CONTENTS

Promega

I. Introduction

The process of introducing nucleic acids into eukaryotic cells by nonviral methods is defined as transfection. Using various chemical, lipid or physical methods, this gene transfer technology is a powerful tool to study gene function and protein expression in the context of a cell. Development of reporter gene systems and selection methods for stable maintenance and expression of transferred DNA have greatly expanded the applications for transfection. Assay-based reporter technology, together with the availability of transfection reagents, provides the foundation to study mammalian promoter and enhancer sequences, *trans*-acting proteins such as transcription factors, mRNA processing, protein:protein interactions, translation and recombination events (Groskreutz and Schenborn, 1997).

Transfection is a method that neutralizes or obviates the issue of introducing negatively charged molecules (e.g., phosphate backbones of DNA and RNA) into cells with a negatively charged membrane. Chemicals like calcium phosphate and DEAE-dextran or cationic lipid-based reagents coat the DNA, neutralizing or even creating an overall positive charge to the molecule (Figure 12.1). This makes it easier for the DNA:transfection reagent complex to cross the membrane, especially for lipids that have a "fusogenic" component, which enhances fusion with the lipid bilayer. Physical methods like microinjection or electroporation simply punch through the membrane and introduce DNA directly into the cytoplasm. Each of these transfection technologies is discussed in the following sections.

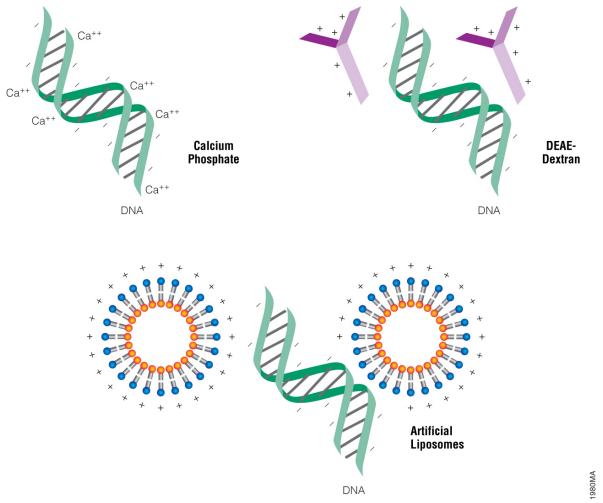

Figure 12.1. Schematic representation of various transfection technologies and how they neutralize the negatively charged DNA. Note that lipid-based reagents also can coat DNA in addition to forming micelles and associating with DNA by attraction as depicted.

This chapter covers general information on transfection techniques and considerations for transfection efficiency and optimization. In addition, we discuss various transfection agents available from Promega as well as general protocols for transfection and specific examples using our transfection reagents. Finally, we review stable transfection and outline a protocol using drug selection.

A. Chemical Reagents

One of the first chemical reagents used to transfer nucleic acids into cultured mammalian cells was DEAE-dextran (Vaheri and Pagano, 1965; McCutchan and Pagano, 1968). DEAE-dextran is a cationic polymer that tightly associates with negatively charged nucleic acids. An excess of positive charge, contributed by the polymer in the DNA:polymer complex, allows the complex to come into closer association with the negatively charged cell membrane. Uptake of the complex is presumably by endocytosis. This method successfully delivers nucleic acids into cells for transient expression; that is, for short-term expression studies of a few days in duration. However, this technique is not generally useful for stable or long-term transfection studies that rely upon integration of transferred DNA into the chromosome (Gluzman, 1981). Other synthetic cationic polymers have been used to transfer DNA into cells, including polybrene (Kawai and Nishizawa, 1984), polyethyleneimine (Boussif *et al.* 1995) and dendrimers (Haensler and Szoka, 1993; Kukowska-Latallo *et al.* 1996).

Calcium phosphate co-precipitation became a popular transfection technique following the systematic examination of this method in the early 1970s (Graham and van der Eb, 1973). The authors examined the performance of various cations and effects of cation concentration, phosphate concentration and pH on transfection. Calcium phosphate co-precipitation is widely used because the components are easily available and inexpensive, the protocol is easy-to-use, and it is effective with many different types of cultured cells. The protocol involves mixing DNA with calcium chloride, adding this in a controlled manner to a buffered saline/phosphate solution and allowing the mixture to incubate at room temperature. The controlled addition generates a precipitate that is dispersed onto the cultured cells. The precipitate is taken up by cells via endocytosis or phagocytosis. Calcium phosphate transfection is routinely used for both transient and stable transfection of a variety of cell types. In addition, calcium phosphate appears to provide protection against intracellular and serum nucleases (Loyter *et al.* 1982).

However, calcium phosphate co-precipitation is prone to variability and is not suited for in vivo gene transfer to whole animals. In addition, small pH changes (± 0.1) can compromise the efficacy of calcium phosphate transfection (Felgner, 1990). Promega offers the calcium phosphate reagent as part of the ProFection® Mammalian Transfection System—Calcium Phosphate (Cat.# E1200).

B. Cationic Lipids

The term "liposome" refers to lipid bilayers that form colloidal particles in an aqueous medium (Sessa and Weissmann, 1968). By 1980, artificial liposomes were being used to deliver DNA into cells (Fraley *et al.* 1980). The next advance in liposomal vehicles was development of synthetic

cationic lipids by Felgner and colleagues (Felgner *et al.* 1987). The cationic head group of the lipid compound associates with negatively charged phosphates on the nucleic acid. Liposome-mediated delivery offers advantages such as relatively high efficiency of gene transfer, ability to transfect certain cell types that are resistant to calcium phosphate or DEAE-dextran, in vitro and in vivo applications, successful delivery of DNA of all sizes from oligonucleotides to yeast artificial chromosomes (Felgner *et al.* 1987; Capaccioli *et al.* 1993; Felgner *et al.* 1993; Haensler and Szoka, 1993; Lee and Jaenisch, 1996; Lamb and Gearhart, 1995), delivery of RNA (Malone *et al.* 1989; Wilson *et al.* 1979), and delivery of protein (Debs *et al.* 1990). Cells transfected by liposome techniques can be used for transient expression studies and long-term experiments that rely on integration of DNA into the chromosome or episomal maintenance. Unlike DEAE-dextran or calcium phosphate chemical methods, liposome-mediated nucleic acid delivery can be used for in vivo transfer of DNA and RNA to animals and humans (Felgner *et al.* 1995).

A lipid with overall net positive charge at physiological pH is the most common synthetic lipid component of liposomes developed for gene delivery (Figure 12.2). Often the cationic lipid is mixed with a neutral lipid such as L-dioleoyl phosphatidylethanolamine (DOPE; Figure 12.3), which can enhance the gene transfer ability of certain synthetic cationic lipids (Felgner *et al.* 1994; Wheeler *et al.* 1996). The cationic portion of the lipid molecule associates with negatively charged nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex (Kabanov and Kabanov, 1995; Labat-Moleur *et al.* 1996), presumably from electrostatic interactions between the negatively charged nucleic acid and positively charged head group of the synthetic lipid. For cultured cells, an overall net positive charge of the liposome/nucleic acid complex generally results in higher transfer efficiencies, presumably because this allows closer association of the complex with the negatively charged cell membrane. Entry of the liposome complex into the cell may occur by endocytosis or fusion with the plasma membrane via the lipid moieties of the liposome (Gao and Huang, 1995). Following cellular internalization, the complexes appear in the endosomes and later in the nucleus. It is unclear how the nucleic acids are released from the endosomes and lysosomes and traverse the nuclear membrane. DOPE is considered a "fusogenic" lipid (Farhood *et al.* 1995), and its role may be to release these complexes from endosomes as well as to facilitate fusion of the outer cell membrane with liposome/nucleic acid complexes. While DNA will need to enter the nucleus, the cytoplasm is the site of action for RNA, protein or antisense oligonucleotides delivered via liposomes.

