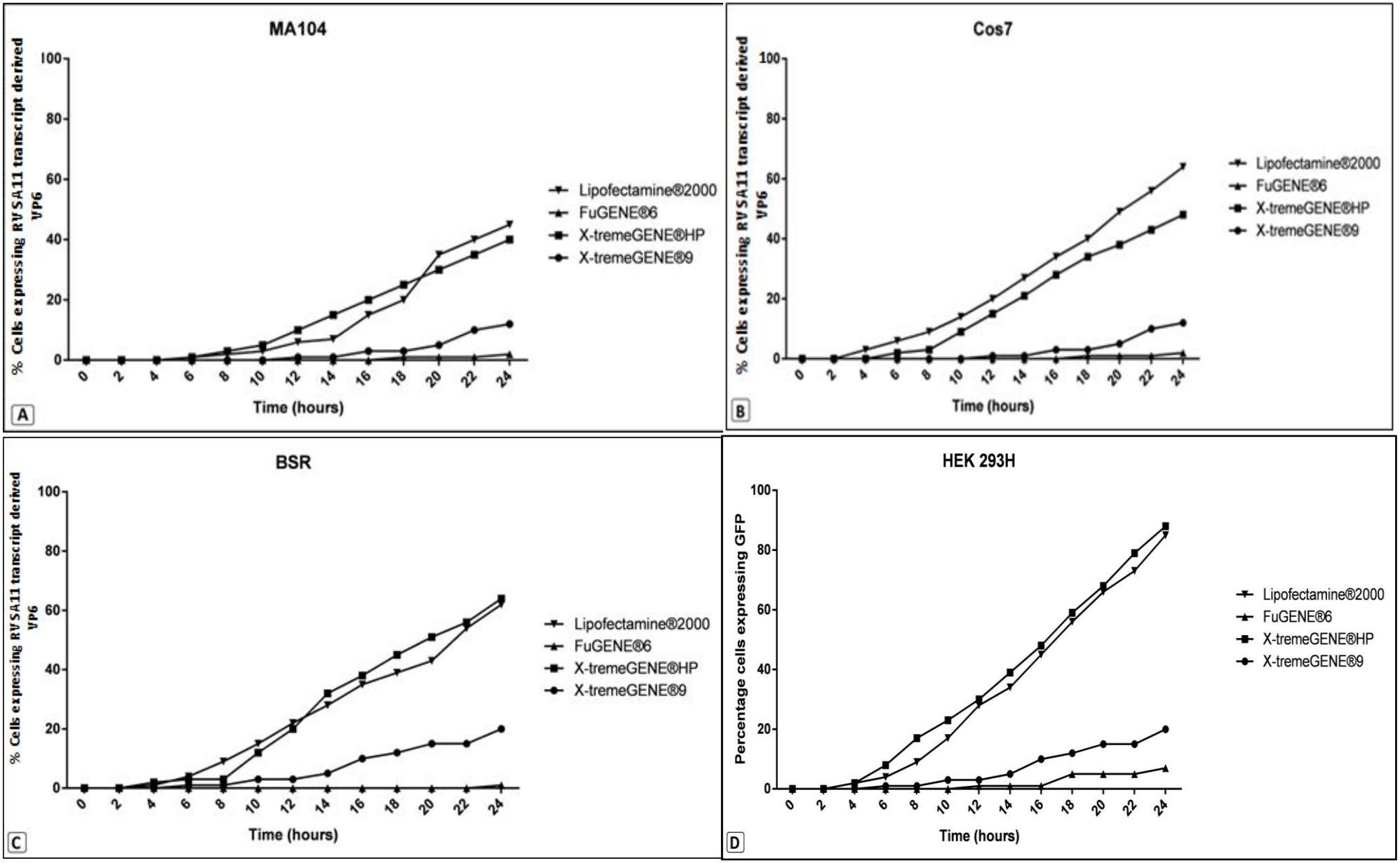

Figure 4.4: ***Efficacy of four different transfection reagents for transfecting rotavirus SA11 transcripts in (A) MA104, (B) COS-7, (C) BSR and (D) HEK 293H cell.*** *The transcript derived expression of rotavirus VP6 was determined.*

4.3.5 Determining the efficacy of eGFP expression with electroporation in four different mammalian cells

Electroporation is a transfection method that utilizes an electrical pulse to permeabilize cell membranes in order for nucleic acids to pass through the membranes into these cells. MA104, COS-7, BSR and HEK 293H cultured cells were electroporated to examine the effects of this transfection method on cell viability (***Figure 4.5(A)***). Cell viability ranged from 60 – 80% after cells were electroporated. The cell viability of COS-7, BSR and HEK 293H cells were just below 80%, while MA104 cell viability was about 60%. Electroporation caused extensive damage to the cultured cells in comparison to the chemical transfection reagents. The pre-programmed electroporation protocols were used for the specific mammalian cell lines and adaption of experimental parameters (data not shown) showed no significance in resolving the lower cell viability.

MA104, COS-7, BSR and HEK 293H cultured cells were also electroporated in the presence of 1 µg eGFP containing plasmid, in order to test the transfection efficiency of the specific cell lines. The expression of eGFP ranged from 20 – 40% (***Figure 4.5B***) and was substantially lower than what was observed for the chemical transfections (between 60 – 95% eGFP expression in most cell types (***Figure 4.3***)). After electroporation, Cos-7 cells showed the best eGFP expression (~40%), followed by BSR (~38%), HEK 293H (~35%) and MA104 (~20%).

