The QIAGEN® Transfection Resource Book

Second Edition

QIAGEN

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Transfection Tools — your online guide to transfection

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Contents

1. Introduction to Transfection

Transfection — the delivery of foreign molecules such as DNA and RNA into eukaryotic cells — has become a powerful tool to study and control gene expression. Cloned genes can be transfected into cells for biochemical characterization, mutational analyses, investigation of the effects of gene expression on cell growth, investigation of gene regulatory elements, and to produce a specific protein for purification.

Many parameters influence transfection efficiency, including cell culture conditions, vector characteristics and quality, and the transfection technology used. *The QIAGEN® Transfection Resource Book* provides useful and practical information on transfection of animal cell cultures with DNA and RNA, including information on high-throughput transfection and optimization of transfection conditions, as well as cell culture guidelines and protocols, vector information, and a troubleshooting guide. This chapter provides an introduction to different types of transfection, general cell culture considerations, and different transfection technologies. More detailed information on transfection of cells with DNA and RNA, as well as information on high-throughput transfection, is provided in later chapters.

Transient and stable transfection

Generally speaking, there are two different types of transfection — transient transfection and stable transfection (1–4). These two types of transfection are suited for different experimental applications, and have different vector requirements.

Transient transfection

In transient transfection, many copies of the nucleic acid of interest (DNA or RNA) are present in the cell following transfection, but the introduced nucleic acid does not integrate into the chromosomes. Transient transfection often results in high levels of expression of the introduced gene, but only for a few days following transfection. Expression of transfected genes can typically be analyzed within 24–96 hours after transfection, depending on the vector used.

Transient transfection can be achieved using both DNA and RNA vectors. For DNA vectors, transient transfection is most efficient when supercoiled plasmid is used.

Transient transfection can be used with genetic reporter systems for analysis of promoter and other regulatory elements, but is generally not suitable for studies using DNA vectors with inducible promoters. See "Genetic Reporter Systems", pages 51–52 for further information.

Stable transfection

Stable (or permanent) transfection is only suitable for transfection of DNA vectors. In this method, the transfected DNA is either integrated into the chromosomal DNA or maintained as an episome. To obtain a stably transfected homogenous cell population, a selectable marker gene (usually a eukaryotic antibiotic-resistance gene) is transfected into cells together with the gene of interest (see "Selection of stably transfected cells", pages 22–23, for further information).

Integration of a DNA vector into chromosomal DNA is a rare event. Approximately one in 10^4 transfected cells will stably integrate DNA; however, the efficiency varies with cell type (2). Integration is most efficient when linear DNA is used.

Stably transfected cells integrate one or a few copies of the introduced DNA, and consequently the level of expression of the transfected gene is generally lower than in transiently transfected cells. However,

depending on the gene and cell type used, the transfected gene will be expressed for long periods of time, and sometimes indefinitely.

Stable transfection can be used with genetic reporter systems for analysis of promoter and other regulatory elements and with vectors containing inducible promoters. Further information on genetic reporter systems and inducible promoters is provided in "Genetic Reporter Systems", pages 51–52, and "Promoters for Gene Expression", pages 49–50. More information on selection of stably transfected cells is provided in "Selection of stably transfected cells", pages 22–23, and a protocol is provided on page 46.

Cell culture considerations for successful transfection

The choice of which cell type to use for a transfection experiment is critical and is influenced by different factors. For example, primary animal cell cultures most closely mimic the in vivo situation but are difficult to work with, while continuous cell lines are easier to work with but have undergone multiple genetic changes and so may not represent the in vivo situation (see "Introduction to animal cell culture", pages 36–37). In addition, the biological properties of a cell type must be taken into consideration. For example, some promoters function differently in different cell types and some cell types are not well suited to particular transfection technologies.

Regardless of the cell type used, a healthy cell culture lays the foundation for successful transfection. We strongly recommend subculturing cells for a minimum of 24 hours before transfection. This ensures normal cell metabolism and increases the likelihood of nucleic acid uptake. Best results are obtained using an appropriate cell confluence level. Contamination with bacteria (e.g., mycoplasma) and fungi should be avoided, since this can drastically alter transfection results. We recommend using cells with a low passage number (<50 splitting cycles) to ensure that the cell genotype does not become altered.

Different cells or cell types have very specific medium, serum, and supplement requirements, and the most suitable medium for each cell type must be determined experimentally. In general, the presence of serum in culture medium enhances transfection. However when transfecting cells with RNA, we recommend performing the transfection procedure in the absence of serum to avoid possible contamination with RNases.

Further information on culturing animal cells can be found in "Animal Cell Culture", pages 36–47. In addition, the Transfection Tools web site — **www.qiagen.com/transfectiontools/** — has a searchable database of cell lines and primary cells successfully transfected using QIAGEN Transfection Reagents that includes information on cell culture conditions used for different cell types.

Transfection technologies

The choice of transfection technology strongly influences transfection results. In general, a transfection technology should be fast and easy to perform, give high transfection efficiencies and reproducible results, and cause minimal cytoxicity.

Classical transfection technologies that are widely used include the DEAE-dextran method, the calcium-phosphate method, electroporation, and liposome-mediated transfection. Each technology has advantages and disadvantages, as discussed in more detail below.

QIAGEN offers two advanced transfection technologies, activated-dendrimer technology and non-liposomal–lipid technology. These newer technologies offer significant advantages over classical methods, providing high transfection

efficiencies and reproducible results. Activated-dendrimer and non-liposomal–lipid technologies are also discussed in more detail below.

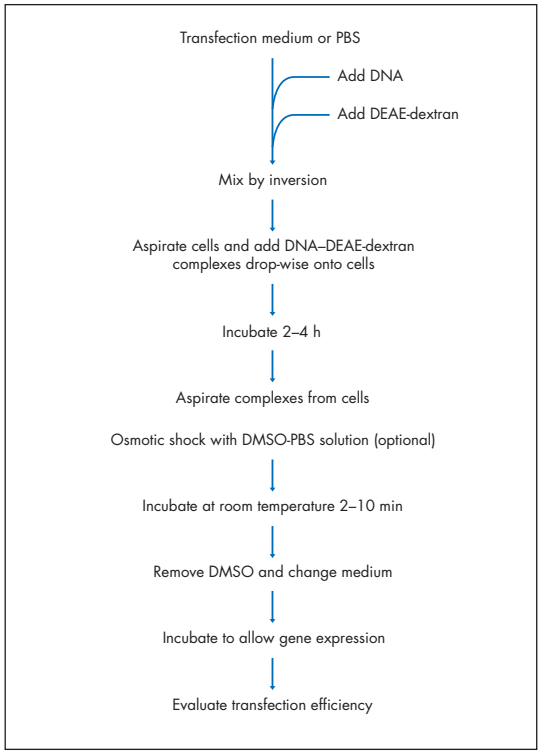
DEAE-dextran

Diethylaminoethyl (DEAE)-dextran was introduced in 1965 (5) and is one of the oldest methods for introducing nucleic acids into cultured mammalian cells. The positively charged DEAE-dextran molecule interacts with the negatively charged phosphate backbone of the nucleic acid. The DNA–DEAE-dextran complexes appear to adsorb onto the cell surface and be taken up by endocytosis. The advantages of this technique are its relative simplicity and reproducibility of results. Disadvantages include cytotoxic effects and the fact that the amount of serum in the culture medium must be temporarily reduced during the transfection procedure. In addition, the DEAE-dextran method is best suited for transient transfection only.

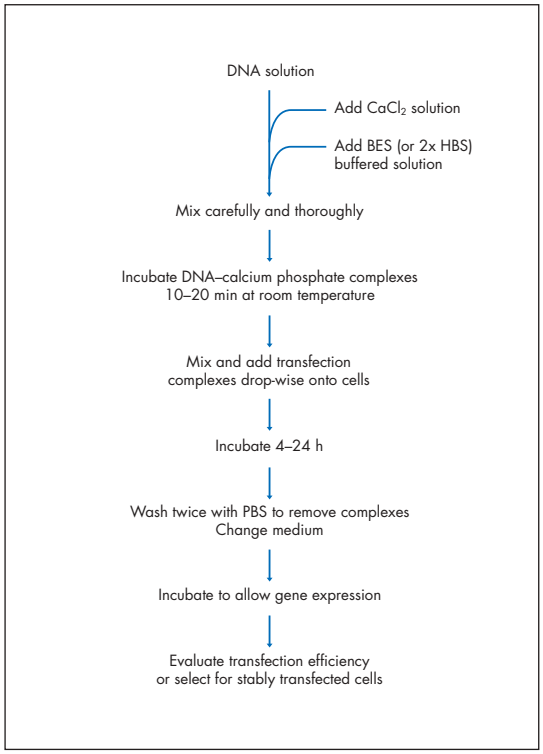
Calcium phosphate

The calcium-phosphate method was first used in 1973 to introduce adenovirus DNA into mammalian cells (6). The principle involves mixing DNA in a phosphate buffer with calcium chloride. The resulting calcium-phosphate–DNA complexes adhere to the cell membrane and enter the cytoplasm by endocytosis. Advantages of calcium-phosphate–based transfection are its easy handling and, compared with the DEAE-dextran method, its much higher suitability for stable transfections. However, a common disadvantage is low reproducibility, which is mainly caused by variation in transfection-complex size and shape. These variations can be caused by minor changes in the pH of the solutions used for the transfection, as well as the manner in which these solutions are combined. A further drawback of the calcium-phosphate method is that some cell types, including primary cells, may resist this form of DNA transfer.

DEAE-Dextran Method*



Calcium-Phosphate Method*



** These flowcharts of commonly used transfection methods are representative of the standard protocols currently cited in the literature and used in most transfection laboratories. Variations of these protocols also exist.*

Electroporation

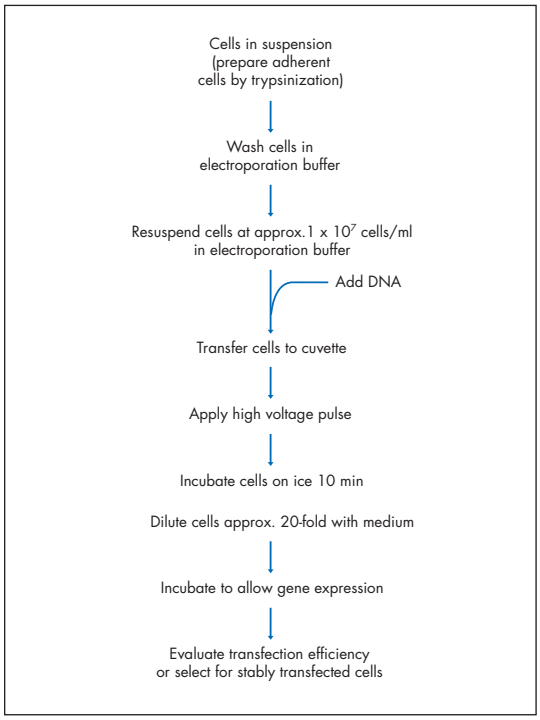
The use of high-voltage pulses to introduce DNA into cultured cells was first established by Wong and Neumann using fibroblasts (7, 8). Cells in a suitable cuvette are subjected to a short high-voltage pulse that causes the membrane potential of the cells to break down. As a result, pores are formed through which macromolecules such as DNA can enter. The main advantage of electroporation is its applicability for transient and stable transfection of all cell types. However, a disadvantage is that approximately 5-fold greater quantities of DNA and cells are needed than in either DEAE-dextran or calcium phosphate methods. A major drawback of electroporation is the high cell mortality that can result in the death of up to 50–70% of the cells. In addition, the optimal settings for voltage, capacitance, pulse length, and gap width are cell-type dependent, and it is necessary to repeat the electroporation experiment a number of times to optimize the electroporation efficiency and cell viability.

Cationic liposomes

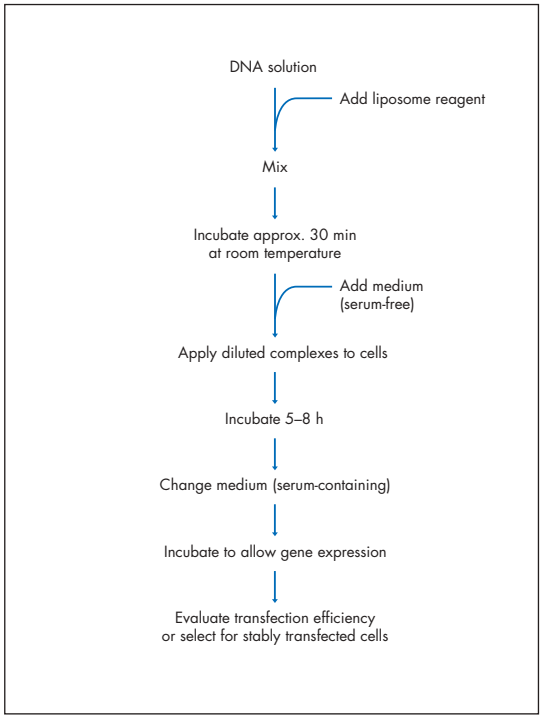
Liposomes were first introduced in 1987 by Felgner and coworkers (9). The liposomes currently in use typically contain a mixture of cationic and neutral lipids organized into lipid bilayer structures. Transfection-complex formation is based on the interaction of the positively charged liposome with the negatively charged phosphate groups of the nucleic acid. The uptake of the liposome–DNA complexes may be mediated by endocytosis.

Compared to the DEAE-dextran and calcium-phosphate methods, liposomes often offer higher transfection efficiency and better reproducibility. However, one drawback of liposome-mediated transfection is that the presence of serum during the transfection procedure often lowers the transfection efficiency. For this reason, serum is often omitted when transfecting with liposomes. In many cases, the absence of serum from the medium increases the cytotoxicity of the liposome. Another drawback of classical liposome-mediated transfection is that results

Electroporation Method*



Liposome-Mediated Transfection*



** These flowcharts of commonly used transfection methods are representative of the standard protocols currently cited in the literature and used in most transfection laboratories. Variations of these protocols also exist.*

can vary widely with cell type. This means that the relative performance of various liposome reagents with a specific cell line may be unpredictable.

