Optimising Transfection Performance

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There are several methods available for the transfection of nucleic acids – each with its own strengths and weaknesses. An increased understanding of the mechanisms by which transfection works will spur further developments in the field, not only from a reagent perspective but also with regard to pharmaceutical applications.

In its simplest form, nucleic acid transfection involves introducing a non-host DNA or RNA into a cell. Early iterations involved chemically mediated methods to form precipitates of optimal particle size that would encapsulate the DNA during uptake. Later advances incorporated lipids and/or primary amine-containing molecules – such as protamine sulphate – for nucleic acid condensation and delivery across the lipid bilayer, with charge nuances affecting both the efficiency of internalisation and later release of DNA by the endosome. Figure 1 demonstrates plasmid DNA uptake by the cell, release into the cytoplasm and subsequent protein expression over a course of time. Viral-based delivery methods – such as lentivirus and retrovirus – which take advantage of the ability to infect cells and deliver nucleic acid cargo, along with electroporation methods in which current opens pores in membranes, have also become popular. Each technique has its strengths and weaknesses, and a number of variables can affect transfection performance. In this article, we will primarily focus on optimisation parameters for lipid-based transfection methodology, and address how insight into these factors can influence performance in pharmaceutical applications.

CELLS & CULTURING METHODS

Cell health and maintenance considerations may seem obvious but are often overlooked when it comes to the optimisation of transfection. Whenever possible, established cell lines should be of low passage number; increased passages may lead to undesirable differentiation typified by morphological changes, modified growth rates and, possibly, decreased protein production or response to stimuli. Instead of cell lines, primary cultures are often used to more closely mimic natural tissues, but typically have a limited growth potential and life span. Cultures should be largely homogeneous (for example, neuronal cultures should be enriched for neurons and suppressed with regard to glial cells) and used as soon as practical. Since each cell type is likely to respond differently to a given transfection reagent or method, optimisation and proper experimental design are necessary to maximise results. Figure 2 demonstrates cell line-dependent differences in transfection efficiency.

Cell density also affects overall transfection efficiency. Current reagents and methods are readily able to cross the lipid bilayer, but have difficulty in endosomal escape and targeting to the nucleus. To achieve transcription and ultimately protein production, nuclear deposition of DNA is required; this is largely dependent on membrane dissolution and reformation during mitosis, meaning that cells have to be actively dividing. For lipid- and chemical-based delivery methods, the best efficiency is often attained at a confluency of 80 per cent, but protocol recommendations may range from 50 to 90 per cent. The optimal density is highly dependent on cell type and reagent specific toxicity, and should be determined empirically. Lentivirus delivery is one of the few methods that does not require actively dividing cells.

Cell culture growth media and choice of supplements can also affect the ability to transfect cells. The American Type Culture Collection (ATCC) provides recommended culture conditions for most cell lines and primary cultures. Serum compatibility should be considered in the selection of lipid-based transfection reagents. Lipoproteins present in the serum may inhibit uptake by competing or interfering with lipid DNA complexes, impeding their fusion with the membrane. Nucleic acid interaction with the lipid component (complex formation) is performed in a serum-free media for a brief period of time (typically 15 minutes) before being applied to the cultured cells. Some reagents are compatible with serum post-complex

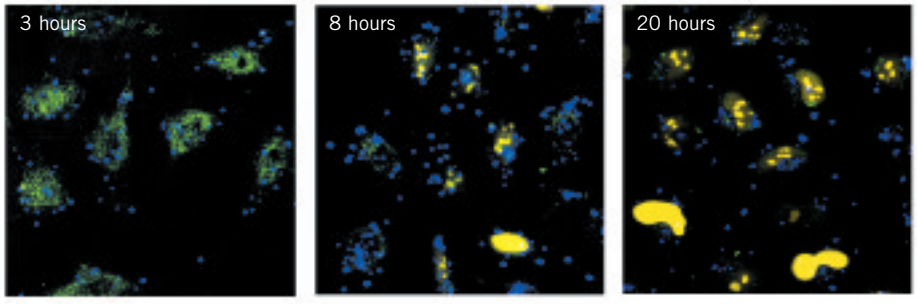


Figure 1: Tracking of plasmid localisation and expression. COS-7 cells were transfected with Cy™5 labelled EYFP-nuc plasmid using a transfection reagent in complete medium. Images were acquired at three, eight and 20 hours post-transfection. The blue staining indicates the cellular localisation of the Cy™5 labelled plasmid, while the yellow signal shows expression of the yellow fluorescent protein (EYFP). The images were acquired using a confocal microscope

formation and can be directly applied to cells, whereas those that are not serum-compatible can be applied to cells in a serum-free media for two to four hours, followed by media replacement or media supplementation with full serum. Transfection may be done in the presence of antibiotics, but is not recommended.