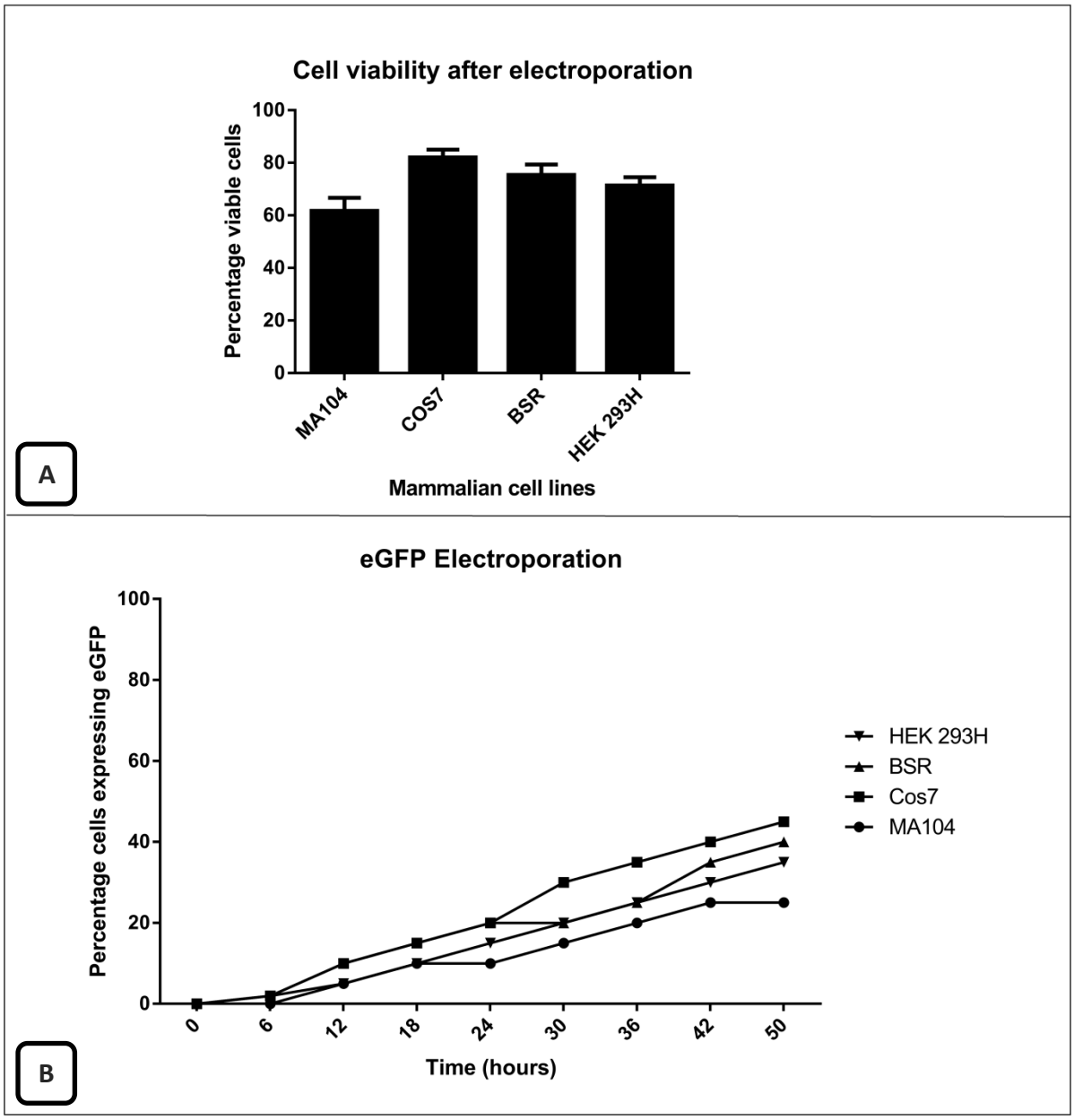

Figure 4.5: ***Four different cell lines were electroporated in the presence of 1 µg eGFP containing plasmid DNA.*** *(A) Cell viability of a variety of mammalian cells 24 hours after electroporation. (B) Percentage cells expressing eGFP over time.*

4.4 Summary

The efficient transfection of both rotavirus transcript and plasmid encoding for rotavirus proteins will be crucial to the success of a rotavirus reverse genetics system. As previously mentioned MA104 cells are notoriously difficult to efficiently transfect with foreign DNA. It is clear from the results obtained in this study supported this notion. 50 hours after transfection, only 7% of the MA104 cells transfected with the FuGENE® 6

transfection reagent expressed eGFP. After optimization, the X-tremeGENE HP- and Lipofectamine ® 2000 transfection reagents were more successful with 67% and 55% of the cultured cells expressing eGFP, respectively (***Figure 4.4***). Although this level of transfection/expression is not entirely desirable, it may be of great advantage when transfecting rotavirus cDNA plasmids and *in vitro* derived transcripts, considering MA104 cells are the most effective cell line for rotavirus propagation. Alternatively, electroporation proved to be ineffective in expressing plasmid derived eGFP and caused considerable cell death.

X-tremeGENE HP transfection regent was also responsible for exceptional expression of eGFP in MA104, COS-7, BSR and HEK 293H cultured cells. Baring in mind that this transfection reagent has a relatively low cytotoxicity and is affordable; the results in this chapter suggests that X-tremeGENE HP may be the best transfection reagent for transfection of rotavirus cDNA expression plasmids and transcripts A suitable cell culture system and correct concentration of the rotavirus mRNA seem to be crucial for rotavirus transcript translation *in vitro*. This is in contrast to the conclusions of Richards and co-workers (Richards *et al,* 2013) although similar experimental methods were followed. The optimised X-tremeGENE HP transfection procedure will be utilized in ***Chapters 5 and 6***.