Virus-mediated gene transfer

Infectious viruses can be modified and used to transfer a nonviral gene into cells (2, 10). For example, the natural ability of retroviruses to integrate into eukaryotic genomes is exploited to stably integrate a single copy of the recombinant viral genome into the host chromosome. Retroviral vectors are a useful tool for stable gene transfer into difficult-to-transfect cells such as primary cells or cells in vivo. However, these vectors are often not well suited for transient infection or introduction of large DNA fragments. Other options for virus-mediated gene transfer include adenovirus-based vectors. These vectors allow transfer of genetic information into non-dividing cells but, as they do not integrate into the host genome, only allow transient expression of introduced genes. Viral vectors based on baculovirus and vaccinia virus can be used for overexpression of proteins. Baculovirus systems are restrictive in that they only allow production of proteins in insect cells. In contrast, vectors based on vaccinia virus can be used with a wide range of mammalian cells and allow introduction of large DNA fragments. However, infected cells die within one or two days so this system is limited to transient protein production.

Procedures using viral-based vectors are often significantly more complicated and time-consuming than other transfection technologies. Additionally, viral vectors are potentially hazardous, and biological safety issues need to be considered carefully.

Biolistic particle delivery

This technique involves precipitating DNA onto microscopic heavy-metal particles (often gold) and then projecting the coated particles into cells using a ballistic device. Once retained by the cell, the DNA is gradually released and expressed. Biolistic bombardment can be used to obtain transient expression in human epithelial, fibroblast, and lymphocyte cell lines, as well as in primary cells (11).

Microinjection

Genes can be delivered into cells by direct microinjection of DNA into cell nuclei. The introduced DNA is occasionally integrated into the chromosomes. The number of cells that can be transfected by microinjection is limited, and the technique is used mainly to introduce genes into oocytes to engineer modified or transgenic animals (12).

Activated dendrimers

Dendrimers are highly branched spherical molecules in which branches terminating at charged amino groups radiate from a central core molecule (13). Due to controlled chemical synthesis, dendrimers have a very precise size and defined shape. Activation of newly synthesized dendrimers involves removal of some of the tertiary amines, resulting in a

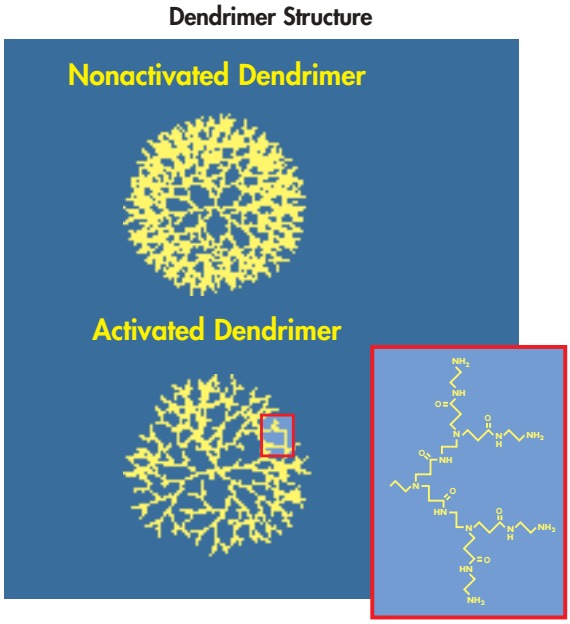


Figure 1. *Schematic diagram of an activated and non-activated dendrimer. A portion of the activated dendrimer molecule is enlarged to show the chemical structure of the molecular branches.*

Activated-Dendrimer–DNA Interaction

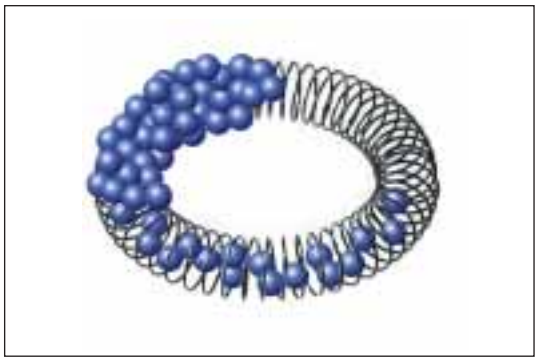


Figure 2. *Model of the activated-dendrimer–DNA complex. Activated dendrimers (purple spheres) interact with DNA (black) to form a ring-like (toroid-like) structure. The upper right section of the illustration shows naked DNA, the lower section shows the interaction between dendrimers and DNA inside the complex, and the upper left section shows the final complete coverage of DNA within the complex.*

molecule with a higher degree of flexibility (Figure 1). Activated dendrimers yield a transfection efficiency 2–3 orders of magnitude higher than non-activated dendrimers (14).

Activated dendrimers assemble DNA into compact structures through the interaction of negatively charged phosphate groups of nucleic acids with the positively charged amino groups of the dendrimers (Figure 2). The resulting activated-dendrimer–DNA complexes possess a net positive charge that enables binding to the negatively charged surface molecules of the cell membrane. The transfection complexes are taken up by nonspecific endocytosis. The reagent buffers the pH of the endosome, leading to pH inhibitition of endosomal nucleases, which ensures stability of the activated-dendrimer–DNA complexes. The defined size and shape of dendrimers ensures consistent transfection-complex formation and reproducibility of transfection results.

QIAGEN offers two activated-dendrimer reagents for efficient and reproducible transfection of cells with DNA — PolyFect and SuperFect Transfection Reagents. These reagents offer significant advantages over classical transfection technologies, such as higher transfection efficiencies (Figure 3), the ability to perform transfection in the presence of serum (Figure 4), and low cytotoxicity. For more information on the use of these reagents, see page 16 (PolyFect Reagent) and page 18 (SuperFect Reagent).

Comparison of PolyFect Reagent and Calcium Phosphate-Mediated Transfection

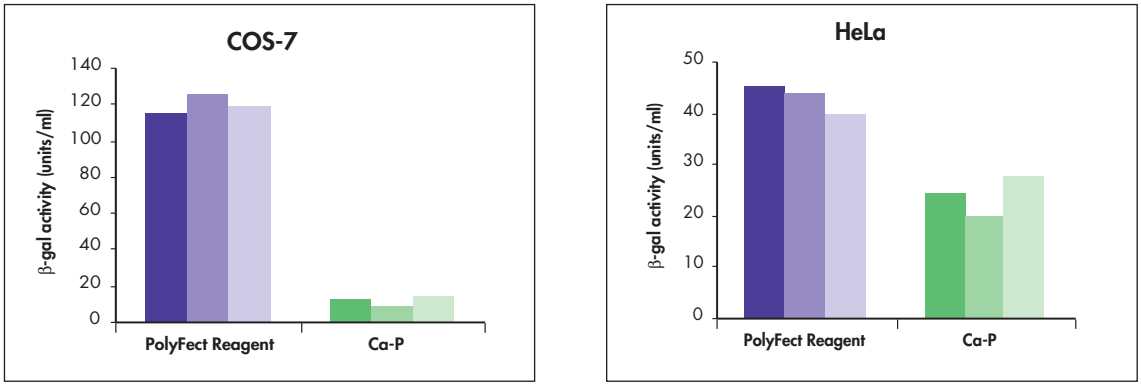


Figure 3. *Comparison of transfection efficiencies obtained using PolyFect Reagent and a calcium phosphate-mediated procedure. COS-7 and HeLa cells were transfected in 6-well plates with a β-galactosidase reporter plasmid using the appropriate protocol. For the calcium phosphate-mediated transfections, 6 µg plasmid DNA was used and the medium was changed after 5 h incubation. Transfections were performed in triplicate. β-galactosidase activity was measured 48 h post-transfection.*

Serum and DNA Quantity vs. Transfection Efficiency

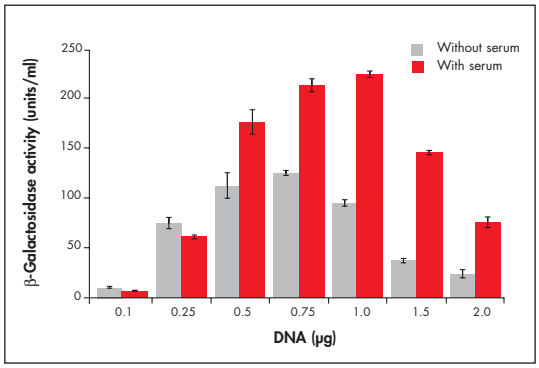


Figure 4. *Influence of serum and DNA quantity on transfection using SuperFect Reagent. 2 x 10^4 COS-7 cells were seeded per well in 96-well plates one day prior to transfection. Cells were transfected using 0.1–2.0 µg of a β-galactosidase reporter plasmid and 3 µl SuperFect Reagent per well, in either the presence or absence of serum. Each bar represents the average efficiency from four replicates assayed 48 h post-transfection.*

Non-liposomal lipids

Non-liposomal lipids represent a further development of the original liposome method introduced in 1987. In contrast to the bilayer structure formed by liposomes, non-liposomal lipids form micellar structures. The positively charged micelle surface can interact with the negatively charged phosphate backbone of nucleic acids. The resulting transfection complex is slightly positively charged, allowing it to bind to the negatively charged surface of the cell membrane.

QIAGEN offers two non-liposomal lipid reagents — TransMessenger Transfection Reagent for transfection of cells with RNA, and Effectene Transfection Reagent for transfection of cells with DNA. In the first step of transfection, the nucleic acid is strongly condensed by interaction of a specific positively charged enhancer with the negatively charged phosphate groups of the nucleic acid. The condensed nucleic acid is then coated with a cationic, non-liposomal lipid micelle. The transfection complex is taken up into the cells by endocytosis. For more information on the use of TransMessenger and Effectene Reagents, see page 17 (Effectene Reagent) and page 28 (TransMessenger Reagent).

2. Transfection of Cells with Plasmid DNA

Plasmid DNA is the most commonly used vector for transfection of cells. Plasmids containing cloned genes can be transfected into cells for a variety of purposes. Transfected cells can be used for biochemical characterization and mutational analyses of genes, investigation of gene regulatory elements, examination of how alterations in gene expression affect cell growth behavior, and production of a specific protein for purification.

This chapter provides useful information for successful transfection of cells with plasmid DNA. Further information on promoters and genetic reporter systems can be found in "Promoters for Gene Expression", pages 49–50, and "Genetic Reporter Systems", pages 51–52.

Vector considerations for plasmid DNA transfection

Vector construct

The configuration (e.g., linear or supercoiled) and size of the plasmid DNA vector influence the efficiency of transfection. Transient transfection is most efficient with supercoiled plasmid DNA. In stable transfection, linear DNA results in lower DNA uptake by the cells relative to supercoiled DNA, but yields optimal integration of DNA into the host genome.

Gene product

Some gene products are toxic to cells. This can cause problems in transient transfection of plasmid DNA if too strong a promoter is used to drive expression of a potentially toxic gene. Toxic gene products are also a problem for selection of stably transfected cells, since cells expressing a gene for antibiotic resistance lose their growth advantage when expressing a gene with negative or toxic effect on cell growth. If the gene product is toxic it will not be possible to obtain stable transfectants with a constitutive promoter. In such cases, stable clones can be obtained by using an inducible promoter system.

Promoter

The choice of promoter is critical for efficient expression of the transfected gene. Some commercially available and widely used promoters for gene expression in mammalian cells include the cytomegalovirus (CMV) and simian virus 40 (SV40) promoters and derivatives, and the pMC1 and phosphoglycerate kinase (PGK) promoters. Promoters are also available that allow gene expression in specific cell types. As mentioned above, a strong promoter is not always the best choice for production of stable transfectants. A weak promoter is sometimes better since strong overexpression of the gene product of interest may have toxic effects on the cells. Alternatively, an inducible promoter can be used to obtain stable transfectants. Further information on promoters is provided in "Promoters for Gene Expression", pages 49–50.

Plasmid DNA quality

Plasmid DNA quality strongly influences the results of transfection experiments (15, 16). The best results are achieved when plasmid DNA of the highest purity is used for transfection. DNA purified with HiSpeed™, QIAGEN®, and QIAfilter™ Plasmid Kits is equivalent in purity to that obtained with 2x CsCl-gradient centrifugation, and is ideally suited for transfection of most cell lines.

One of the most important parameters to consider for transfection is endotoxin contamination. Endotoxins, also known as lipopolysaccharides

(LPS), are cell membrane components of Gram-negative bacteria (e.g., *E. coli*) that are released during the lysis step of plasmid preparation. Due to their chemical structure and properties, endotoxins are often copurified with plasmid DNA. The presence of endotoxins in plasmid DNA can result in sharply reduced transfection efficiencies (Figure 5), particularly in primary cells and sensitive cell lines (15, 16). Endotoxins also interfere with transfection of hematopoietic cells, such as macrophages and B-cells, by causing nonspecific activation of immune responses (17, 18). To avoid low transfection efficiencies and misinterpretation of experimental results, it is important to use plasmid DNA that is free of endotoxin contamination.

For transfection of endotoxin-sensitive cells (e.g., primary cells, suspension cells, and hematopoietic cells) or for highest transfection efficiencies and lowest cytotoxicity, we recommend using DNA purified with EndoFree® Plasmid Kits. These kits efficiently remove bacterial lipopolysaccharide molecules during the plasmid purification procedure, ensuring optimal transfection results (Figures 5 and 6).