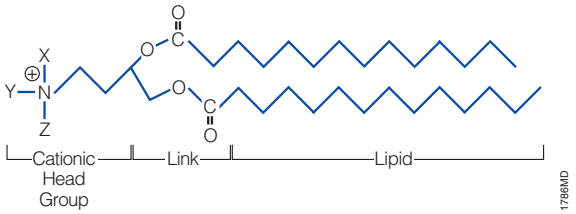

Figure 12.2. The general structure of a synthetic cationic lipid. X, Y and Z represent a number of possible chemical moieties, which can differ, depending on the specific lipid.

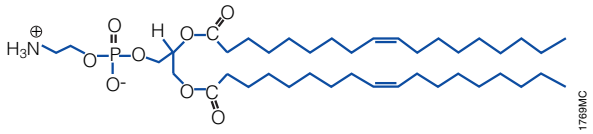

Figure 12.3. Structure of the neutral lipid DOPE.

Promega offers the FuGENE® HD Transfection Reagent (Cat.# E2311), a novel nonliposomal transfection reagent with wide application in different cell types and low toxicity, FuGENE® 6 Transfection Reagent (Cat.# E2691, a nonliposomal formulation that transfects a wide variety of cell lines with high efficiency and low toxicity, and the TransFast™ Transfection Reagent (Cat.# E2431), which uses a polycationic head group attached to a lipid backbone structure to deliver nucleic acids into eukaryotic cells. The best transfection reagent and conditions for a particular cell type must be empirically and systematically determined because inherent properties of the cell influence the success of any specific transfection method.

C. Physical Methods

Physical methods for gene transfer were developed and used beginning in the early 1980s. Direct microinjection into cultured cells or nuclei is an effective although laborious technique to deliver nucleic acids into cells by means of a fine needle (Cappechi, 1980). This method has been used to transfer DNA into embryonic stem cells that are used to produce transgenic organisms (Bockamp *et al.* 2002) and to introduce antisense RNA into *C. elegans* (Wu *et al.* 1998). However, the apparatus is costly and the technique extremely labor-intensive, thus it is not an appropriate method for studies that require a large number of transfected cells.

Electroporation was first reported for gene transfer studies in mouse cells (Wong and Neumann, 1982). This technique is often used for cell types such as plant protoplasts, which are difficult to transfect by other methods. The mechanism is based on the use of an electrical pulse to perturb the cell membrane and form transient pores that allow passage of nucleic acids into the cell (Shigekawa and Dower, 1988). The technique requires fine-tuning and optimization of pulse duration and strength for each type of cell used. In addition, electroporation often requires more cells than chemical methods because of substantial cell death, and extensive optimization often is required to balance transfection efficiency and cell viability. More modern

instrumentation allows nucleic acid delivery to the nucleus and successful transfer of DNA and RNA to primary and stem cells.

Another physical method of gene delivery is biolistic particle delivery, also known as particle bombardment. This method relies upon high-velocity delivery of nucleic acids on microprojectiles to recipient cells by membrane penetration (Ye *et al.* 1990). This method is successfully employed to deliver nucleic acid to cultured cells as well as to cells in vivo (Klein *et al.* 1987; Burkholder *et al.* 1993; Ogura *et al.* 2005). Biolistic particle delivery is relatively costly for many research applications, but the technology also can be used for genetic vaccination and agricultural applications.

D. Viral Methods

While transfection has been used successfully for gene transfer, the use of viruses as vectors has been explored as an alternative method to deliver foreign genes into cells and as a possible in vivo option. Adenoviral vectors are useful for gene transfer due to a number of key features: 1) they rapidly infect a broad range of human cells and can achieve high levels of gene transfer compared to other available vectors; 2) adenoviral vectors can accommodate relatively large segments of DNA (up to 7.5kb) and transduce these transgenes in nonproliferating cells; and 3) adenoviral vectors are relatively easy to manipulate using recombinant DNA techniques (Vorburger and Hunt, 2002). Other vectors of interest include adeno-associated virus, herpes simplex virus, retroviruses and lentiviruses, a subset of the retrovirus family. Lentiviruses (e.g., HIV-1) are of particular interest because they are well studied, can infect quiescent cells, and can integrate into the host cell genome to allow stable, long-term transgene expression (Anson, 2004).

As with all gene transfer methods, there are drawbacks. For adenoviral vectors, packaging capacity is low, and production is labor-intensive (Vorburger and Hunt, 2002). With retroviral vectors, there is the potential for activation of latent disease and, if there are replication-competent viruses present, activation of endogenous retroviruses and limited transgene expression (Vorburger and Hunt, 2002; Anson, 2004).

II. General Considerations

A. Reagent Selection

With so many different methods of gene transfer, how do you choose the right transfection reagent or technique for your needs? Any time a new parameter, like a new cell line, is introduced, the optimal conditions for transfection will need to be determined. This may involve choosing a new transfection reagent. For example, one reagent may work well with HEK-293 cells, but a second reagent is a better choice when using HepG2 cells. Promega offers the FuGENE® HD Protocol Database to help identify a protocol for your cell line when using the FuGENE® HD Transfection Reagent. A drop-down menu allows you to search the database by cell line, plate type and number of cells to be transfected. The Transfection Assistant also offers a drop-down menu to select cell lines and either FuGENE® HD or FuGENE® 6 Transfection Reagent for transfection conditions. The conditions should be considered only guidelines since you may need to optimize the transfection conditions for your specific application. See Optimization of Transfection Efficiency (Section IV) and General Transfection Protocol (Section VI) for details.

B. Transient Expression versus Stable Transfection

Another parameter to consider is the time frame of the experiment you wish to conduct. Is it short- or long-term? For instance, determining which promoter deletion constructs can still function as a promoter can be accomplished with a transient transfection experiment, while establishing stable expression of an exogeneously introduced gene construct will require a longer term experiment.

Transient Expression

Cells are typically harvested 24–72 hours post-transfection for studies designed to analyze transient expression of transfected genes. The optimal time interval depends on the cell type, research goals and specific expression characteristics of the transferred gene. Analysis of gene products may require isolation of RNA or protein for enzymatic activity assays or immunoassays. The method used for cell harvest will depend on the end product assayed. For example, expression of the firefly luciferase gene in the pGL4.10[*luc2*] Vector (Cat.# E6651) is generally assayed 24–48 hours post-transfection, whereas the pGL4.12[*luc2CP*] Vector (Cat.# E6671) with its protein degradation sequences can be assayed in a shorter time frame (e.g., 3–12 hours), depending on the research goals and the time it takes for the reporter gene to reach steady state. For more information on luminescent reporter genes like firefly luciferase, see the *Protocols and Applications Guide* chapter on bioluminescent reporters.