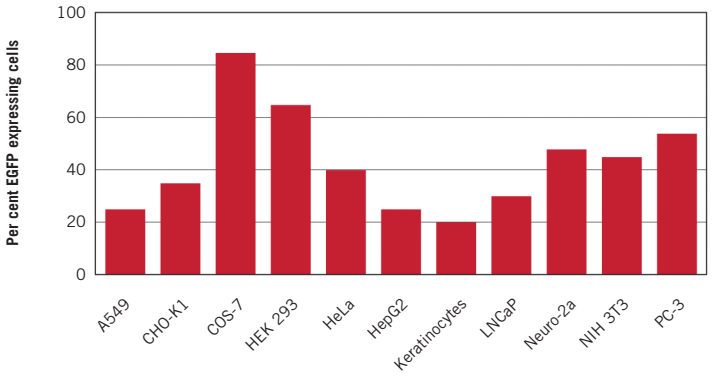
OPTIMISED CHARGE RATIO FOR DELIVERY

For lipid-based delivery, protocols typically provide a range of reagent-to-nucleic acid ratios; the reason for this relates to charge considerations and is two-fold. First, the negative charge contributed by phosphates within the nucleic acid requires an offsetting positive charge by the reagent and/or condensing agent – for example, protamine sulphate or polyethylenimine (PEI) – during complex formation. Charge should be sufficient for good complex formation, yet lend itself to release upon endosomal escape. Secondly, the reagent must provide the ability to transport across the lipid bilayer through neutralisation or decreasing the electrostatic repulsion imparted on the DNA by the negatively charged membrane. The optimal reagent to nucleic acid ratio is highly cell type-dependent. As a starting point, it is recommended to vary the transfection reagent while keeping a constant plasmid DNA concentration (for example, 1:1, 3:1 and 5:1 ratios of volume to mass). Additional benefits may be derived by maintaining the ratio and increasing the amount of plasmid added (see Figure 3).

TOXICITY

Reagents have inherent properties that cause differing levels of toxicity to the cells. Toxicity liability should be determined through measurement of Lactate Dehydrogenase (LDH) release levels, or by conducting assays with colorimetric substrates such as (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) with proper controls. At times, the goal of the experiment will dictate whether transfection efficiency or enhanced cell viability is more important.

The nucleic acid used should be free of mycoplasma, endotoxin and other impurities, which can affect cell health and hence lower transfection efficiency. While high efficiencies can be obtained with in-house purified DNA, pharmaceutical companies have increasingly moved toward outsourcing large-scale DNA preparation to companies that specialise in the production of injection-grade nucleic acid.



PHARMACEUTICAL APPLICATIONS

From drug discovery to biopharmaceutical manufacturing, transfection has a role in the drug development process. Efficiency, output and cost are considerations that may have an impact on the choice of approach, and are especially important as it pertains to RNAi studies and screening, biopharmaceutical production and stem cell applications. Each will be discussed briefly below with suggested parameters to consider for optimisation.

Gene Silencing

siRNA-mediated knockdown has become commonplace as a tool for drug discovery and validation. Genome-wide screens are now being used to identify putative genes and pathways involved in disease states. Since siRNA targets mRNA within the cytoplasm, siRNA delivery has a lower barrier to entry than plasmid DNA that requires nuclear localisation. Gene expression silencing of 90 per cent or greater can normally be achieved. Electroporation is recommended for genome-wide screens due to the need for higher throughput in 96-well or 384-well plate liquid handlers, while smaller culture requirements can easily be met by lipid-based transfection of siRNA. For optimisation, the lipid reagent is typically held constant at the manufacturer's recommended concentration, and the molar amount of siRNA to be used is varied (for example, 5nM to 50nM). Electroporation often requires more siRNA for delivery per sample and is not generally recommended for large cultures due to the costs of the synthetic siRNA and technology limitations. Silencing

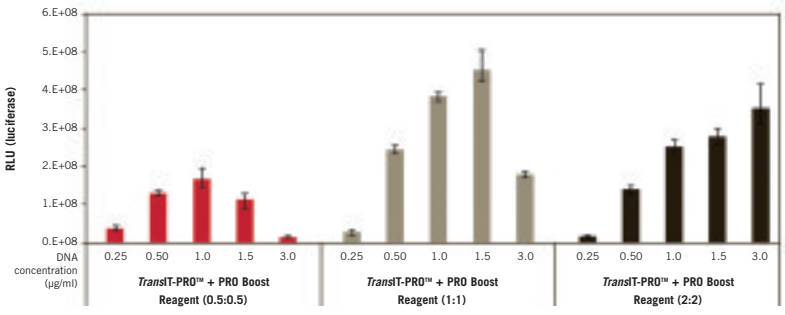


Figure 2: Representative cell lines were transfected with an EGFP expression vector using a lipid transfection reagent in complete growth medium without medium changes. Transfection efficiency was determined using flow cytometry at 48 hours post-transfection