Plasmid Purity and Quantity vs. Transfection Efficiency

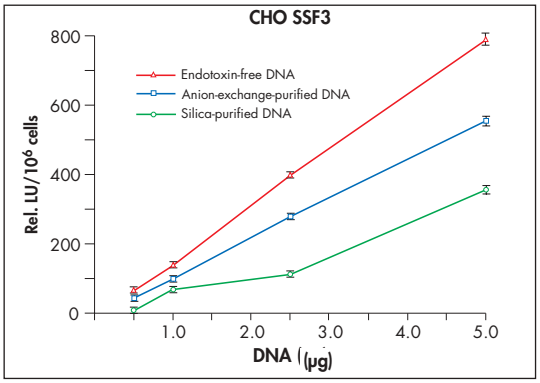


Figure 5. *Effect of the amount and quality of DNA (a luciferase reporter plasmid) on transfection efficiency in CHO SSF variants grown in suspension under serum-free conditions. Each point represents the mean of three independent experiments; bars represent standard deviations;* ***Rel. LU****: relative light units. Data kindly provided by M. O. Zang-Gandor, Novartis AG, Basel, Switzerland.*

Plasmid Purification Methods vs. Transfection Efficiency

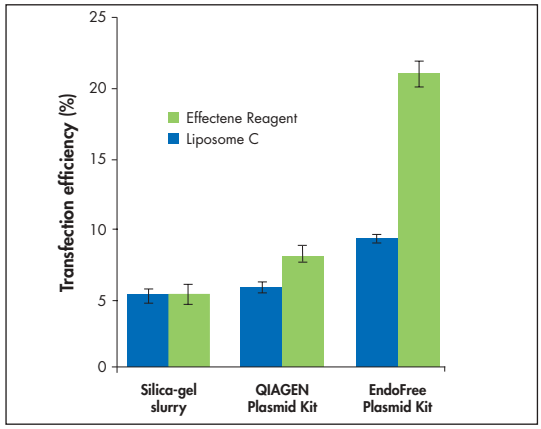


Figure 6. *Primary rabbit gastric parietal cells were transfected with a GFP reporter plasmid prepared using the methods indicated. Transfections were performed using Effectene Reagent or a commonly used liposome reagent (Liposome C). The bars represent the percentage of total cells expressing GFP as determined by scoring the number of green fluorescent cells 48 h post-transfection. Data represent the average of 6–9 replicate dishes using more than 2 different DNA preparations for each purification method. Data kindly provided by J. Parente and C. Chew, Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA, USA.*

Plasmid DNA transfection technology

The choice of transfection technology strongly influences transfection results. As discussed in Chapter 1, classical transfection methods (i.e., DEAE-dextran, calcium phosphate, electroporation, and liposome-mediated transfection) have associated advantages and disadvantages that need to be considered when choosing a transfection technology. QIAGEN offers three advanced reagents for highly efficient transfection of different cell types with DNA — PolyFect, Effectene, and SuperFect Transfection Reagents. These reagents offer significant advantages over classical transfection technologies, including a fast and easy procedure, transfection in the presence of serum, and low cytotoxicity.

Table 1 provides recommendations for choosing which QIAGEN Transfection Reagent to use with

different cell types. In addition, the Transfection Tools web site — **www.qiagen.com/transfectiontools/** — has a searchable database of cell lines and primary cells successfully transfected using QIAGEN Transfection Reagents that includes information on the transfection conditions (e.g., amount of nucleic acid used, cell culture conditions, etc.).

PolyFect Transfection Reagent

PolyFect Transfection Reagent is specifically developed and optimized for transfection of the commonly used cell lines COS-7, NIH/3T3, HeLa, 293, and CHO, and consistently delivers high transfection efficiencies (Figures 7 and 8).

Table 1. QIAGEN Transfection Reagent Selection Guide

Application	Recommended transfection reagent	Page
RNA transfection		
	TransMessenger Transfection Reagent	28
DNA transfection		
COS-7, NIH/3T3, HeLa, 293, or CHO cells	PolyFect Transfection Reagent	16
Primary cells or sensitive cell lines	Effectene Transfection Reagent	17
Other cell lines	Effectene Transfection Reagent or SuperFect Transfection Reagent	17 18

High Transfection Efficiencies with PolyFect Reagent

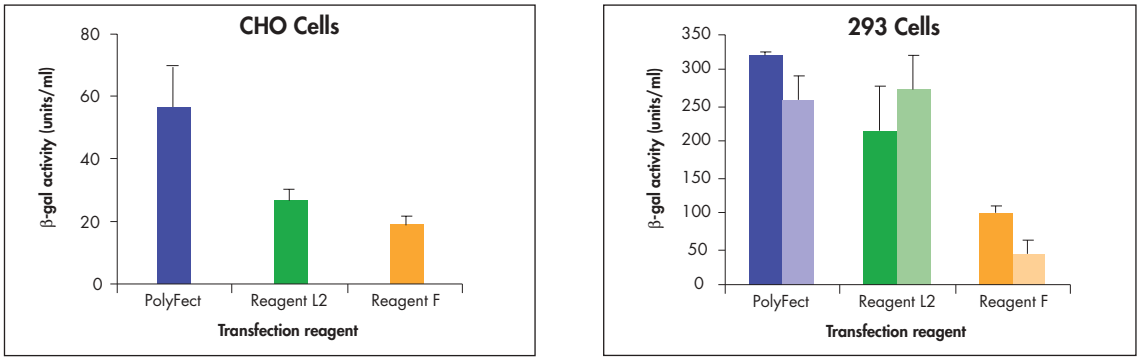


Figure 7. *Comparison of transfection efficiencies for PolyFect Reagent and two lipid-based reagents. Cells cultured in 6-well plates were transfected with a β–galactosidase reporter plasmid. The appropriate protocol was used for transfection with PolyFect Reagent. Optimized protocols were used for transfection with Reagents L2 and F; these were: 10 µl Reagent L2 with 3 µg DNA and 15 µl Reagent F with 2 µg DNA for CHO cells, and 10 µl Reagent L2 with 2 µg DNA and 7 µl Reagent F with 3 µg DNA for 293 cells. All transfections were performed in triplicate. Two independent transfection experiments are shown for 293 cells.*

PolyFect Reagent with HeLa Cells

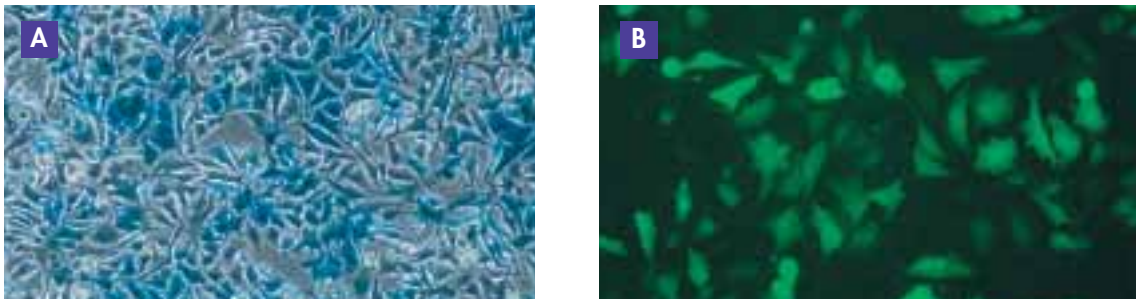


Figure 8. *Expression of A β-galactosidase and B green fluorescent protein (GFP) in HeLa cells. Cells were cotransfected in 6-well plates with β-galactosidase and GFP reporter plasmids using PolyFect Transfection Reagent and the HeLa cell protocol. Expression was visualized by X-gal staining or fluorescence microscopy 2 days post-transfection.*

PolyFect Reagent is Extremely Stable

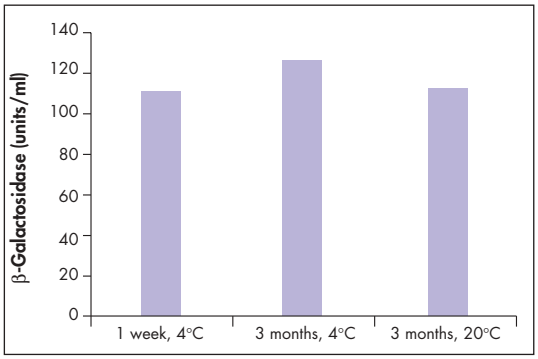
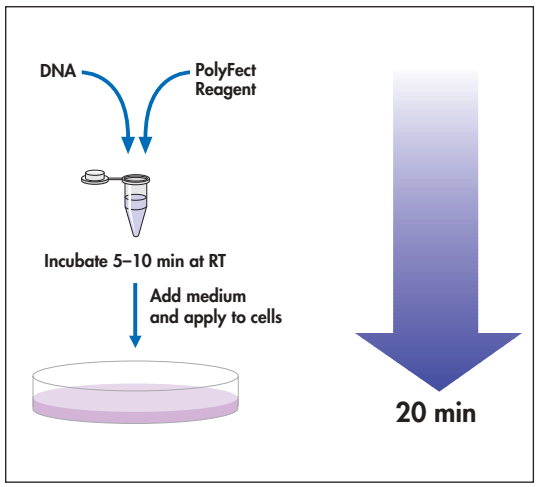


Figure 9. *PolyFect Reagent was stored for the indicated times at the indicated temperature, and then used in transfection experiments. HeLa-S3 cells were transfected with a β-galactosidase reporter plasmid in 6-well plates using the optimized HeLa-S3 protocol available from QIAGEN Technical Services.*

In contrast to many liposomal reagents, PolyFect Reagent enables transfection in the presence of serum without lowering transfection efficiency. In addition, the reagent is extremely stable (Figure 9). PolyFect Reagent is supplied with optimized, cell-specific protocols for a number of different cell culture formats, so optimization experiments are not required.

PolyFect Reagent is provided as a ready-to-use solution of specifically designed activated-dendrimers (see "Activated dendrimers", page 11). The optimized transfection protocols are fast and simple — just add PolyFect Reagent to the DNA solution, mix, incubate for 5–10 minutes, add growth medium (which can contain serum and antibiotics), and pipet the PolyFect–DNA complexes onto the cells (see flowchart). The cells are then incubated for expression of the transfected gene. No post-transfection removal of complexes or medium change/addition is necessary, making the procedure fast and easy.

PolyFect Procedure



Effectene Transfection Reagent

Effectene Transfection Reagent is suitable for transient and stable transfection of a broad range of cell types, and yields significantly higher transfection efficiencies than many widely used lipid-based reagents (Figure 10) and classical technologies such as electroporation (Figure 11). Effectene Reagent is particularly effective for primary cells (Figure 7, page 16, and Figure 12) as transfection can be performed in the presence of serum and using low amounts of DNA (Figure 13), resulting in minimal cytotoxicity.

Comparison of Effectene Reagent and Lipid-Based Reagents

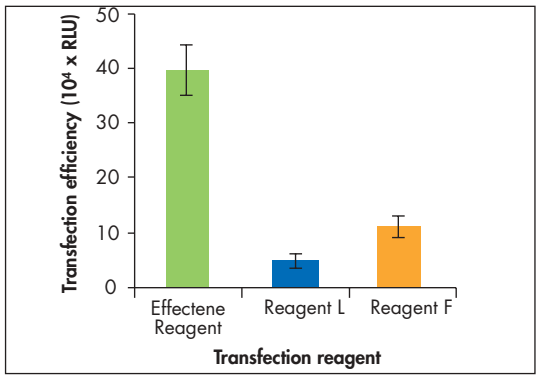


Figure 10. *Comparison of transfection efficiencies using Effectene Reagent and two commonly used lipid-based reagents. Murine teratocarcinoma F9 cells (5 x 10^5) were transfected in 6-well plates with a luciferase reporter plasmid using optimized conditions based on the manufacturer's instructions for each reagent. Transfection efficiencies were determined by measuring luciferase activity 48 h post-transfection, and are given as relative light units (**RLU**). (Data kindly provided by I. Clavereau, D. Petitprez, and I. Van Seuningen, Unité INSERM 377, Place de Verdun, Lille Cedex, France.)*

Comparison of Effectene Reagent and Electroporation

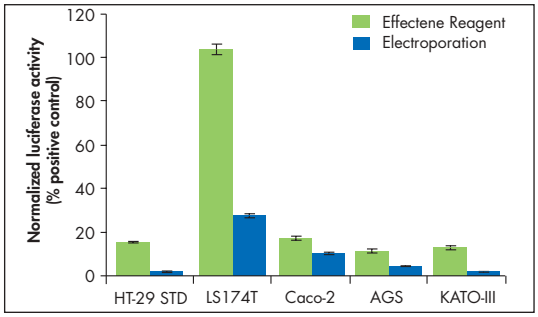


Figure 11. *Colon (HT-29 STD, LS174T, and Caco-2) and gastric (AGS and KATO-III) carcinoma cell lines were transfected with a luciferase reporter plasmid using either Effectene Reagent or electroporation. Luciferase activity (measured 48 h post-transfection) was normalized and is expressed as a percentage of the activity obtained with a second, positive control luciferase reporter plasmid. Each bar represents the mean and standard deviation from duplicate assays from at least three separate experiments. Data kindly provided by I. Clavereau, D. Petitprez, and I. Van Seuningen, Unité INSERM 377, Place de Verdun, Lille Cedex, France. Data excerpted from Clavereau et al., QIAGEN News 2000 No. 1, 3.*

Effectene Reagent is a non-liposomal lipid formulation (see "Non-liposomal lipids", page 13) that is used in conjunction with a special DNA-condensing enhancer and optimized buffer to achieve high transfection efficiencies. The enhancer first condenses the DNA molecules and Effectene

Effectene Reagent with Primary Cells

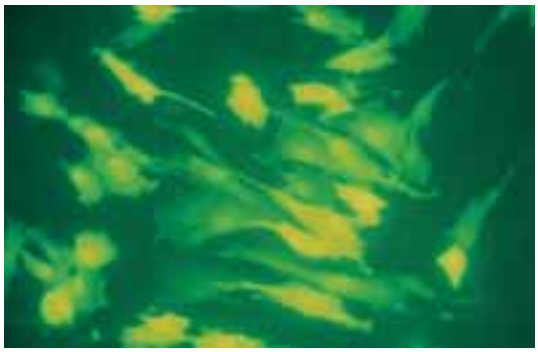


Figure 12. *Expression of green fluorescent protein (GFP) in primary rabbit aortic smooth muscle cells transfected using Effectene Reagent. 1 x 10^5 cells were seeded one day prior to transfection, and transfections were performed in 6-well plates using 0.4 µg of a GFP reporter plasmid and 10 µl Effectene Reagent per well. Cells were viewed 24 h post-transfection by fluorescence microscopy. Approximately 40% of the cells were transfected, as determined by FACS® analysis. (Data kindly provided by K. Veit, 2nd Medical Clinic, Dept. Clinical Pharmacology, Mainz, Germany.)*