When performing a transient transfection, you can choose between a standard or reverse transfection protocol. In a standard transfection protocol, the cells are plated on day 1, transfected on day 2 and assayed on day 3 or 4. In a reverse transfection protocol, cells are added directly to a plate containing the transfection reagent/DNA mix and assayed on day 2 or 3. Because the cells are added directly to the DNA, this process reduces the experimental time by one day and allows for high-throughput transfection of DNA in a plate- or microarray-format. For more information on reverse transfection including a protocol, read the PubHub article on the subject.

Stable Transfection

The goal of stable, long-term transfection is to isolate and propagate individual clones containing transfected DNA that has integrated into the cellular genome. Distinguishing nontransfected cells from those that have taken up exogenous DNA involves selective screening. This screening can be accomplished by drug selection when an appropriate drug-resistance marker is included in the transfected DNA. Alternatively, morphological transformation can be used

as a selectable trait in certain cases. For example, bovine papilloma virus vectors produce a morphological change in transfected mouse CI127 cells (Sarver *et al*. 1981).

Before using a particular drug for selection purposes, you will need to determine the amount of drug necessary to kill untransfected cells. This may vary greatly among cell types. Consult Ausubel *et al.* 1995 for additional information about designing experiments to test various drug concentrations and determine the amount needed to select resistant clones (i.e., generate a kill curve).

When drug selection is used, cells are maintained in nonselective medium for 1–2 days post-transfection, then replated in selective medium containing the drug. The use of selective medium is continued for 2–3 weeks, with frequent changes of medium to eliminate dead cells and debris, until distinct colonies can be visualized. Individual colonies can be isolated by cloning cylinders, selected and transferred to multiwell plates for further propagation in the presence of selective medium. Individual cells that survive the drug treatment expand into clonal groups that can be individually propagated and characterized. For a protocol to select transfected cells by antibiotics, see Stable Transfection (Section VII).

Several different drug-selection markers are commonly used for long-term transfection studies. For example, cells transfected with recombinant vectors containing the bacterial gene for neomycin phosphotransferase [e.g., pCI-neo Mammalian Expression Vector (Cat.# E1841)] can be selected for stable transformation in the presence of the neomycin analog G-418 (Cat.# V8091; Southern and Berg, 1982). Similarly, expression of the hygromycin B phosphotransferase gene from the transfected vector [e.g., pGL4.14 [*luc2*/Hygro] Vector (Cat.# E6691)] will confer resistance to the drug hygromycin B (Blochlinger and Diggelmann, 1984).

An alternative strategy is to use a vector carrying an essential gene that is defective in a given cell line. For example, CHO cells deficient in dihydrofolate reductase (DHFR) gene expression do not survive without added nucleosides. However, these cells, when stably transfected with DNA expressing the DHFR gene, will synthesize the required nucleosides and survive (Stark and Wahl, 1984). An additional advantage of using DHFR as a marker is that gene amplification of DHFR and associated transfected DNA occurs when cells are exposed to increasing doses of methotrexate, resulting in multiple copies of the plasmid in the transfected cell (Schimke, 1988).

C. Type of Molecule Transfected

Plasmid DNA is most commonly transfected into cells, but other macromolecules can be transferred as well. For example, short interfering RNA (siRNA; Hong *et al.* 2004; Snyder *et al.* 2004; Klampfer *et al.* 2004), oligonucleotides (Labroille *et al.* 1996; Berasain *et al.* 2003; Lin *et al.* 2004), RNA (Shimoike *et al.* 1999; Ray and Das, 2004) and even proteins (Debs *et al*. 1990; Lin *et al.* 1993) have been successfully introduced into cells via transfection methods. However, conditions that work for plasmid DNA transfer will likely need to be optimized when using other macromolecules. In all cases, the agent transfected needs to be of high quality and relatively pure. Nucleic acids need to be free of proteins, other contaminating nucleic acids and chemicals (e.g., salts from oligo synthesis). Protein should be pure and in a solvent that is not detrimental to cell health. For additional information on plasmid DNA quality, see DNA Quality and Quantity (Section III.D).

D. Assay for Transfection

After cells are transfected, how will you determine success? Plasmids containing reporter genes can be used to easily monitor transfection efficiencies and expression levels in the cells. An ideal reporter gene product is one that is unique to the cell, can be expressed from plasmid DNA and can be assayed conveniently. Generally, reporter gene assays are performed 1–3 days after transfection; the optimal time should be determined empirically. For a discussion of luminescent reporter gene options, see the *Protocols and Applications Guide* chapter on bioluminescence reporters. A direct test for the protein of interest, such as an enzymatic assay, may be another method to assess transfection success.

In the case of siRNA, success may be measured using a reporter gene or assaying mRNA (e.g., RT-PCR) or protein target levels (e.g., Western blotting). For additional siRNA-specific reporter options, see the *Protocols and Applications Guide* chapter on RNA interference.

If multiple assays will be performed, make sure the techniques you choose are compatible with all assay chemistries. For example, if lysates are made from transfected cells, the lysis buffer used ideally would be compatible with all subsequent assays. In addition, if cells are needed for propagation after assessment, make sure to retain some viable cells for passage after the assay.

III. Factors Influencing Transfection Efficiency

With any transfection reagent or method, cell health, degree of confluency, number of passages, contamination, and DNA quality and quantity are important parameters that can greatly influence transfection efficiency. Note that with any transfection reagent or method used, some cell death will occur.

A. Cell Health

Cells should be grown in medium appropriate for the cell line and supplemented with serum or growth factors as needed for viability. Contaminated cells and media (e.g., contaminated with yeast or mycoplasma) should never be used for transfection. If cells have been compromised in any way, discard them and reseed from a frozen, uncontaminated stock. Make sure the medium is fresh if any components are unstable. Medium lacking necessary factors can harm cell growth. Be sure the 37°C incubator is supplied with CO_2 at the correct percentage (usually 5–10%) and kept at 100% relative humidity.

If there are any concerns about what type of culture medium or CO_2 levels are needed for your cell line of interest, consult the American Type Culture Collection [ATCC] web site.

B. Confluency

As a general guideline, transfect cells at 40–80% confluency. Too few cells will cause the culture to grow poorly without cell-to-cell contact. Too many cells results in contact inhibition, making cells resistant to uptake of foreign DNA. Actively dividing cells take up introduced DNA better than quiescent cells.

C. Number of Passages

Keep the number of passages low (<50). In addition, the number of passages for cells used in a variety of experiments should be consistent. Cell characteristics can change over time with immortalized cell lines, and cells may not respond to the same transfection conditions after repeated passages, resulting in poor expression.

D. DNA Quality and Quantity

Plasmid DNA for transfections should be free of protein, RNA, chemical and microbial contamination. Suspend ethanol-precipitated DNA in sterile water or TE buffer to a final concentration of 0.2–1mg/ml. The optimal amount of DNA to use in the transfection will vary widely, depending on the type of DNA, transfection reagent, target cell line and number of cells.

IV. Optimization of Transfection Efficiency

You will need to optimize specific transfection conditions to achieve the desired transfection efficiencies. Important parameters to consider are the charge ratio of cationic lipid transfection reagent to DNA, amount of transfected nucleic acid, length of time cells are exposed to the transfection reagent and presence or absence of serum. Reporter genes are useful to determine optimal conditions. The transfection efficiency achieved using any transfection reagents varies depending on the cell type being transfected and transfection conditions used.