Figure 3: Luciferase protein expression was compared at varying plasmid DNA concentrations (0.25 to 3µg/ml) and using three different ratios of transfection reagent and boost (0.5:0.5, 1:1 and 2:2) per µg of DNA. Transfections were performed in 24-well deep well shaker blocks using CHO-S cells cultured in BD Select™CD1000 media (2ml/well). Cells were plated at a density of 0.5 x 10^6 cells/ml at the time of transfection, harvested 24 hours post-transfection and assayed using a conventional luciferase assay

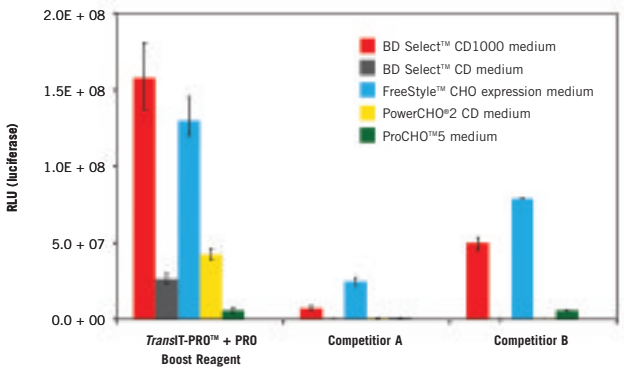


Figure 4: CHO-S cells were adapted to five representative growth media and then transfected with a plasmid encoding luciferase using a Mirus reagent and two competitor reagents according to the manufacturer's protocol. Transfections were performed in 24-well deep well shaker blocks using 1µg plasmid DNA per ml of culture, and 0.5 x 10^6 cells/ml at the time of transfection

can also be achieved by the use of short-hairpin RNA (shRNA) through normal plasmid delivery or viral integration. Proper use of controls is essential to determine that the siRNA/shRNA does not exhibit off-target effects yet still targets the gene of interest. Cell density, passage number, culture conditions, quality of the siRNA and concentration of the siRNA/shRNA are all parameters that can affect knockdown efficiencies.

Biotherapeutic Production

The expansion of biotherapeutics, most notably monoclonal antibodies and recombinant proteins, has created the need for rapid methods to produce protein, display correct co- and post-translational modifications, and create enough protein yield to facilitate further assay development and validation. Most commonly, HEK-293 and CHO cells are used for this purpose because they are easily adapted to serum-free conditions in suspension cultures and generate high-level protein production. Often researchers start with transient transfection methods during the optimisation stage while stable clones are being screened for large-scale manufacture. Optimisation of transient transfection involves cell selection, media compatibility and reagent to DNA ratio, with the goal of having high yield at reasonable cost. While standard HEK 293 and CHO cells are available from ATCC, many pharmaceutical companies are utilising strains or variants that have enhanced secretion capabilities or suppressed apoptotic liability. Targeted proteins or variants may exhibit differential protein expression profiles requiring reagent to nucleic acid optimisation and selection of ideal media growth conditions. The selection of reagent and cell culture media can greatly impact transfection performance and overall protein yield (see Figure 4). For optimisation, a range of reagent to plasmid ratios at selected culture densities is suggested, with choice of reagent impacted by media compatibility and protein yield.

Another consideration is cost versus yield. If small amounts of protein are needed for the testing, validation and QC assay development, then costs can be conserved by using a cationic polymer (for example, linear PEI) as a delivery agent, especially in 293 cells. Often though, higher yields are desired in which case lipid-based reagents may be preferred. The scale of cultures used (typically litre scale) does not lend itself to conventional electroporation methods.

Stem Cells

The interest in using induced pluripotent stem (iPS) cells for therapeutic applications is creating a new need for higher efficiency transfection reagents and vector delivery systems. Current efficiencies are very low (in the neighbourhood of 0.001 to 0.0001 per cent colony formation) and have, at an early stage, depended heavily on viral delivery of the necessary transcription factors to induce pluripotency. The use of viruses has raised concerns due to the likelihood of inducing cell-altering changes upon viral integration into the genome. Alternative methods that do not involve genome perturbation have been developed, such as the use of episomal vectors or the introduction of transcription factors through serial transfection, but both approaches are still restricted by low efficiencies. Purely from a colony-screening standpoint, resource and reagent costs are substantial and better efficiency is desired. Transfection may also be involved in later steps, such as selection for differentiation along given pathways (using a plasmid expressing a protein providing drug resistance under a tissue-specific promoter), or introduction of plasmids encoding relevant reporters or differentiation modulators. Cell density and proper reagent to plasmid DNA ratio are the other two parameters best suited for optimisation.

CONCLUSION

While transfection reagents and methods have advanced since their first use in 1973, there are many challenges that remain. Hurdles to overcome include achieving better efficiencies without inducing toxicity, and the ability to transfect almost any cell type including non-dividing cells. Increased understanding of transfection mechanisms will spur further developments in the field, not only from a reagent perspective, but also with regard to pharmaceutical applications.

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