Serum and DNA Quantity vs. Transfection Efficiency

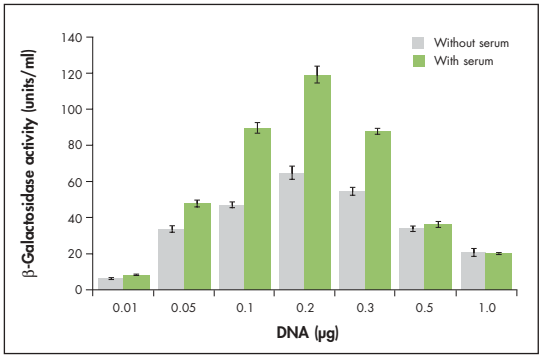


Figure 13. *Influence of serum and DNA quantity on transfection using Effectene Reagent. 2 x 10^4 COS-7 cells were seeded per well in 96-well plates one day before transfection. Cells were transfected using 0.01–1.0 µg of a β–galactosidase reporter plasmid and 0.08–8.0 µl Enhancer (DNA: Enhancer ratio of 1:8) and 2 µl Effectene Reagent, in either the presence or absence of serum. Each bar represents the average efficiency from four replicates 48 h post-transfection.*

Reagent subsequently coats them with cationic lipids, providing a particularly efficient way of transferring DNA into eukaryotic cells. Due to the low cytotoxicity of Effectene Reagent, removal of Effectene–DNA complexes after transfection is not necessary for most cell lines. As Effectene Reagent is very efficient in delivering DNA into cells, only one-fifth of the quantity of DNA required for most liposome reagents is usually sufficient (Figure 13), allowing more experiments to be performed with valuable DNA samples.

SuperFect Transfection Reagent

SuperFect Transfection Reagent is suitable for transient and stable transfection of a broad range of cell lines and in many cases, yields significantly higher transfection efficiencies than commonly used liposomes (Figures 14 and 15). In addition, SuperFect Reagent allows transfection in the presence of serum without lowering transfection efficiencies (see Figure 4, page 13).

SuperFect Reagent consists of activated-dendrimer molecules with a defined spherical architecture (see "Activated dendrimers", page 11). The

High Transfection Efficiencies with SuperFect Reagent

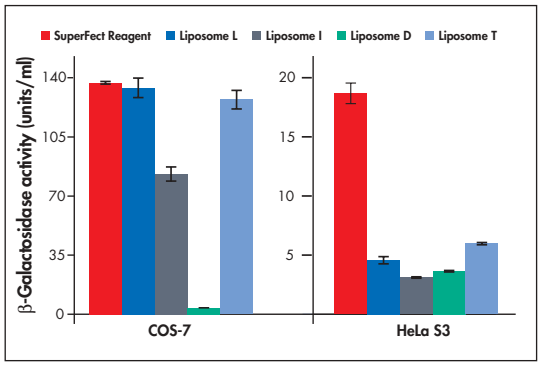


Figure 14. *Comparison of transfection efficiencies obtained using SuperFect Reagent and four of the most commonly used liposome reagents. COS-7 and HeLa S3 cells (as indicated) were transfected in 96-well format using 0.5 µg of a β-galactosidase reporter plasmid. 2 x 10^4 cells were seeded per well one day prior to transfection. Transfection efficiencies are given as β-galactosidase units per ml. Each bar represents the average efficiency from 4 replicates.*

Transfection of Neuronal PC-12 Cells Using SuperFect Reagent

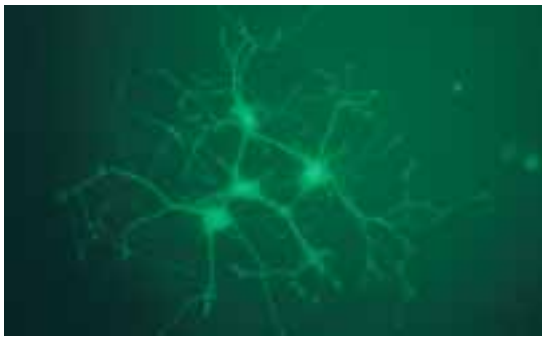


Figure 15. *Expression of green fluorescent protein (GFP) in differentiated PC-12 cells 5 days post-transfection. 10^4–10^5 cells previously stimulated with 50 ng/ml NGF were plated per 60 mm dish one day prior to transfection. Transient transfections were performed in 2 ml low-serum growth medium (DMEM plus 0.05% FBS) using 3 µg of a GFP reporter plasmid and 15 µl SuperFect Reagent. (Data kindly provided by K. Kelly-Spratt, University of Texas Southwestern Medical Center, Dallas, TX, USA.)*

reagent is provided as a ready-to-use solution that is simply mixed with the DNA solution, incubated for 5–10 minutes, mixed with growth medium (which can contain serum and antibiotics), and added directly to the cells. After a 2–3 hour incubation, a medium change is performed and the cells are then incubated for expression of the transfected gene.

Optimization of plasmid DNA transfection

A number of different transfection parameters can be modified to obtain optimal results. We recommend careful optimization of the parameters below for every cell type and vector combination used. Once optimized, these parameters should be kept constant in all future experiments to help ensure reproducible results.

Effectene and SuperFect Transfection Reagents are provided with protocols that provide high transfection efficiencies in many cell lines. However, we recommend optimization of the parameters below to achieve the optimal transfection efficiency. PolyFect Transfection Reagent is optimized for high-efficiency transfection of commonly used cells lines. The reagent is provided with optimized, cell-specific protocols for transfection of COS-7, NIH/3T3, HeLa, 293, and CHO cells in a variety of cell culture formats, so no optimization experiments are required.

Cell density at the time of complex addition

Too low or too high a cell density at the time of complex addition can result in poor uptake of transfection complexes and/or insufficient expression of the transfected gene. The optimal confluence should be determined for every new cell line to be transfected, and kept constant in future experiments. This is achieved by counting cells prior to seeding and by keeping the time period between seeding and transfection constant. For adherent cells, the optimal confluency at the time of transfection-complex addition is normally 40–80% (Figure 16). For suspension cells, we recommend splitting the cells the day prior to transfection to ensure that the cells will be in optimal physiological condition for the transfection procedure. Table 2 provides guidelines for the number of adherent and

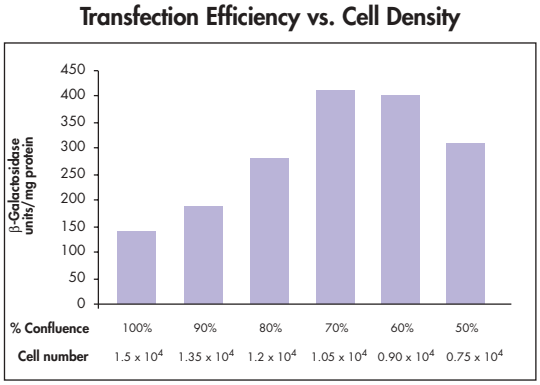


Figure 16. *Transfections were performed in 96-well format using 0.1 µg of a β-galactosidase reporter plasmid, 0.8 µl Enhancer, and 1 µl Effectene Reagent per well. The indicated numbers of HeLa-S3 cells were seeded one day prior to transfection to provide cell densities of approximately 50–100% at the time of transfection. Transfection efficiencies and protein content were measured from four replicates 48 h after transfection.*

suspension cells to seed in different cell culture formats for transfection experiments.

Amount of DNA

The optimal amount of DNA is influenced by the characteristics of the transfected plasmid, for example, the promoter, origin of replication, and plasmid size. Toxic effects may result if a plasmid encoding a toxic protein or too much plasmid with a high expression rate is used. Conversely, if insufficient plasmid with a low expression rate is used, gene expression may be too low. Therefore, optimization of plasmid DNA amount should be performed for every new plasmid and/or new cell type used. Examples of pipetting schemes for optimizing transfection of adherent and suspension cells in 60 mm dishes with SuperFect Reagent or Effectene Reagent are provided in Tables 3 and 5, respectively.

Transfection reagent to DNA ratio

When working with charge-based transfection methods (i.e., calcium phosphate-, lipid-, liposome-, or activated-dendrimer–mediated), the DNA:transfection reagent ratio is a critical parameter to optimize. The overall charge of the transfection complexes is determined by the ratio of reagent to DNA. Optimal binding of transfection complexes to the negatively charged groups on the cell surface requires an appropriate net positive charge. Therefore, DNA:transfection reagent ratio is an extremely important parameter to optimize for every new cell type and DNA construct used. Examples of pipetting schemes for optimizing transfection with SuperFect Reagent and Effectene Reagent are provided in Tables 3 and 5, respectively. Blank Tables 4 and 6 are provided for the creation of personal pipetting schemes. As a starting point for optimization, we recommend using a DNA:SuperFect Reagent ratio of 1:5, and a DNA:Effectene Reagent ratio of 1:25.

Table 2. Recommended number of cells per culture vessel for transfection

Culture format	Adherent cells	Suspension cells	
	Number to seed the day prior to transfection*†	Number to seed on day of transfection*	Volume of medium (µl)
96-well plate	0.5–2.0 x 10^4	0.5–2.0 x 10^5	100
48-well plate	1.0–4.0 x 10^4	1.0–3.5 x 10^5	150
24-well plate	2.0–8.0 x 10^4	2.0–7.0 x 10^5	350
12-well plate	0.4–2.0 x 10^5	0.5–1.5 x 10^6	800
6-well plate	0.9–4.0 x 10^5	1.0–3.5 x 10^6	1600
60 mm dish	2.0–8.0 x 10^5	2.5–7.5 x 10^6	4000
100 mm dish	0.5–2.5 x 10^6	0.5–2.0 x 10^7	7000

* *Actual values depend on cell type and size.*

† *The volume of medium used to seed adherent cells the day before transfection is not critical. When seeding adherent cells, use a volume of medium suitable for your cell culture format.*

Table 3. Example of a pipetting scheme for optimizing the transfection of adherent cells in 60 mm dishes using SuperFect Reagent

DNA (µg)	Ratio of DNA to SuperFect Reagent		
	1:2	1:5	1:10
2.5	2.5 µg DNA 5 µl SuperFect	2.5 µg DNA 12.5 µl SuperFect	2.5 µg DNA 25 µl SuperFect
5.0	5 µg DNA 10 µl SuperFect	5 µg DNA 25 µl SuperFect	5 µg DNA 50 µl SuperFect
10.0	10 µg DNA 20 µl SuperFect	10 µg DNA 50 µl SuperFect	10 µg DNA 100 µl SuperFect

Table 4. Blank table for your personal pipetting scheme for SuperFect Reagent

DNA (µg)	Ratio of DNA to SuperFect Reagent		
	1:2	1:5	1:10
	µg DNA µl SuperFect	µg DNA µl SuperFect	µg DNA µl SuperFect
	µg DNA µl SuperFect	µg DNA µl SuperFect	µg DNA µl SuperFect
	µg DNA µl SuperFect	µg DNA µl SuperFect	µg DNA µl SuperFect

Table 5. Example of a pipetting scheme for optimizing the transfection of adherent cells in 60 mm dishes using Effectene Reagent

DNA (µg)	Ratio of DNA to Effectene Reagent		
	1:10	1:25	1:50
0.5	0.5 µg DNA 4 µl Enhancer 5 µl Effectene	0.5 µg DNA 4 µl Enhancer 12.5 µl Effectene	0.5 µg DNA 4 µl Enhancer 25 µl Effectene
1.0	1 µg DNA 8 µl Enhancer 10 µl Effectene	1 µg DNA 8 µl Enhancer 25 µl Effectene	1 µg DNA 8 µl Enhancer 50 µl Effectene
2.0	2 µg DNA 16 µl Enhancer 20 µl Effectene	2 µg DNA 16 µl Enhancer 50 µl Effectene	2 µg DNA 16 µl Enhancer 100 µl Effectene

Table 6. Blank table for your personal pipetting scheme for Effectene Reagent

DNA (µg)	Ratio of DNA to Effectene Reagent		
	1:10	1:25	1:50
	µg DNA µl Enhancer µl Effectene	µg DNA µl Enhancer µl Effectene	µg DNA µl Enhancer µl Effectene
	µg DNA µl Enhancer µl Effectene	µg DNA µl Enhancer µl Effectene	µg DNA µl Enhancer µl Effectene
	µg DNA µl Enhancer µl Effectene	µg DNA µl Enhancer µl Effectene	µg DNA µl Enhancer µl Effectene

Incubation period with DNA–reagent complexes

Effectene Reagent

For adherent and suspension cells, results with Effectene Reagent show that in most cases, removal of transfection complexes is not necessary. However, if cytotoxicity is observed, the Effectene–DNA complexes should be removed 6–18 hours after addition, the cells washed carefully with PBS, and fresh medium should be added.

SuperFect Reagent

For adherent cells, optimal results with SuperFect Reagent are typically obtained by choosing incubation periods of 2–3 hours. If sensitive adherent cells demonstrate extensive cell death after incubation with SuperFect–DNA complexes, we recommend reducing the exposure time of cells to complexes to 1 hour. If transfection efficiency is lower than expected, and cytotoxicity is acceptably low, an increase in incubation time of up to 16 hours may be considered. For suspension cells, experiments have shown that in most cases, removal of SuperFect–DNA complexes is not necessary. However, if toxicity is observed, the transfection complexes should be removed by centrifugation after a 2–3-hour incubation period.