A. Charge Ratio of Cationic Transfection Reagent to DNA

The amount of positive charge contributed by the cationic lipid component of the transfection reagent should equal or exceed the amount of negative charge contributed by the phosphates on the DNA backbone, resulting in a net neutral or positive charge on the multilamellar vesicles associating with the DNA. Charge ratios of 1:1 to 2:1 TransFast™ Reagent:DNA have worked well with various cultured cells, but ratios outside of this range may be optimal for other cell types or applications. See the *TransFast™ Transfection Reagent Technical Bulletin* #TB260 for more details.

B. DNA or RNA

The optimal amount of DNA or RNA will vary depending on the type of nucleic acid, number of cells, culture dish size and target cell line used. For example, HEK-293 cells are optimally transfected with 0.25µg of pGL3-Control Vector (Cat.# E1741) using TransFast™ Reagent at a 2:1 ratio in a 24-well plate. In contrast, the same cells are optimally transfected with 0.55µg of DNA using the FuGENE® HD Transfection Reagent at a 3:1 ratio in the same well size. For other cell lines, we suggest testing the DNA amounts given in Table 12.1.

Increasing the quantity of transfected DNA significantly may not yield better results. In fact, if initial transfection results are satisfactory, a reduced DNA quantity can be tested (while keeping the optimal reagent:DNA ratio constant). Often a range of DNA concentration is suitable for transfection. However, if the DNA concentration is below or above this range, transfection efficiencies will decrease. If there is too little DNA, the experimental response may not be present. If there is too much DNA, the excess can be toxic to cells. Calibrate the system using a test plasmid with reporter gene function.

C. Time

Traditionally, transfection reagents must be in contact with cells for some period of time, then additional medium is added or the medium is replaced to help minimize toxic effects of the reagent. The optimal transfection time depends on the cell line, transfection reagent and nucleic acid used. For the FuGENE® HD Transfection Reagent, which is one of the more gentle methods of DNA transfection into cells, there is no need to add additional medium or replace the medium after transfection.

For initial tests with liposomal reagents that require adding or replacing the medium, use a one-hour transfection interval, and test transfection times of 30 minutes to 4 hours (Figure 12.4) or even overnight, depending on the reagent used. Monitor cell morphology during the transfection interval, particularly when cells are maintained in serum-free medium because some cell lines lose viability under these conditions. The transfection time with the TransFast™ Reagent is usually significantly shorter than that required with other cationic lipid compounds and can be decreased to as little as 30 minutes with certain cell lines. In addition to saving time, this shortened transfection time may significantly reduce the risk of cell death.

Table 12.1. Suggested DNA Amounts to Use for Optimization.

Transfection Reagent	DNA Amount to Test	Reagent:DNA Ratios to Test	Culture Dish Size
FuGENE® 6 Transfection Reagent	0.04–0.2µg	4:1, 3.5:1, 3:1, 2.5:1, 2:1 and 1.5:1	96-well plate
FuGENE® HD Transfection Reagent	0.04–0.2µg	4:1, 3.5:1, 3:1, 2.5:1, 2:1 and 1.5:1	96-well plate
TransFast™ Reagent	0.25, 0.50, 0.75, 1µg	2:1 and 1:1	24-well plate

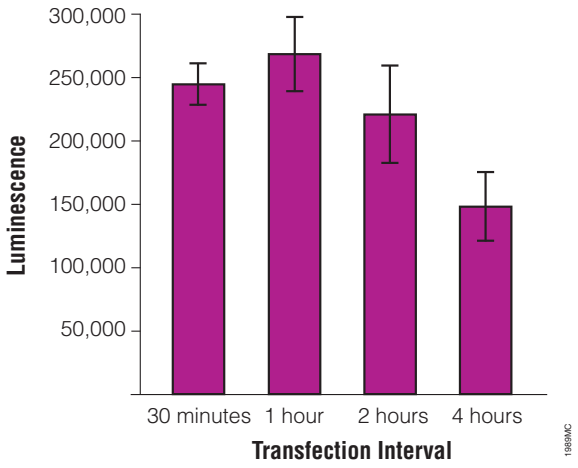

Figure 12.4. Effect of transfection interval on transfection of CHO cells using TransFast™ Reagent. CHO cells were transfected with 250ng of pGL3-Control DNA using TransFast™ Reagent at a 2:1 reagent:DNA charge ratio for various times in the absence of serum. All transfections were performed in 24-well plates, and cell lysates were harvested 2 days post-transfection. The results represent the mean of 6 replicates and are expressed as relative light units (RLU) per well.

D. Serum

Transfection protocols often require serum-free conditions for optimal performance because serum can interfere with many commercially available transfection reagents. The TransFast™ and FuGENE® HD Transfection Reagents can be used in transfection protocols in the presence of serum, allowing transfection of cell types or applications that require continuous exposure to serum (e.g., primary cells). Note that best results are obtained when variability is minimized among lots of serum.

E. Co-Transfection and Dual-Reporter Assays

While many people use a single reporter gene for their experimental system, a dual-reporter system has distinct advantages. A second reporter gene allows expression to be normalized for transfection efficiency and cell number. Small perturbations in growth conditions for transfected cells can dramatically affect gene expression. A second reporter helps to determine if the effects are due to the treatment of the cells or a response from the experimental reporter.

The Dual-Glo™ Luciferase Assay System (Cat.# E2920, E2940, E2980) is an efficient means of quantitating luminescent signal from two reporter genes in the same sample. In this system, firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferase activities are measured sequentially from a single sample in a homogeneous format. In the Dual-Glo™ System, both reporters yield linear assay responses (with respect to the amount of enzyme) and exhibit no endogenous activity in experimental host cells. In addition, the extended half-life of the reporter signals are ideal for use with multiwell assay formats.

The various Promega *Renilla* luciferase vectors can be used as control vectors when co-transfected with a firefly luciferase vector into which the promoter of interest is cloned. Alternatively, the firefly vector may be used as the control vector and the *Renilla* luciferase vector as the experimental construct. In a co-transfection experiment, it is important to realize that *trans* effects between promoters on co-transfected plasmids can potentially affect reporter gene expression (Farr and Roman, 1992). This is primarily of concern when either the control or experimental reporter vector or both contain very strong promoter/enhancer elements. The occurrence and magnitude of such effects will depend on several factors: 1) the combination and activities of genetic regulatory elements present on the co-transfected vectors; 2) the amount and relative ratio of experimental vector to control vector introduced into cells; and 3) the cell type transfected.

To help ensure independent genetic expression of experimental and control reporter genes, preliminary co-transfection experiments should be performed to optimize both the vector DNA amount and ratio of co-reporter vectors. Because the Promega *Renilla* luciferase vectors are designed for optimal expression, it is possible to use very small quantities of these vectors to provide low-level, constitutive co-expression of *Renilla* luciferase activity. This means that the ratio of firefly and *Renilla* luciferase vectors to test can range from 1:1 to 100:1 (or greater) to determine the optimal expression. The key to a dual-reporter system is to maximize expression of the experimental reporter while minimizing that of the control reporter. However, the expression level of the control reporter should be three standard deviations above background to be significant.