Incubation time following transfection

It is important to allow sufficient time (sometimes up to 72 hours) for expression of the transfected gene(s) and to note that this time varies both with the protein being expressed and with cell type. If the optimal time point for analysis of cells following transfection is not known, it may be necessary to perform a time-course experiment.

Controls

To check for optimal cell growth conditions, include a negative control (no DNA, no transfection reagent). To establish that the reporter assay is working properly, include a positive control (parallel transfection with established transfection method). To determine whether there are insert-related problems, transfect a plasmid without the insert of interest.

Selection of stably transfected cells

Transfection using DNA vectors allows generation of stable (or permanent) transfectants, that is, cells in which the transfected DNA is either integrated into the chromosomal DNA or maintained as an episome. Approximately one in 10^4 transfected cells will stably integrate DNA, although the efficiency

varies with cell type (2). Integration is most efficient when linear DNA is used.

To obtain a stably transfected homogenous cell population, a selectable marker gene (usually a eukaryotic antibiotic-resistance gene) is transfected into cells together with the gene of interest. The selectable marker can either be present in the same vector as the gene of interest, or cotransfected in a separate vector. In the latter case, the molar ratio of gene of interest to selectable marker is typically in the range of 5:1 to 10:1 to ensure that any cell that contains the selectable marker also contains the gene of interest.

Frequently used selectable markers are the genes encoding aminoglycoside phosphotransferase (APH; *neoR* gene) or hygromycin B phosphotransferase (HPH). Other selectable markers include the genes encoding adenosine deaminase (ADA), dihydrofolate reductase (DHFR), thymidine kinase (TK) or xanthine-guanine phosphoribosyl transferase (XGPRT; *gpt* gene). In some cases, stable transfection causes a morphological change that can be used as a selectable trait.

After transfection, cells are generally maintained in nonselective medium for up to 2 days. After this time, the cells are harvested and transferred to a selective medium containing the relevant concentration of the appropriate antibiotic. The selective medium is changed over a period of 2–3 weeks or more to eliminate dead cells and debris. Stable transfectants appear as distinct antibiotic-resistant colonies, which can be isolated and subcloned to multiwell plates for further propagation in selective medium. A protocol for selection of stably transfected cells is provided on page 46.

The selection process can be accelerated by either cotransfecting a plasmid expressing a membrane-anchored selection tag together with the plasmid carrying the gene of interest, or transfecting a plasmid that expresses both the selection tag and the gene of interest. After transfection, the cell population is incubated with magnetic beads coated with a molecule (e.g., an antibody) that binds to the selection tag expressed at the surface of transfected cells. Cells bound to the beads can then be purified from the remaining, untransfected cell population. Magnetic-bead procedures select only those cells that have been transiently transfected. Further selection is necessary to obtain stably transfected cells (usually a small portion of those transiently transfected; see above).

3. Transfection of Cells with Oligonucleotides

The most common application for transfection of cells with oligonucleotides is "antisense" experiments, where an oligonucleotide is introduced into cells to prevent translation of a specific mRNA species. The sequence of an antisense oligonucleotide is complementary to a portion of the target mRNA, allowing it to hybridize to the mRNA. The DNA–RNA duplex is recognized by the cell and degraded, thereby preventing translation of the mRNA into a protein. In addition to research applications, antisense technology also has therapeutic potential, for example, to prevent expression of a specific disease-associated gene.

This chapter provides information for transfection of cells with oligonucleotides. For information on transfection of cells with siRNA oligonucleotide duplexes, see "Transfection of Cells with RNA", pages 26–31, and "Guidelines for RNA Interference (RNAi) Experiments", pages 53–55.

Vector considerations for oligonucleotide transfection

Oligonucleotides for antisense experiments are often synthesized with modified bases that increase their stability. For example, 2'-O-methyl oligoribonucleotides have increased stability compared to regular oligodeoxynucleotides when bound to RNA. "S"-oligos contain internal phosphorothioate groups, in which one of the oxygen atoms within the phosphate backbone is replaced by a sulfur atom, and are resistant against most exonucleases and endonucleases. Both oligodeoxynucleotides and 2'-O-methyl oligoribonucleotides can be phosphorothioated. The structure of an oligonucleotide for transfection will depend on the application.

Oligonucleotide quality

Oligonucleotides used for transfection experiments should be highly pure, that is, free of synthesis salts and other contaminants. Suppliers of custom oligonucleotides, such as QIAGEN Operon® (**www.operon.com**), can produce high-quality, HPLC-purified oligodeoxynucleotides as well as S-oligos and 2'-O-methyl oligoribonucleotides for transfection experiments. Alternatively, residual salts can be removed following oligonucleotide synthesis using the QIAquick® Nucleotide Removal Kit to give oligonucleotides suitable for use in transfection experiments.

Oligonucleotide transfection technology

As with transfection of plasmid DNA, the choice of transfection technology influences transfection results when using oligonucleotide vectors. Both Effectene and SuperFect Transfection Reagents from QIAGEN have been used to successfully transfect cells with oligonucleotides (Figures 17 and 18). For more information on these reagents, see "Plasmid DNA transfection technology", pages 15–19.

Optimization of oligonucleotide transfection

As with transfection of plasmid DNA vectors, a number of different parameters can be modified to obtain optimal results when using oligonucleotide vectors. Once optimized, these parameters should be kept constant in all future experiments to help ensure reproducible results.

The general principles for optimization of oligonucleotide transfection are similar to those for plasmid DNA transfection. See "Optimization of plasmid DNA transfection", pages 19–22, for optimization guidelines.

Transfection of Oligonucleotides Using Effectene Reagent

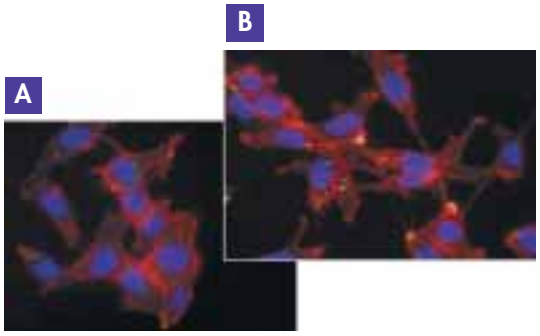


Figure 17. *C6 glioblastoma cells (1 x 10^4) were transfected with either naked FITC-labeled CD44 antisense oligodeoxynucleotide (ODN) or with Effectene–ODN complexes. Intracellular accumulation and distribution of the transfected ODNs was analyzed 16 h post-transfection by immunofluoresence* **A** *Naked ODNs (2 µg).* **B** *ODNs (0.04 µg) complexed with Effectene Reagent. Transfected ODNs are green in the photos. (Data kindly provided by G. Beutel and M. Schott, Institute of Neuropathology, Hanover Medical School, Hanover, Germany.)*

Transfection of Oligonucleotides Using SuperFect Reagent

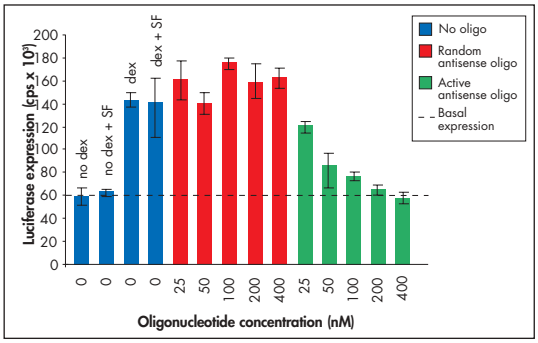


Figure 18. *Transfection of antisense oligonucleotides into NIH/3T3 cells using SuperFect Reagent (**SF**). Cells were transiently transfected with an H-ras/luciferase construct, induced with dexamethasone (**dex**), and then transfected with different amounts of either a random antisense oligonucleotide, or an antisense oligonucleotide with luciferase-inhibitory activity. Each column represents the mean of three replicates. Data kindly provided by L. J. Miraglia, Department of Structural Biology, ISIS Pharmaceuticals, Carlsbad, CA, USA.*

4. Transfection of Cells with RNA

Transfection of cells with RNA rather than DNA provides an alternative approach that offers new possibilities for transfection experiments. RNA transfection has been used for a number of specific applications, including transfection of non-dividing cells (19–21), direct studies of RNA viruses (22–24), RNA translation studies (25–26), and studies using antisense RNA (27–28). Transfection of RNA is also used in the RNA interference (RNAi) method, which allows genes to be efficiently "switched off" by introduction of double-stranded RNA into cells (Figure 19; references 29–31). In addition, RNA transfection could be useful for studying cells that are not efficiently transfected with plasmid DNA. Transfected RNA sequences are expressed in the absence of transcription and in a promoter-independent manner, and protein expression usually occurs sooner following transfection of RNA than following transfection of DNA.

A variety of RNA molecules can be used as vectors for transfection, for example, in vitro transcribed RNA, viral RNA, RNA oligos, siRNA (see also "Guidelines for RNA Interference (RNAi) Experiments", pages 53–55), and ribozymes. Use of RNA vectors for transfection involves considerations that do not apply to DNA vectors. As discussed below, a number of different factors affect the efficiency of RNA transfection, and should be considered carefully before beginning transfection procedures.

Vector considerations for RNA transfection

mRNA structure

The structure of the RNA molecule used for transfection can influence transfection efficiency. Messenger RNA (mRNA) species in eukaryotic cells have a number of features that influence their expression, including the cap, poly-A tail, and internal ribosomal entry site (IRES). These features, discussed in more detail below, may be required for efficient translation of an RNA vector in some cell types, but may hinder expression in other cell types.

mRNA usually terminates at the 5' end with a cap, a modified nucleotide that enhances mRNA stability and is necessary in some cell types to ensure optimal translation. A cap can be incorporated into an in vitro-transcribed RNA by adding an RNA cap structure analog to the transcription reaction.

The 3' end of mRNA usually terminates with a poly-A

RNAi Using TransMessenger Transfection Reagent

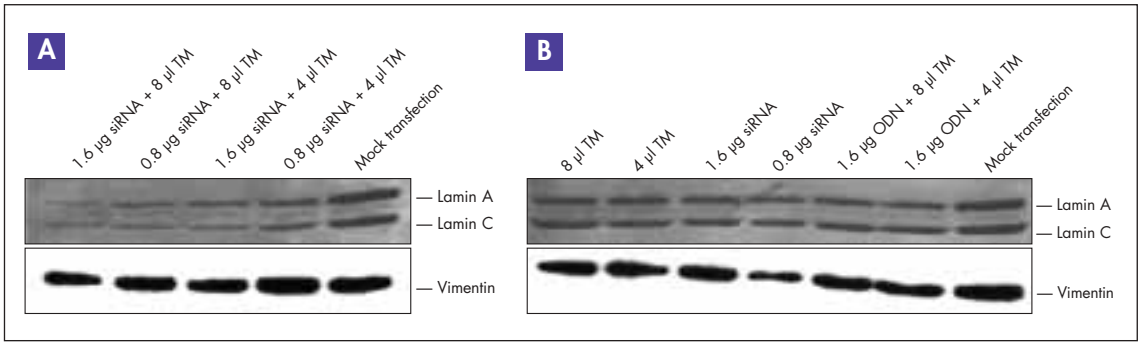


Figure 19. *Western blots of transfected HeLa-S3 cell extracts using lamin A/C- or vimentin-specific monoclonal antibodies. Cells were transfected using the indicated reagents.* ***TM****: TransMessenger Transfection Reagent;* ***siRNA****; short interfering RNA;* ***ODN****: scrambled 24mer oligodeoxynucleotide.* **A** *Lamin A/C expression was significantly reduced in cells that had been transfected with TransMessenger Reagent-siRNA complexes.* **B** *Expression was not suppressed in cells that were untransfected, transfected using TransMessenger Reagent or siRNA alone, or transfected with a scrambled ODN–TransMessenger Reagent complex.*

tail, a stretch of adenylate residues added 3' to the mRNA coding sequence that also stabilizes the mRNA. A poly-A tail can be incorporated into an in vitro-transcribed RNA by use of a relevant DNA template or added post-transcriptionally using a poly(A) polymerase.

An IRES is a structural element with a specific sequence that helps the ribosome to access the mRNA in the absence of a 5' cap, thereby enhancing translation. However in some cell types, an RNA species containing an IRES will not be efficiently translated due to a lack of specific proteins necessary for translation initiation at an IRES in these cells.

siRNA structure for RNAi

The structure and sequence of RNA duplexes that will be used for RNAi is critical for effective silencing of gene expression in transfected cells. These double-stranded short interfering RNA (siRNA) molecules should target the open reading frame of the target mRNA, have a GC content of around 50%, and ideally have the sequence motif AA(N_{19})TT (although AA(N_{21}) or CA(N_{21}) will also work). Further information on RNAi and the design of siRNA is provided in "Guidelines for RNA Interference (RNAi) Experiments", pages 53–55.

Gene product

Some gene products are toxic to cells, especially if they are overexpressed. Therefore, the amount of RNA transfected should be optimized for every new RNA and/or new cell type used (see "Amount of RNA", page 30).

RNA quality

RNA purity

Optimal transfection results are achieved when RNA of the highest purity is used for transfection. Therefore only RNA of the highest quality, which is free of contaminating DNA and proteins, should be used. RNA purified with RNeasy® Kits and Oligotex® mRNA Kits is highly recommended for transfection.

We strongly recommend checking RNA quality before starting the transfection procedure. RNA concentration and purity can be determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) using a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to ~40 µg RNA per milliliter. RNA integrity and size can be checked by denaturing agarose-gel electrophoresis and ethidium bromide staining. The RNA should appear as a sharp band on the stained gel. If the band is not sharp but appears as a smear containing smaller sized RNAs, it is likely that the RNA has suffered major degradation during its preparation. The functionality of the transcript can be determined by in vitro translation.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when no DNA is visible on an agarose gel. For RNA transfection, digestion of the purified RNA with RNase-free DNase is recommended. On-column DNase digestion can be performed during RNA purification using RNeasy Kits.