Additionally, experimental treatments may sometimes undesirably affect control reporter expression. This compromises the accuracy of experimental data interpretation; typically this occurs through sequences in the vector backbone, promoter or reporter gene itself. For this reason, Promega offers different promoter elements with either the same vector backbone, such as that of the pGL4.7 Vector series, or a choice of vector backbones, available with the synthetic *Renilla* luciferase vectors (phRL and phRG), to select the most reliable co-reporter vector for your system. In fact, due to extremely complicated cellular experimental conditions, testing several vectors is sometimes required before finding the best internal control for a particular experimental situation.

The strength of the promoter in your cell system is an important consideration. A more moderately expressing promoter like thymidine kinase [TK; e.g., pGL4.74[*hRluc*/TK] Vector (Cat.# E6921)] may be preferable to SV40 or CMV. Stronger promoters may exhibit more *trans* effects, cross-talk or regulatory problems. However, adjusting the ratio of experimental vector to control vector (e.g., using 100:1 or 200:1) may eliminate some of these issues.

For a discussion of other dual-reporter assays and vector offerings, see the *Protocols and Applications Guide* chapter on bioluminescent reporters and the complete listing of Promega vectors.

V. Promega Transfection Products

The following sections discuss the various Promega transfection reagents.

A. ProFection® Mammalian Transfection System

The ProFection® Mammalian Transfection System—Calcium Phosphate (Cat.# E1200) is a simple system containing two buffers: $CaCl_2$ and HEPES-buffered saline.

A precipitate containing calcium phosphate and DNA is formed by slowly mixing a HEPES-buffered phosphate solution with a solution containing calcium chloride and DNA. The DNA precipitate is distributed onto eukaryotic cells and enters cells through an endocytic-type mechanism. Calcium phosphate transfection may be used to produce long-term stable transfectants. It also works well for transient expression of transfected genes and can be used with most adherent cell lines.

Special Usage Notes:

- To increase transfection efficiency for some cell types, additional treatments such as glycerol (Frost and Williams, 1978; Wilson and Smith, 1997), dimethyl sulfoxide (DMSO; Lowy *et al.* 1978; Lewis *et al.* 1980), chloroquine (Luthman and Magnusson, 1983) and sodium butyrate (Gorman *et al.* 1983) may be added during incubation with the calcium phosphate/DNA precipitate. These treatments are thought to disrupt the phagocytic vacuole membrane, allowing the DNA to be released to the cytoplasm (Felgner, 1990). Since each of these chemicals is toxic to cells, transfection conditions for individual cell types, including reagent concentration and exposure time, must be carefully optimized.
- Alternatively, glycerol or DMSO may be added briefly (4–6 hours) after adding the DNA precipitate (Ausubel *et al.* 1995).

Additional Resources for the ProFection® Mammalian Transfection System

Technical Bulletins and Manuals

TM012 *ProFection® Mammalian Transfection System Technical Manual*

Citations

Haÿ, E. *et al.* (2004) Bone morphogenetic protein receptor IB signaling mediates apoptosis independently of differentiation in osteoblastic cells. *J. Biol. Chem.* **279**, 1650–58.

MC3T3-E1 (mouse preosteoblast) cells were plated at 5,000 cells/cm^2 in 100mm dishes the day before transfection. Cells were co-transfected with 15µg of plasmids expressing either wildtype or dominant negative bone morphogenetic protein-2 (BMP-2) and pSV-β-Galactosidase Control Vector at a 10:1 ratio in the presence of serum for 16 hours using the ProFection® Mammalian Transfection System—Calcium Phosphate. After the incubation, the medium was changed and the expression assessed 72 hours post-transfection by counting the number of β-galactosidase-positive cells and assessing the expression of BMP-2 by Western blot analysis.

PubMed Number: 14576167

McNamara-Schroeder, K.J. *et al.* (2001) The *Drosophila* U1 and U6 gene proximal sequence elements act as important determinants of the RNA polymerase specificity of small nuclear RNA gene promoters in vitro and in vivo. *J. Biol. Chem.* **276**, 31786–92.

Drosophila S2 cells were transfected with a U6 maxigene plasmid and a control firefly luciferase construct (derived from the pGL2 Basic Vector) using the ProFection® Mammalian Transfection System—Calcium Phosphate. Expression of the maxigene was confirmed by isolating total RNA and performing primer extension analysis with the aid of the Primer Extension System. Luciferase assays were performed on an aliquot of cells after lysis with Reporter Lysis Buffer.

PubMed Number: 11431466

B. TransFast™ Transfection Reagent

The TransFast™ Transfection Reagent (Cat.# E2431) comprises the synthetic cationic lipid, (+)-N,N [bis (2-hydroxyethyl)-N-methyl-N- [2,3-di(tetradecanoyloxy) propyl] ammonium iodide (Figure 12.5), and the neutral lipid DOPE.

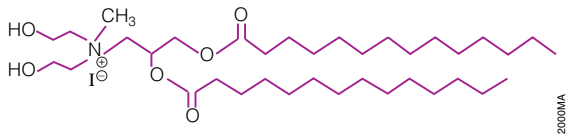

Figure 12.5. Structure of the TransFast™ cationic lipid.

The TransFast™ Reagent is supplied as a dried lipid film that forms multilamellar vesicles upon hydration with water. The TransFast™ Transfection Reagent delivers nucleic acid into eukaryotic cells in vitro and in vivo (Bennett *et al.* 1997) and performs well with many cell lines including NIH/3T3, CHO, HEK-293, K562, PC12, Jurkat and insect Sf9 cells. The TransFast™ Reagent combines the advantages of cationic liposome-mediated transfection with speed and ease-of-use to transfect cells for transient and stable expression.

Special Usage Notes:

- The TransFast™ Reagent can be used in the presence of serum, allowing transfection of cell types that are serum-sensitive, such as primary cell cultures.
- Prepare the TransFast™ Reagent the day before transfection, because it needs to be frozen before the initial use.
- There are separate protocols to transfect adherent and suspension cells using the TransFast™ Reagent.

Additional Resources for TransFast™ Transfection Reagent

Technical Bulletins and Manuals

TB260 *TransFast™ Transfection Reagent Technical Bulletin*

Promega Publications

Transfecting a human neuroblastoma cell line with Monster Green™ Fluorescent Protein

Using bioluminescent reporter genes to optimize shRNA target sites for RNAi of the *bcr/abl* gene

A comparison of pCI-neo Vector and pcDNA4/HisMax Vector

TransFast™ Transfection Reagent update

An efficient new transfection reagent for eukaryotic cells: TransFast™ Transfection Reagent

Citations

Boggs, K. and Reisman, D. (2007) C/EBPbeta participates in regulating transcription of the p53 gene in response to mitogen stimulation. *J. Biol. Chem.* **282**, 7982–90.

To explore further the role of C/EBPb isoforms in regulating p53 expression during the cell cycle, the 1.7kb murine p53 promoter was cloned into the pGL3-Basic Vector. Using TransFast™ Reagent, Swiss3T3 and 6629 (C/EBPb-null) cells were transfected using 0.1–0.75µg of pGL3-1.7-kb p53 promoter construct with or without co-transfection of 0.25µg of C/EBPb-2 and 50ng of pRL-TK Vector as an internal control. Twenty-four hours post-transfection, cells were harvested and assayed for luciferase activity, normalizing reporter activity to *Renilla* luciferase. Site-directed mutagenesis was used to mutate or delete the –972/–953 *cis*-acting element carrying the C/EBPb-binding site within the p53 promoter, and 0.1–0.75µg of each mutant construct was transfected into Swiss3T3 cells with or without co-transfection of 0.25µg of C/EBPb-2 and 50ng of pRL-TK Vector. The cells were harvested 24 hours post-transfection and assayed for reporter activity, normalizing to pRL-TK Vector activity.

PubMed Number: 17244625

Guindalini, C. *et al.* (2006) A dopamine transporter gene functional variant associated with cocaine abuse in a Brazilian sample. *Proc. Natl. Acad. Sci. U S A* **103**, 4552–7.

The authors investigated the effect of various polymorphisms in the dopamine transporter gene (*SLC6A3*) on susceptibility to cocaine addiction. Genotyping of various polymorphisms in cocaine abusers and control subjects revealed a potential association of the int8 VNTR with cocaine abuse. Seven alleles of the int8 VNTR were sequenced. Various allelic sequences then were cloned into a modified phRL-SV40 *Renilla* luciferase reporter vector and transfected into the mouse SN4741 cell line, which expresses the dopamine transporter, and the effects on reporter activity were monitored. Sequences of two alleles then were cloned into a pGL3 Promoter Vector construct and transfected into JAP cells. The cells were challenged with various amounts of cocaine or KCl and forskolin, and the effect on reporter activity was monitored. The TransFast™ Reagent was used for transfections at a 2:1 reagent:DNA ratio.

PubMed Number: 16537431

C. FuGENE® HD Transfection Reagent

The FuGENE® HD Transfection Reagent (Cat.# E2311) is a novel, nonliposomal formulation to transfect DNA into a wide variety of cell lines with high efficiency and low toxicity. The protocol does not require removal of serum (including up to 100% serum) or culture medium and does not require washing or changing of medium after introducing the reagent:DNA complex. Additionally, the FuGENE® HD reagent supports transfection in chemically defined media and does not contain any animal derived components. For more information about transfection conditions using the FuGENE® HD Transfection Reagent, visit the FuGENE® HD Protocol Database.

Additional Resources for the FuGENE® HD Transfection Reagent

Technical Bulletins and Manuals

TM328 *FuGENE® HD Transfection Reagent Technical Manual*

Promega Publications

Reverse Transfection Using FuGENE® 6 and FuGENE® HD

Optimize Transfection of Cultured Cells

Citations

Jiwani, S. *et al.* (2013) Bone morphogenetic protein receptor IB signaling mediates apoptosis independently of differentiation in osteoblastic cells. *J. Bacteriol.* **195**, 708–16.

The authors transiently transfected 2 × 10^5 HeLa cells grown on coverslips in a six-well plate using the FuGENE® HD Transfection Reagent, 2.5µg of DNA and a reagent:DNA ratio of 8:2.5.

PubMed Number: 23204471

de Jong, M.F. *et al.* (2013) Sensing of bacterial type IV secretion via the unfolded protein response *mBio* **epub**, e00418–12.

FuGENE® HD Transfection Reagent was used to transiently transfect HeLa cells seeded on 12mm coverslips in 24-well plates. Cells were seeded at a concentration of 5 × 10^4 cells/well and transfected after 24 hours using a 2:1 ratio of FuGENE® HD reagent to DNA (4µl of reagent, 2µg of DNA). Transiently transfected cells were used for confocal microscopy. For luciferase assays, HEK293 cells were seeded into 48-well plates at 40% confluency, and 24 hours later, FuGENE® HD reagent was used to transfect the cells with one of a variety of constructs.

PubMed Number: 23422410

D. FuGENE® 6 Transfection Reagent

The FuGENE® 6 Transfection Reagent (Cat.# E2691) is a nonliposomal reagent that transfects DNA into a wide variety of cell lines with high efficiency and low toxicity. The protocol does not require removal of serum or culture medium and does not require washing or changing of medium after introducing the reagent/DNA complex. For more information about transfection conditions using the FuGENE® 6 Transfection Reagent, visit the Transfection Assistant.

Additional Resources for the FuGENE® 6 Transfection Reagent

Technical Bulletins and Manuals

TM350 *FuGENE® 6 Transfection Reagent Technical Manual*

Promega Publications

Reverse Transfection Using FuGENE® 6 and FuGENE® HD

Citations

Quaynor, S.D. *et al.* (2013) Delayed puberty and estrogen resistance in a woman with estrogen receptor α variant. *N. Engl. J. Med.* **369(2)**, 164-71.

In this study, the FuGENE® 6 Transfection Reagent was used to perform transient transfection of COS-7 cells. Plasmids containing mutated or non-mutated copies of the estrogen receptor gene ESR1 were transfected together with luciferase reporter constructs containing estrogen response elements upstream of the luciferase gene. Transactivation of the estrogen response element was reduced in the mutated estrogen receptor compared with the nonmutated receptor.

PubMed Number: 23841731

Raponi, M. *et al.* (2012) Evolutionary constraint helps unmask a splicing regulatory region in BRCA1 exon 11. *PLOS ONE* **7**, e37255.

In this study, MCF7 human breast adenocarcinoma cells were transfected with plasmid constructs using FuGENE® 6 Transfection Reagent. Cells were grown to 50% confluence and transfected with 2µg of DNA at a 3:2 ratio of FuGENE® 6 reagent:DNA.

PubMed Number: 22615956

VI. General Transfection Protocol

A. Preparation of Cells for Transfection

Trypsinization Procedure to Remove Adherent Cells

Trypsinizing cells prior to subculturing or cell counting is an important technique for successful cell culture. The following technique works consistently well when passaging cells.

Materials Required:

- 1X trypsin-EDTA solution
- 1X PBS or 1X HBSS
- adherent cells to be subcultured
- appropriate growth medium (e.g., DMEM) with serum or growth factors or both added
- culture dishes, flasks or multiwell plates, as needed
- hemocytometer

1. Prepare a sterile trypsin-EDTA solution in a calcium- and magnesium-free salt solution such as 1X PBS or 1X HBSS. The 1X solution can be frozen and thawed for future use, but trypsin activity will decline with each freeze-thaw cycle. The trypsin-EDTA solution may be stored for up to 1 month at 4°C.
2. Remove medium from the tissue culture dish. Add enough PBS or HBSS to cover the cell monolayer: 2ml for a 150mm flask, 1ml for a 100mm plate. Rock the plates to distribute the solution evenly. Remove and repeat the wash. Remove the final wash. Add enough trypsin solution to cover the cell monolayer.
3. Place plates in a 37°C incubator until cells just begin to detach (usually 1–2 minutes).
4. Remove the flask from the incubator. Strike the bottom and sides of the culture vessel sharply with the palm of your hand to help dislodge the remaining adherent cells. View the cells under a microscope to check whether all cells have detached from the growth surface. If necessary, cells may be returned to the incubator for an additional 1–2 minutes.
5. When all cells have detached, add medium containing serum to cells to inactivate the trypsin. Gently pipet cells to break up cell clumps. Cells may be counted using a hemocytometer and/or distributed to fresh plates for subculturing.