Prevention of RNase contamination

Ribonucleases (RNases) are very stable and active enzymes that do not generally require cofactors to function. Since RNases are difficult to inactivate and minute amounts are sufficient to destroy RNA, plasticware, glassware, or solutions should only be used after eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases during the transfection procedure. In order to create and

maintain an RNase-free environment when working with RNA, the precautions listed below must be followed throughout the procedure.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds, and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin. Change gloves frequently and keep tubes closed. Ensure that all glassware, plasticware, and solutions (including water and culture medium) are RNase-free.

Disposable plasticware

The use of sterile, disposable, plastic tubes throughout the procedure is recommended. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Serum

In general, the presence of serum in culture medium enhances transfection. However, to avoid potential contamination with RNases, we recommend not using serum during the initial incubation of transfection reagent–RNA complexes with cells as this can lead to lowered transfection efficiencies. If transfection in the presence of serum is necessary, it is important to use a high-quality lot of serum that is validated to have no detectable RNase activity.

RNA transfection technology

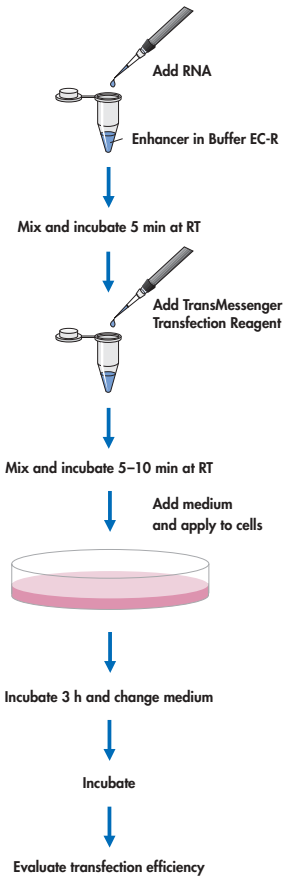
As with transfection using DNA vectors, the choice of transfection technology strongly influences transfection results when using RNA. While classical transfection methods (i.e., DEAE-dextran, calcium phosphate, electroporation, and liposome-mediated transfection) can be used for transfection of RNA, care should be taken that the reagents used are free of RNases and that the method is suitable for RNA vectors. It should be kept in mind that techniques developed for transfection of DNA vectors may not work efficiently for RNA vectors.

TransMessenger Transfection Reagent

TransMessenger Transfection Reagent from QIAGEN is the first reagent specifically developed for transfection of cells with RNA. Strict quality control is performed to test for an absence of RNase activity, lot-to-lot consistency, and low endotoxin levels (<10 EU/ml). These rigorous standards eliminate reagent variables that can adversely affect the efficiency of RNA transfection.

TransMessenger Reagent provides a fast and easy procedure (see flow chart) and allows high

TransMessenger Procedure



TransMessenger Reagent with HeLa Cells

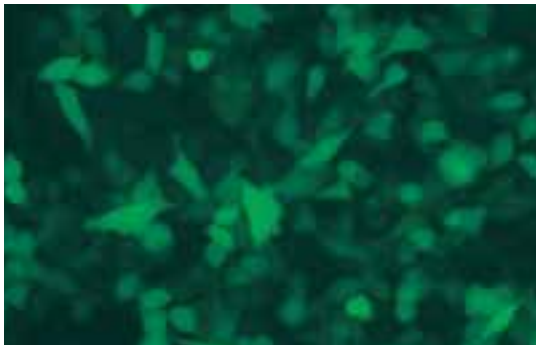


Figure 20. *Expression of green fluorescent protein (GFP) in HeLa-S3 cells. 8 x 10^4 cells were seeded into 48-well plates and transfected 24 h later with 0.5 µg of an in vitro-transcribed GFP-encoding RNA (transcribed from P7ASP-GFP/Mlu) using 1 µl Enhancer R and 2.5 µl TransMessenger Reagent. Cells were analyzed 24 h post-transfection by fluorescence microscopy. Approximately 50% of the cells were successfully transfected. (P7ASP-GFP/Mlu kindly provided by J. Bogenberger, Stanford University Blood Center, Palo Alto, CA, USA.)*

TransMessenger Reagent with Mature Dendritic Cells

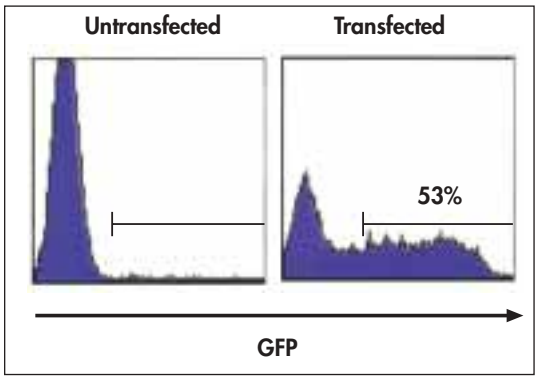


Figure 21. *Mature dendritic cells were transfected with 1 µg of a GFP-encoding RNA using 12 µl Enhancer-R and 6 µl TransMessenger Reagent. Cells were analyzed for GFP expression by flow cytometry. Data kindly provided by M. Liao, C. Yee, Y. Li. and P. Greenberg, Fred Hutchinson Cancer Research Center, Seattle, WA, USA.*

transfection efficiencies in a wide range of cell types (Figures 20 and 21). The reagent is a lipid-based formulation that is used in conjunction with a specific RNA-condensing enhancer and an optimized buffer. RNA molecules are condensed by the enhancer and then coated by TransMessenger Reagent for efficient transfer into eukaryotic cells.

All TransMessenger Reagent components are provided as ready-to-use solutions. To generate TransMessenger–RNA transfection complexes, the RNA is simply mixed with Enhancer R and Buffer EC-R and incubated for 5 minutes at room temperature. TransMessenger Reagent is added and the solution is incubated for a further 5–10 minutes. The complexes are mixed with medium and added directly to the cells. Following a 3 hour incubation, the medium is changed and the cells are incubated until they are ready for analysis.

Optimization of RNA transfection

We recommend careful optimization of a number of critical parameters for every cell type and RNA combination used. Once optimized, these parameters should be kept constant in all future experiments with each particular combination.

Cell density at the time of complex addition

The optimal confluency for transfection of adherent cells with RNA is usually 80–90%. The optimal confluency should be determined for every new cell type to be transfected and kept constant in future experiments. This is achieved by counting cells prior to seeding and by keeping the time period between seeding and transfection (minimum 24 hours) constant. This will ensure that the cell density is not too high and that the cells are in optimal physiological condition on the day of transfection. Table 7 provides a guide for the number of adherent cells to seed in different cell culture formats for RNA transfection experiments.

Table 7. Recommended number of cells to seed for different culture formats

Culture format	Suggested no. of adherent cells to seed*
96-well plate	2–3 x 10^4
48-well plate	4–8 x 10^4
24-well plate	8–10 x 10^4
12-well plate	2–3 x 10^5
6-well plate	4–6 x 10^5

** Be sure that cells are seeded a minimum of 24 hours before transfection. Actual cell number used depends on cell type and size. Sufficient cells should be seeded such that the culture is 80–90% confluent on the day of transfection. The volume of medium used is not critical. Use a volume suitable for the cell culture format.*

Amount of RNA

The amount of RNA is a critical factor for successful transfection (Figure 22). Toxic effects may arise if too much RNA is used, while conversely, expression levels may be too low if insufficient RNA is used. Therefore, the amount of RNA transfected should be optimized for every new RNA and/or new cell type used.

Transfection reagent to RNA ratio

When working with charge-based transfection methods (i.e., calcium phosphate-, lipid-, liposome-, or activated-dendrimer–mediated), the ratio of RNA to transfection reagent is a critical parameter to optimize. The overall charge of the transfection complexes is determined by the ratio of reagent to RNA. Optimal binding of transfection complexes to the negatively charged groups on the cell surface requires a slightly positive net charge. Therefore, RNA:transfection reagent ratio is an extremely important parameter to optimize for every new cell type and RNA construct used (Figure 23). Example pipetting schemes for optimizing transfection with TransMessenger Reagent are provided in Table 8. Blank Table 9 is provided for the creation of personal pipetting schemes. As a starting point for optimization, we recommend using an RNA:TransMessenger ratio of 1:4.

Amount of RNA vs. Transfection Efficiency

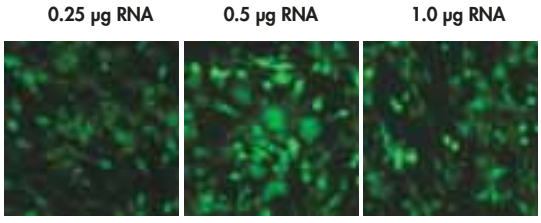


Figure 22. *Optimization experiments in CHO-K1 cells. 2 x 10^4 cells were seeded in quadruplicate into 96-well plates and transfected 24 h later with an in vitro-transcribed GFP-encoding RNA using increasing amounts of RNA with 1.5 µl TransMessenger Reagent, as described in the TransMessenger* Transfection Reagent Handbook. *Cells were photographed 24 h post-transfection.*

Amount of TransMessenger Reagent vs. Transfection Efficiency

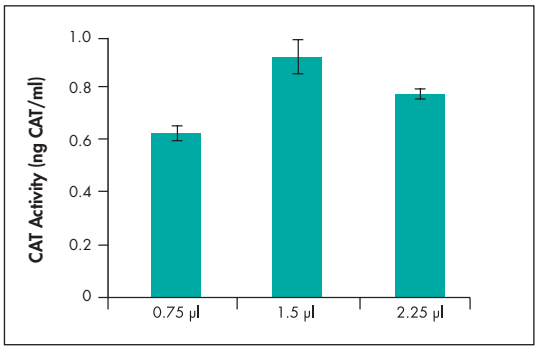


Figure 23. *Optimization experiments in CHO-K1 cells. 2 x 10^4 cells were seeded in quadruplicate into 96-well plates and transfected 24 h later with an in vitro-transcribed CAT-encoding RNA using increasing amounts of TransMessenger Reagent with 0.25 µg RNA, as described in the TransMessenger Transfection Reagent Handbook. CAT activity was measured 24 h post-transfection.*

Table 8. Example of a pipetting scheme for optimizing the transfection of adherent cells in 6-well plates using TransMessenger Reagent

Amount of RNA	Ratio of RNA to TransMessenger Transfection Reagent 1:2	1:4	1:8
1 µg	1 µg RNA 2 µl Enhancer R 2 µl TransMessenger Reagent	1 µg RNA 2 µl Enhancer R 4 µl TransMessenger Reagent	1 µg RNA 2 µl Enhancer R 8 µl TransMessenger Reagent
2 µg	2 µg RNA 4 µl Enhancer R 4 µl TransMessenger Reagent	2 µg RNA 4 µl Enhancer R 8 µl TransMessenger Reagent	2 µg RNA 4 µl Enhancer R 16 µl TransMessenger Reagent
4 µg	4 µg RNA 8 µl Enhancer R 8 µl TransMessenger Reagent	4 µg RNA 8 µl Enhancer R 16 µl TransMessenger Reagent	4 µg RNA 8 µl Enhancer R 32 µl TransMessenger Reagent

Table 9. Blank table for your personal pipetting scheme for TransMessenger Reagent

Amount of RNA	Ratio of RNA to TransMessenger Transfection Reagent 1:	1:	1:
µg	µg RNA µl Enhancer R µl TransMessenger Reagent	µg RNA µl Enhancer R µl TransMessenger Reagent	µg RNA µl Enhancer R µl TransMessenger Reagent
µg	µg RNA µl Enhancer R µl TransMessenger Reagent	µg RNA µl Enhancer R µl TransMessenger Reagent	µg RNA µl Enhancer R µl TransMessenger Reagent
µg	µg RNA µl Enhancer R µl TransMessenger Reagent	µg RNA µl Enhancer R µl TransMessenger Reagent	µg RNA µl Enhancer R µl TransMessenger Reagent

Incubation period with RNA–reagent complexes

TransMessenger Reagent

For adherent cells, optimal results with TransMessenger Reagent are typically obtained by choosing incubation periods of 3 hours, after which the TransMessenger–RNA complexes should be removed, the cells washed carefully with PBS, and fresh medium added. If no cytotoxic effects are observed, the incubation time can be increased up to 4 hours.

Incubation time following transfection

Compared to DNA transfections, optimal expression levels of RNA vectors may be obtained earlier following transfection. For example, cells transfected with a CAT reporter RNA are typically incubated for approximately 24 hours after transfection for maximal levels of protein expression. The appropriate incubation time will vary with the RNA vector used and with cell type. If the optimal time point for analysis of a particular RNA vector in a particular cell type is not known, it may be necessary to perform a time-course experiment.

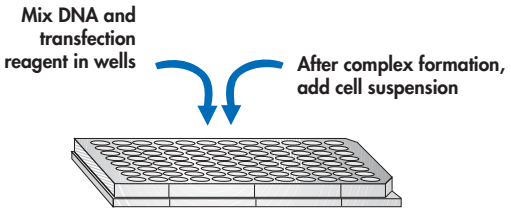
5. High-Throughput Transfection

The application of recombinant DNA technology to fields such as drug discovery and development has led to an increased need for high-throughput transfection. High-throughput transfection methods should require only minimal handling and provide excellent reproducibility and minimal cytotoxicity.

Transfection procedure

To maximize the number of plates processed in a single day, high-throughput transfection methods need to be fast, simple to perform, and involve only a minimum number of steps. QIAGEN has developed rapid transfection protocols for COS-7, NIH/3T3, HeLa, HeLa-S3, 293, and CHO cells in 96-well plates using PolyFect or Effectene Transfection Reagent. These optimized, cell-specific protocols allow same-day plating and transfection of cells — the DNA is simply mixed with the transfection reagent in the wells of a 96-well plate, and then the cells are added (see flowchart).

Rapid Transfection Procedure



Both PolyFect and Effectene Reagent show robust, reproducible, high-efficiency transfection in 96-well plates (Figure 24). Unlike many other transfection reagents, PolyFect and Effectene Reagent allow transfection in the presence of serum without lowering transfection efficiency. For most cell lines, post-transfection removal of transfection complexes or change/addition of medium is not necessary, making these reagents ideal for high-throughput applications and easily adaptable to automated transfection procedures. For more information on these reagents, please see "Plasmid DNA transfection technology", pages 15–19.

High-Throughput Transfection Using PolyFect and Effectene Reagents

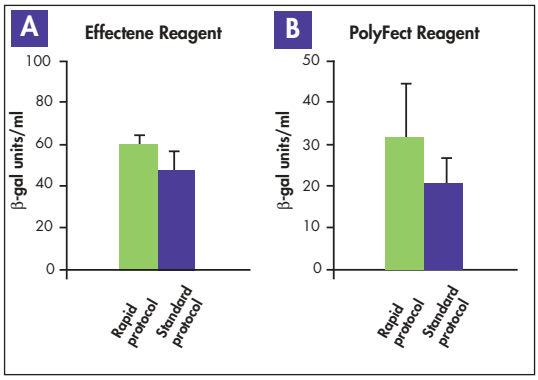


Figure 24. *High transfection efficiencies using rapid and standard Effectene and PolyFect protocols.* **A** *HeLa cells cultured in 96-well plates were transfected with a β-galactosidase reporter plasmid using the standard (0.1 µg DNA, 0.5 µl Effectene Reagent) or rapid Effectene protocol. β-galactosidase (β-gal) activity was measured 48 h post-transfection.* **B** *CHO cells cultured in 96-well plates were transfected with a β-galactosidase reporter plasmid using the standard or rapid PolyFect protocol. In the standard protocol, a medium change was performed after 3 h. β-gal activity was measured 48 h post-transfection using 1/5 of the well volume.*

Preparation of DNA

High-throughput transfection experiments often require a reliable method for high-throughput, small-scale purification of plasmid DNA. The QIAwell® Plasmid Purification System allows minipreparation of ultrapure plasmid DNA that shows excellent performance in transfection of sensitive cell types. Alternatively, the QIAprep® Turbo System can be used for minipreparation of high-purity plasmid DNA that is suitable for transfection-based screening of less sensitive cell lines (e.g., COS-7).

6. Troubleshooting Guide

The information below may be helpful if lower transfection efficiencies or higher cytotoxicity than expected is observed. Troubleshooting information can also be found in the *PolyFect, Effectene, SuperFect, and TransMessenger Transfection Reagent Handbooks*. Printed copies of these handbooks are available from QIAGEN, or an electronic version can be downloaded as a PDF file from **www.qiagen.com/transfectiontools/**. In addition, the scientists at QIAGEN Technical Services are always happy to answer any questions you may have about the information and protocols in this book, or molecular biology applications in general (see inside front cover for contact information).

Low transfection efficiency

Low transfection efficiencies may be due to a number of factors, including:

- Suboptimal transfection reagent to nucleic acid ratio
- Insufficient amount of transfection complex
- Suboptimal incubation time for gene expression
- Adverse vector affects (e.g., toxic gene products)
- Suboptimal cell density at the time of transfection
- Poor DNA/RNA quality
- Reporter assay problems

Optimization of transfection conditions, in particular, the amount of vector, the ratio of reagent to nucleic acid, and the amount of transfection complex, should help improve transfection efficiency. We recommend following these steps for optimization:

1. Vary the ratio of nucleic acid to reagent and determine the ratio that gives the highest transfection efficiency.

2. If cytotoxicity is low using this ratio, then, keeping the ratio constant, increase the overall amount of complex used.

3a. If the cytotoxicity remains low but the transfection efficiency is still too low, the time for which the transfection complexes are incubated with the cells can be increased.

3b. If transfection efficiency is increased but cytotoxicity is also increased, reduce the time for which the transfection complexes are incubated with the cells and carefully wash cells 2–3 times with PBS (see "Reagents and Solutions", page 48) after incubation.

In addition, be sure that the cells are at an optimal density for transfection and that the vector is highly pure. Following transfection, ensure that the cells are incubated for an appropriate time to express the transfected gene.

We recommend performing positive and negative controls with every transfection to help identify the source of any problems. Chapters 2 and 3 provide information on optimization of transfection as well as factors to consider for successful transfection of DNA and RNA vectors, respectively.

Excessive cell death

Excessive cell death following transfection could be due to:

- Excessive exposure of cells to transfection complexes
- Use of too much transfection complex
- Stressed cells
- Adverse vector affects

Some cell types may be very sensitive to the amount of transfection complex used and/or the length of time for which they are exposed to the complexes. Reducing the time of incubation with transfection complexes may help reduce cytotoxic effects. After the transfection complexes have been removed, wash the cells gently with PBS (see "Reagents and Solutions", page 48) or complete medium. If cell death continues after reducing exposure times, decrease the amount of complex used (but keep the ratio of transfection reagent to nucleic acid constant). We recommend following these steps to reduce cell death:

1. After removal of transfection complexes from the cells, gently wash the cells 2–3 times with PBS (see "Reagents and Solutions", page 48).

2. If cytotoxicity is still high, keep the ratio of nucleic acid to reagent constant but lower the amount of complex used for transfection.

3a. If cytotoxicity is still observed, reduce the time for which the cells are incubated with the transfection complexes.

3b. If cytotoxicity is reduced but the transfection efficiency is also reduced, optimize the ratio of nucleic acid to reagent (see "Low transfection efficiency", page 33).

In addition, it is important not to stress cells during the transfection procedure with temperature shifts or long periods without medium during washing steps. We recommend performing transfection in the presence of serum so that cells are not deprived of necessary growth factors and nutrients. Note, however, that serum is a potential source of RNases, and generally should not be used when incubating RNA vector-transfection complexes with cells. If serum must be used during the RNA transfection procedure, we recommend first testing the serum for absence of RNase contamination.

Optimization of the amount of vector used for transfection may help if the vector encodes a toxic protein or contains a strong promoter.

We recommend performing positive and negative controls with every transfection to help identify the source of any problems. Chapters 2 and 3 provide information on optimization of transfection as well as factors to consider for successful transfection of DNA and RNA vectors, respectively.

Variable transfection efficiencies in replicate experiments

Variable transfection efficiencies in replicate experiments are usually due to cell culture-related factors, such as:

- Inconsistent cell confluency in replicate experiments
- Mycoplasma contamination
- Use of cells at a high passage number
- Serum variability

It is important to maintain the same transfection conditions between replicate experiments to obtain consistent results. Count cells prior to seeding to ensure that the same number of cells is seeded in each experiment. In addition, keep incubation times between seeding and addition of transfection complexes constant. Variations in serum quality can also lead to variation in transfection efficiency. We recommend assessing a small lot of serum from a reputable supplier with a control cell line. Once a given lot has been determined to give satisfactory, reproducible results, additional serum from the same lot should be purchased and used in all future experiments. Mycoplasma contamination adversely affects transfection efficiency. Variations in the growth behavior of mycoplasma-infected cells will lead to different transfection efficiencies between different experiments. See "Cell culture contamination", pages 38–39, for further information on mycoplasma contamination and eradication.

Cells that have been passaged extensively tend to change their growth behavior, morphology, and transfectability. When cells with high passage numbers are used for replicate experiments, decreased transfection efficiency may be observed in later transfections. We recommend using cells with a low passage number (<50 splitting cycles). See "Animal Cell Culture", pages 36–47, for further information on cell culture.

Appendix A: Animal Cell Culture

A healthy cell culture lays the foundation for successful transfection. Different cell types have different characteristics as well as very specific medium, serum, and supplement requirements. Optimization of cell culture conditions is necessary to ensure that cells are healthy and in optimal condition for transfection. In addition, contamination with bacteria, mycoplasma, or fungi must be avoided, since this can drastically alter transfection results.

Introduction to animal cell culture

Depending on their origin, animal cells grow either as an adherent monolayer or in suspension. Different cell cultures differ in their proliferative potential, with three general categories being recognized — primary, finite, and continuous cell cultures. Different cell types vary greatly with respect to their growth behavior and nutritional requirements.

Adherent cells

Adherent cells are anchorage-dependent and usually propagate as a monolayer attached to the cell culture vessel. This attachment is essential for proliferation — many adherent cell cultures will cease proliferating once they become confluent (i.e., when they completely cover the surface of the cell culture vessel), and some will die if they are left in this confluent state for too long. Most cells derived from tissues are anchorage-dependent.

Suspension cells

Suspension cells can survive and proliferate without being attached to a substratum. Hematopoietic cells (derived from blood, spleen, or bone marrow) as well as some transformed cell lines and cells derived from malignant tumors can be grown in suspension.

Primary cell cultures

Primary cell cultures come from the outgrowth of migrating cells from a piece of tissue or from tissue that is disaggregated by enzymatic, chemical, or mechanical methods. Primary cultures are formed from cells that survive the disaggregation process, attach to the cell culture vessel (or survive in suspension), and proliferate. Primary cells are morphologically similar to the parent tissue. These cultures are capable of only a limited number of cell divisions, after which they enter a nonproliferative state called senescence and eventually die out. Adherent primary cells are particularly susceptible to contact inhibition, that is, they will stop growing when they have reached confluency. At lower cell densities, however, the normal phenotype can be maintained. Primary cell culture is generally more difficult than culture of continuous cell lines.

Primary cell cultures are sometimes preferred over continuous cell lines in experimental systems. Primary cells are considered by many researchers to be more physiologically similar to in vivo cells. In addition, cell lines cultured for extended periods of time can undergo phenotypic and genotypic changes that can lead to discrepancies when comparing results from different laboratories using the same cell line. Furthermore, many cell types are not available as continuous cell lines. For information on isolating cells for primary cell culture, please see "General considerations for primary cell culture", pages 41–42.

Finite cell cultures

Finite cell cultures are formed after the first subculturing (passaging) of a primary cell culture. These cultures will proliferate for a limited number of

cell divisions, after which they will senesce. The proliferative potential of some human finite cell cultures can be extended by introduction of viral transforming genes (e.g., the SV40 transforming-antigen genes). The phenotype of these cultures is intermediate between finite cultures and continuous cultures. The cells will proliferate for an extended time, but usually the culture will eventually cease dividing, similar to senescent primary cells. Use of such cells is sometimes easier than use of primary cell cultures, especially for generation of stably transfected clones.

Continuous cell lines

Finite cell cultures will eventually either die out or acquire a stable, heritable mutation that gives rise to a continuous cell line that is capable of unlimited proliferative potential. This alteration is commonly known as in vitro transformation or immortalization and frequently correlates with tumorigenicity. Rodent primary cell cultures form continuous cell lines relatively easily, either spontaneously or following exposure to a mutagenic agent. In contrast, human primary cell cultures rarely, if ever, become immortal in this way and require additional genetic manipulation to form a continuous cell line. However, cell cultures derived from human tumors are often immortal.

Continuous cell lines are generally easier to work with than primary or finite cell cultures. However, it should be remembered that these cells have undergone genetic alterations and their behavior in vitro may not represent the in vivo situation.

Safety and handling considerations

When working with potentially hazardous material, it is important to be aware of the possible risks associated with both the material and the experimental protocol. All cell cultures are considered a biohazard because of their potential to harbor an infectious agent (e.g., a virus). The degree of hazard depends on the cells being used and the experimental protocol. Primary cell cultures in particular should be handled carefully as these cultures have a high risk of containing undetected viruses. Although commonly used cell lines are generally assumed to be free of infectious agents, care should still be exercised when working with these cell lines as it is possible that they contain infectious agents, such as latent viruses. Cell cultures used to study specific viruses should be assumed to have the same degree of hazard as the virus under study.

We recommend handling all material as potentially infectious to ensure the safest possible working environment. Adherence to good laboratory practice when working with cell cultures is essential to reduce the risk of exposure of the worker to any potentially infectious agent(s) in the cell culture. Work should be performed in an approved laminar flow hood using aseptic technique, and the creation of aerosols should be avoided (see below). After the work is complete, all waste media and equipment (i.e., used flasks, pipets, etc) should be disinfected by autoclaving or immersion in a suitable disinfectant according to institutional and regional guidelines.

Legislation and regulatory guidelines

Before undertaking any work with human or animal tissue (e.g., to establish a primary cell culture), it is necessary to ensure that the nature of the work conforms to the appropriate medical-ethical and animal-experiment legislation and guidelines. It may be necessary to seek approval from the relevant regulatory authorities and/or individuals.

Aseptic technique and minimization of aerosols

Aseptic technique and the proper use of laboratory equipment are essential when working with cell

cultures. Always use sterile equipment and reagents, and wash hands, reagent bottles, and work surfaces with a biocide before beginning work. Creation of aerosols should be avoided — aerosols represent an inhalation hazard, and can potentially lead to cross-contamination between cultures. To avoid aerosols, use TD (to deliver) pipets, and not TC (to contain, also called "blowout") pipets; use pipets plugged with cotton; do not mix liquids by rapidly pipetting up and down; do not use excessive force to expel material from pipets; and do not bubble air through liquids with a pipet. Avoid releasing the contents of a pipet from a height into the receiving vessel. Expel liquids as close as possible to the level of liquid of the receiving vessel, or allow the liquid to run down the sides of the vessel.

Proper use of equipment can also help minimize the risk of aerosols. For example, when using a centrifuge, ensure the vessel to be centrifuged is properly sealed, avoid drops of liquid near the top of the vessel, and use centrifuge buckets with caps and sealed centrifuge heads to prevent contamination by aerosols.

Laminar flow hoods

For the most efficient operation, laminar flow hoods should be located in an area of the laboratory where there is minimal disturbance to air currents. Avoid placing laminar flow hoods near doorways, air vents, or locations where there is high activity. Hoods are often placed in dedicated cell culture rooms.