Typically, cells are subcultured in preparation for transfection the next day. The subculture should bring the cells of interest to the desired confluency for transfection. As a general guideline, plate 5×10^4 cells per well in a 24-well plate or 5.5×10^5 cells for a 60mm culture dish for ~80% confluency the day of transfection. Change cell numbers proportionally for different size plates (see Table 12.2).

Table 12.2. Area of Culture Plates for Cell Growth.

Size of Plate	Growth Area[a] (cm^2)	Relative Area[b]
24-well	1.88	1X
96-well	0.32	0.2X
12-well	3.83	2X
6-well	9.4	5X
35mm	8.0	4.2X
60mm	21	11X
100mm	55	29X

[a]This information was calculated for Corning® culture dishes.

[b]Relative area is expressed as a factor of the total growth area of the 24-well plate recommended for optimization studies. To

determine the proper plating density, multiply 5 × 10^4 cells by this factor.

B. Preparation of DNA for Transfection

High-quality DNA free of nucleases, RNA and chemicals is as important for successful transfection as the reagent chosen. See the *Protocols and Applications Guide* chapter on DNA purification for information about purifying transfection-quality DNA.

In the case of a reporter gene carried on a plasmid, a promoter appropriate to the cell line is needed for gene expression. For example, the CMV promoter works well in many mammalian cell lines but has little functionality in plants. The best reporter gene is one that is not endogenously expressed in the cells. Firefly luciferase, *Renilla* luciferase, click beetle luciferase, chloramphenicol aceyltransferase and β-galactosidase fall into this category. Vectors for all five reporters are available from Promega. See the Reporter Vectors web page for more information on our wide array of reporter plasmids.

C. Optimization of Transfection

In previous sections, we discussed factors that influence transfection success. Here we present a method to optimize transfection of a particular cell line with a single transfection reagent. For more modern lipid-based reagents such as the FuGENE® HD Transfection Reagent, we recommend using 100ng of DNA per well of a 96-well plate at reagent:DNA ratios of 4:1, 3.5:1, 3:1, 2.5:1, 2:1 and 1.5:1. Figure 12.6 outlines a typical optimization matrix. When preparing the FuGENE® HD Transfection Reagent:DNA complex, the incubation time may require optimization; we recommend 0–15 minutes. Incubations longer than 30 minutes may result in decreased transfection efficiency. See Technical Manual #TM328.

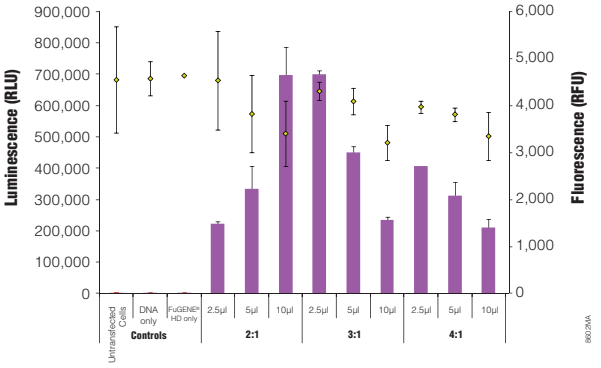

Figure 12.6. Transfection optimization using the FuGENE® HD Transfection Reagent. FuGENE® HD Transfection Reagent: DNA complexes were formed by combining 2µg of pGL4.13[*luc2*/SV40] Vector, enough FuGENE® Reagent to achieve the indicated ratios and cell culture medium to a final volume of 100µl. To HEK-293 cells in a 96-well plate (2 × 10^4 cells/100µl/well), 2µl, 5µl or 10µl of this complex was added per well. Control wells contained untransfected cells, cells transfected with DNA only (no FuGENE® HD Reagent) or cells transfected with FuGENE® HD Transfection Reagent only (no DNA). Firefly luciferase activity was measured using the ONE-Glo™ Luciferase Assay System, and cell viability was determined using the CellTiter-Fluor™ Cell Viability Assay. Luminescence and fluorescence were quantified using the GloMax®-Multi+ Detection System, and results are expressed as relative light units and relative fluorescence units (RFU), respectively.

For traditional reagents, such as the TransFast™ Reagent, we recommend testing various amounts of transfected DNA (0.25, 0.5, 0.75 and 1µg per well in a 24-well plate) at two charge ratios of lipid reagent to DNA (2:1 and 4:1; see Figure 12.7 and Technical Bulletin #TB260). This brief optimization can be performed using a transfection interval of one hour under serum-free conditions. One 24-well culture plate per reagent is required for the brief optimization with adherent cells (3 replicates per DNA amount).

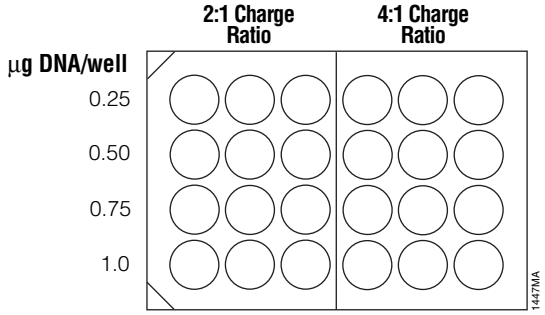

Figure 12.7. Suggested plating format for initial optimization of cationic lipid transfection conditions.

A more thorough optimization can be performed to screen additional charge ratios, time points and effects of serum-containing medium at the DNA amounts found to be optimal during initial optimization studies. One hour or two hours for the transfection interval is optimal for

many cell lines. In some cases, however, it may be necessary to test charge ratios and transfection intervals outside of these ranges to achieve optimal gene transfer.

Both DEAE-dextran and calcium phosphate work well with larger cell cultures (e.g., 100mm culture dish or T75 flask). General guidelines for DNA amount and time for calcium phosphate-mediated transfection are given in Table 12.3.

Table 12.3. Guidelines for Calcium Phosphate Transfection.

Size of Culture Dish	Amount of DNA Transfected	Incubation Time with Transfection Complex[a]
60mm	6–12µg DNA	4–16 hours
100mm	10–20µg	4–16 hours

[a]If the cells are sensitive to the reagent, incubate for no more than 4 hours. Incubation time can be longer but will need to be optimized for the individual cell line.

Some transfection methods require removal of medium with reagent after incubation; others do not. Read the technical literature accompanying the selected transfection reagent to learn which method is appropriate for your system. However, if there is excessive cell death during transfection, consider decreasing time of exposure to the transfection reagent, decreasing the amounts of DNA and reagent added to cells, plating additional cells and removing the reagent after the incubation period and adding complete medium.

D. Endpoint Assay

Many transient expression assays use lytic reporter assays like the Dual-Luciferase® Assay System (Cat.# E1910) or Bright-Glo™ Assay System (Cat.# E2610) 24 hours post-transfection. However, the assay time frame can vary (24–72 hours after transfection), depending on protein expression levels. Reporter-protein assays use colorimetric, radioactive or luminescent methods to measure enzyme activity present in a cell lysate. Some assays (e.g., Luciferase Assay System) require that cells are lysed in a buffer after removing the medium, then mixed with a separate assay reagent to determine luciferase activity. Others are homogeneous assays (e.g., Bright-Glo™ Assay System) that include the lysis reagent and assay reagent in the same solution and can be added directly to cells in medium. Examine the reporter assay results and determine where the greatest expression (highest reading) occurred. These are the conditions to use with your constructs of interest.

Other assays include histochemical staining of cells (determining the percentage of cells that are stained in the presence of the reporter gene substrate; Figure 12.8), fluorescence microscopy (Figure 12.9) or cell sorting if using a fluorescent reporter like the Monster Green® Fluorescent Protein phMGFP Vector (Cat.# E6421).

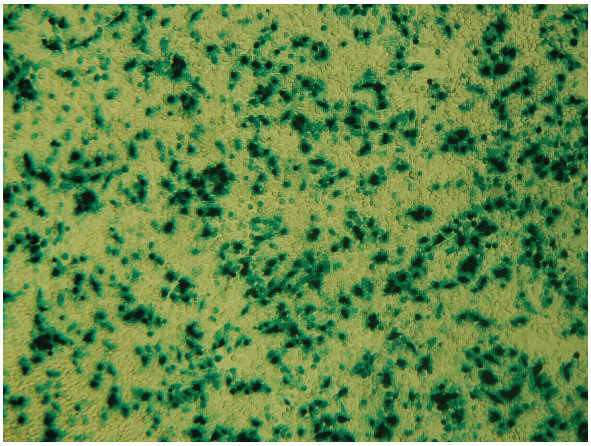

Figure 12.8. Histochemical staining of RAW 264.7 cells for β-galactosidase activity. RAW 264.7 cells were transfected using 0.1µg DNA per well and a 3:1 ratio of FuGENE® HD to DNA. Complexes were formed for 5 minutes prior to applying 5µl of the complex mixture to 50,000 cells/well in a 96-well plate. Twenty-four hours post-transfection, cells were stained for β-galactosidase activity using X-gal. Data courtesy of Fugent, LLC.

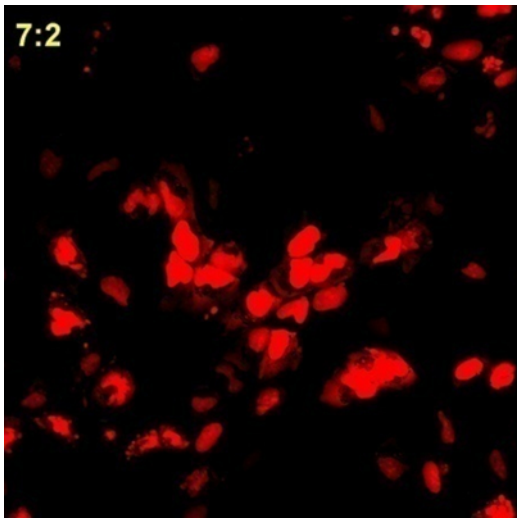

Figure 12.9. Fluorescent microscopy of U-2 OS cells transfected with a HaloTag®-NLS$_3$ Vector using the FuGENE® HD Transfection Reagent. U-2 OS cells were transiently transfected with 0.5µg of the HaloTag®-NLS$_3$ Vector, which encodes the HaloTag® protein with three copies of a nuclear localization signal (NLS), at a 3.5:1 FuGENE® HD Reagent:DNA ratio. Twenty-four hours post-transfection, cells were labeled using the HaloTag® TMR Ligand and the live-cell imaging protocol described in the *HaloTag ® Technology: Focus on Imaging Technical Manual* #TM260. The resulting fluorescence was visualized by microscopy.

Assaying relative expression using the HaloTag® technology provides new options for rapid, site-specific labeling of proteins in living cells and in vitro. The ability to create labeled HaloTag® fusion proteins with a wide range of optical properties and functions allows researchers to image and localize labeled HaloTag® protein fusions in live- or fixed-cell populations and isolate and analyze

HaloTag® protein fusions and protein complexes. Several ligands are available for this system with new options being added regularly. For more information on this labeling technology, see the *Protocols and Applications Guide* chapter on cell labeling.

VII. Stable Transfection

A. Selection of Stably Transfected Cells

Optimization for stable transfection begins with successful transient transfection. However, cells should be transfected with a plasmid containing a gene for drug resistance, such as neomycin phosphotransferase (*neo*). As a negative control, transfect cells using DNA that does not contain the drug-resistance marker.

1. Prior to transfection, determine the selective drug concentration required to kill untransfected cells (kill curve; Ausubel *et al.* 1995).
2. Forty-eight hours after transfection, trypsinize adherent cells and replate at several different dilutions (e.g., 1:100, 1:500) in medium containing the appropriate selection drug. For effective selection, cells should be subconfluent since confluent, nongrowing cells are very resistant to the effects of antibiotics like G-418.
3. For the next 14 days, replace the drug-containing medium every 3 to 4 days.
4. During the second week, monitor cells for distinct "islands" of surviving cells. Drug-resistant clones can appear in 2–5 weeks, depending on the cell type. Cell death should occur after 3–9 days in cultures transfected with the negative control plasmid.
5. Transfer individual clones by standard techniques (e.g., using cloning cylinders) to 96-well plates, and continue to maintain cultures in medium containing the appropriate drug.

Table 12.4 provides an overview of commonly used antibiotics to select and maintain stable transfectants.

B. Calculating Stable Transfection Efficiency

The following procedure may be used to determine the percentage of stable transfectants obtained.

Note: The stained cells will not be viable after this procedure.

Materials Required:

- methylene blue
- methanol
- cold deionized water
- light microscope

1. After approximately 14 days of selection in the appropriate drug, monitor the cultures microscopically for the presence of viable cell clones. When distinct "islands" of surviving cells are visible and nontransfected cells have died out, proceed with Step 2.
2. Prepare stain containing 2% methylene blue in 50–70% methanol.
3. Remove the growth medium from cells by aspiration.
4. Add to cells sufficient stain to cover the bottom of the dish.
5. Incubate for 5 minutes.
6. Remove the stain, and rinse gently under deionized cold water. Shake off excess moisture.
7. Allow the plates to air-dry. The plates can be stored at room temperature.
8. Count the number of colonies, and calculate the percent of transfectants based on the cell dilution and original cell number.

For further information on stable transfections, see Ausubel *et al.* 1995.

Table 12.4. Antibiotics Used to Select Stable Transfectants.

Antibiotic	Resistance Gene	Working Concentration	Stock Solution
G-418 or Geneticin®	aminoglycoside (e.g., neomycin) phosphotransferase	G-418 is often used for initial selection at 500µg/ml with a range of 50–1,000µg/ml	50mg/ml in either water or 100mM HEPES (pH 7.3); the latter buffer helps maintain culture medium pH
Hygromycin (Hygro)	*hph*	10–400µg/ml	100mg/ml in water
Puromycin (Puro)	*pac*	1–10µg/ml	10mg/ml in water or HEPES buffer (pH 7.0)

VIII. Composition of Solutions

1X HBSS (Hanks Balanced Salt Solution)

5mM KCl
0.3mM KH_2PO_4
138mM NaCl
4mM $NaHCO_3$
0.3mM Na_2HPO_4
5.6mM D-glucose

The final pH should be 7.1.

1X PBS

137mM NaCl
2.7mM KCl
4.3mM Na_2HPO_4
1.47mM KH_2PO_4

The final pH should be 7.1.

1X Trypsin-EDTA solution

0.05% trypsin (w/v)
0.53mM EDTA

Dissolve in a calcium- and magnesium-free salt solution such as 1X PBS or 1X HBSS.

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