Keep laminar flow hoods clean, and avoid storing equipment inside the hood. Before starting work, disinfect the work surface of the hood as well as the outside of any bottles (e.g., by wiping with a biocide), and then place everything needed for the cell culture procedure in the hood. Arrange equipment, pipets, waste containers, and reagent bottles so that used items are not placed near clean items, and avoid passing used items over clean items. Place used items (e.g., pipets) in a container inside the hood, and disinfect or seal before removing from the hood.

Cell culture contamination

The presence of microorganisms can inhibit cell growth, kill cells, and lead to inconsistent results. Contamination of cell cultures can occur with both cell culture novices and experts. Potential contamination routes are numerous. For example, cultures can be infected through poor handling, from contaminated media, reagents, and equipment (e.g., pipets), and from microorganisms present in incubators, refrigerators, and laminar flow hoods, as well as on the skin of the worker and in cultures coming from other laboratories.

Bacteria, yeasts, fungi, molds, mycoplasmas, and other cell cultures are common contaminants in animal cell culture. Adherence to good laboratory practice (see above) will help prevent contamination of the cell culture with microbial or other animal cells. To safeguard against accidental cell culture loss by contamination, we recommend freezing aliquots of cultured cells to re-establish the culture if necessary (see page 46).

Microbial contamination

The characteristic features of microbial contamination are presented in Table 10. The presence of an infectious agent sometimes can be detected by turbidity and a sharp change in the pH of the medium (usually indicated by a change in the color of the medium), and/or cell culture death. However, for some infections, no turbidity is observed and adverse affects on the cells are not easily observed.

Cell cultures should be routinely evaluated for contamination. Mycoplasmal infections are one of the more common and difficult-to-detect infections;

Table 10. Characteristic features of microbial contamination

Characteristic	Bacteria	Yeast	Fungi
Change in pH	pH drops with most infections	pH changes with heavy infections	pH sometimes increases
Cloudy medium: Under microscope (100–400x)	Shimmering in spaces between cells; rods or cocci may be observed	Round or ovoid particles that bud off smaller particles	Thin filamentous mycelia; sometimes clumps of spores

their detection and eradication are described in further detail below.

Mycoplasmal infection — detection

Mycoplasmas are small, slow-growing prokaryotes that lack a cell wall and commonly infect cell cultures. They are generally unaffected by the antibiotics commonly used against bacteria and fungi. Furthermore, as mycoplasma do not overgrow cell cultures and typically do not cause turbidity, they can go undetected for long periods of time and can easily spread to other cell cultures. The negative effects of mycoplasmal contamination include inhibition of metabolism and growth, as well as interference with nucleic acid synthesis and cell antigenicity. Acute infection causes total deterioration of the cell culture, sometimes with a few apparently resistant colonies that may, in fact, also be chronically infected. There are three main approaches to detect mycoplasma — Hoechst 33258 staining (1, 4) mycoplasma-specific DNA probes (Fisher Scientific), and PCR-based assays (e.g., the VenorGeM® Mycoplasma Detection Kit from Minerva Biolabs). Alternatively, a PCR-based, mycoplasma-testing service is offered by the ATCC or Microbiological Associates on a fee-for-service basis.

Mycoplasmal infection — eradication

The best action to take with a culture containing chronic mycoplasmal infection is to discard it by either autoclaving or incineration. Only if the cell culture is absolutely irreplaceable should eradication be attempted. This process should be performed by experienced personnel in an isolated hood that is not used for cell culture, preferably in a separate room. Elimination of mycoplasma is commonly achieved by treatment with various commercially available antibiotics such as Mynox® Mycoplasma Elimination Reagent (Minerva Biolabs), a quinolone derivative (Mycoplasma Removal Agent), enrofloxacin (Baytril®), and a combination of tiamulin and minocycline (BM-Cyclin). Treatment procedures and appropriate antibiotic concentrations can be found in the suppliers' instructions and in references 1 and 32.

Cross-contamination of cell lines

Cross-contamination of one cell culture with fast-growing cells from another culture (such as the cell line HeLa) presents a serious risk. To avoid cross-contamination, only use cell lines from a reputable cell bank; only work with one cell line at a time in the hood; use different pipets, bottles of reagents, and bottles of medium for different cell lines; and check cells regularly for the correct morphological and growth characteristics.

Cell culture conditions

Media and serum

The choice of cell culture medium is extremely important, and significantly affects the success of cell culture experiments. Different cell types have highly varied and specific growth requirements, and the

most suitable medium for each cell type must be determined experimentally. Common basal media include Eagle minimal essential medium (MEM), Dulbeccos modified Eagle medium (DMEM), RPMI 1640, and Ham F10. These contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and are available as either a powder or as a liquid from various commercial suppliers.

Basal media are usually supplemented just before use with serum, L-glutamine, and antibiotics and/or fungicides to give complete medium (also called growth medium). Serum is a partially undefined material that contains growth and attachment factors, and may show considerable variation in the ability to support growth of particular cells. Fetal calf serum (FCS) is the most often used, but for some applications less expensive sera such as horse or calf serum can be used. Different serum batches should be tested to find the best one for each cell types. L-glutamine is an unstable amino acid that, with time, converts to a form that cannot be used by cells, and should be added to medium just before use. Antibiotics and fungicides can be used as a supplement to aseptic technique to prevent microbial contamination. The working concentration of commonly used antibiotics and fungicides is provided in Table 11. Some cell types, particularly primary cells, require additional supplements to attach to the cell culture vessel and proliferate, such as collagen and fibronectin, hormones such as estrogen, and growth factors such as epidermal growth factor and nerve growth factor.

Media, serum, and supplements should be tested for sterility prior to use by incubation of a small aliquot at 37°C for 48 hours. If microbial growth has occurred after this incubation, the medium or supplement should be discarded.

Incubation conditions

The incubation conditions used to culture cells are also important. Cell cultures should be incubated in an incubator with a tightly regulated temperature (e.g., a water-jacketed incubator) and CO_2 concentration. Most cell lines grow at 37°C and 5% CO_2 with saturating humidity, but some cell types require incubation at lower temperatures and/or lower CO_2 concentrations.

Cell culture vessel

The choice of growth vessel can influence the growth of adherent cells. Sterile, disposable dishes and flasks that have been treated to allow

Table 11. Commonly used antibiotics and fungicides for animal cell culture

Additive	Working concentration	Effective against	Stability at 37°C
Antibiotic			
Penicillin	50–100 U/ml	Gram-positive bacteria	3 days
Streptomycin	50–100 µg/ml	Gram-negative bacteria	3 days
Kanamycin	100 µg/ml	Gram-positive and Gram-negative bacteria; mycoplasma	5 days
Gentamycin	5–50 µg/ml	Gram-positive and Gram-negative bacteria; mycoplasma	5 days
Fungicide			
Nystatin	100 U/ml	Yeasts and molds	3 days
Amphotericin B	0.25–2.5 µg/ml	Yeasts and molds	3 days

Adapted from reference 2

attachment of animal cells to the growing surface are available commercially.

Cell banking

For some cell cultures, especially those that are valuable, it is common practice to maintain a two-tiered frozen cell bank: a master cell bank and a working cell bank. The working cell bank comprises cells from one of the master bank samples, which have been grown for several passages before storage. If and when future cell samples are needed, they are taken from the working cell bank. The master cell bank is used only when absolutely necessary. This ensures that a stock of cells with a low passage number is maintained, and avoids genetic variation within the cell culture (see below).

Culture instability

The growth rate of cells that have been repeatedly subcultured may sometimes unexpectedly decrease, and the cytotoxicity of, for example, a transfection process may unexpectedly increase. This instability can result from variations in cell culture conditions, genomic variation, and selective overgrowth of constituents of the cell population. We recommend using cells with a low passage number (<50 splitting cycles). To safeguard against instability in continuous cell lines, avoid senescence or transformation in finite cell lines, and maintain consistency in transfection experiments. We recommend creating cell banks by freezing aliquots of cells to recall into culture if and when necessary.

General considerations for primary cell culture

As described above (page 36), primary cell cultures are derived either by outgrowth of migrating cells from a small piece of tissue (primary explant technique), or by enzymatic or mechanical disaggregation of tissue. Primary cell culture is generally more demanding than culture of established cell lines. A comprehensive overview of primary cell isolation and culture is provided in references 1 and 4. The following general factors should be considered when isolating primary cells for culture.

Regulatory guidelines

As described above (page 37) any work with human or animal tissue must conform to the appropriate medical-ethical and animal-experiment legislation and guidelines. It may be necessary to seek approval from the relevant regulatory authorities and/or individuals.

Biohazards

Human and primate material and biopsy and autopsy specimens should be handled in a Class II biohazard hood. All media and apparatus should be disinfected after use by autoclaving or immersion in a suitable disinfectant according to your institution's guidelines.

Starting material

The quality and freshness of the starting material is critical for the success of primary culture. Embryonic tissue is easier to disaggregate, yields more cells, and proliferates more rapidly in primary culture than adult tissue. The morphology of the tissue should be examined carefully and documented.

Choice of technique

Mechanical and proteolytic enzymatic disaggregation of tissue usually yield a high number of cells that are more representative of the whole tissue in a shorter time than the primary explant technique. However, it is important to minimize the exposure of cells to proteolytic enzymes to ensure maximum cell viability.

Aseptic technique

Sterile conditions should be used as soon as possible in the isolation procedure. Outside the lab, the site of the dissection should be sterilized with a suitable disinfectant to minimize contamination, and all dissection instruments should be sterile. Working quickly, the tissue should be removed aseptically, stored in medium, and transported to the lab as soon as possible. In the lab, the tissue should be chopped finely with minimum damage in a suitable biohazard hood. Any fat or necrotic tissue should be removed.

Growth conditions

Primary cells have highly varied and specific growth requirements. While some primary cells require hardly any additional media supplements to assist cell attachment and proliferation, others will attach and proliferate only when provided with a cocktail of specific media supplements. Common supplements include attachment factors such as collagen and fibronectin, hormones such as estrogen, and growth factors such as epidermal growth factor and nerve growth factor. The choice of growth vessel can also be important.

Essential protocols for cell culture

Establishment and maintenance of animal cell cultures require standardized approaches for media preparation, feeding, and passaging (or subculturing) of the cells. Cultures should be examined regularly to check for signs of contamination and to determine if the culture needs feeding or passaging.

The cell culture protocols below have been adapted from the following sources: *Culture of Animal Cells, A Manual of Basic Technique* (1), *Current Protocols in Molecular Biology* (2), and *Cells: A Laboratory Manual* (4). These protocols are examples of methods for general cell culture, and have not been rigorously validated and optimized by QIAGEN. There are many alternative protocols in current use.

IMPORTANT: Potentially biohazardous materials (e.g., cells, culture medium, etc.) should be sterilized before disposal, and disposed of according to your institution's guidelines.

Cell thawing

1. **Heat a water bath to 37°C, and prewarm the growth medium into which the cells will be plated.**
2. **Add prewarmed growth medium to an appropriately sized cell culture vessel.**
   The size of the cell culture vessel depends on the cell type as well as the cell density.
3. **Remove a vial of frozen cells from liquid nitrogen, and place in the water bath until thawed.**
   IMPORTANT: Wear protective goggles and gloves when thawing vials that have been stored in liquid nitrogen. Vials may explode when removed from liquid nitrogen.
   IMPORTANT: Proceed to step 4 as soon as the cells have thawed. Do not allow the cells to warm up before transferring them into growth medium.
4. **Wash the outside of the vial with a suitable disinfectant.**
5. **Slowly pipet the thawed cell suspension into the cell culture vessel containing prewarmed growth medium. Swirl the vessel gently to mix the cells with the medium.**
   Note: Immediate removal of DMSO may sometimes be necessary, especially for suspension cells, primary cells, and sensitive cell types. For such cell types, pipet the thawed cell suspension into a sterile centrifuge tube containing prewarmed medium, centrifuge at 200 x *g* for 2 min, aspirate the supernatant, resuspend the cells in fresh growth medium, and

then transfer to an appropriate cell culture vessel.

IMPORTANT: Thoroughly mix the cells in the cell culture vessel to ensure even distribution of the cells throughout the vessel.

6. **Incubate cells overnight under their usual growth conditions.**
7. **The next day, replace the growth medium.**

Trypsinizing cells

Trypsinization is a technique that uses the proteolytic enzyme trypsin to detach adherent cells from the surface of a cell culture vessel. This procedure is performed whenever the cells need to be harvested (e.g., for passaging, counting, or for collecting cells for the isolation of biomolecules such as DNA or RNA).

1. **Aspirate the medium and discard.**
2. **Wash cells with PBS or HBSS (see "Reagents and Solutions", page 48), aspirate, and discard.**

   The volume of PBS or HBSS should be approximately the same as the volume of medium used for culturing the cells.
3. **Repeat step 2.**
4. **Add enough prewarmed 1x trypsin–EDTA solution (see "Reagents and Solutions", page 48) to cover the monolayer, and rock the flask/dish 4–5 times to coat the monolayer.**
5. **Place the flask/dish in a CO_2 incubator at 37°C for 1–2 min.**
6. **Remove flask/dish from incubator and firmly tap the side of the flask/dish with palm of hand to assist detachment.**

   If cells have not dislodged, return the flask/dish to the incubator for a few more minutes.

   IMPORTANT: Do not leave cells in 1x trypsin–EDTA solution for extended periods of time. Do not force the cells to detach before they are ready to do so, or clumping may occur.

   Overly confluent cultures, senescent cells, and some cell lines may be difficult to trypsinize. While increasing the time of trypsin exposure may help to dislodge resistant cells, some cell types are very sensitive to trypsin and extended exposure may result in cell death. In addition, some cell lines will resist this treatment and will produce cell clumps. Such cell clumps can be broken up by repeatedly pipetting the cells up and down in a syringe with a needle attached. This should be performed as gently as possible to avoid damaging the cells.
7. **Once dislodged, resuspend the cells in growth medium containing serum.**

   Use medium containing the same percentage of serum as used for growing the cells.

   Note: The serum inactivates trypsin activity.
8. **Gently pipet the cells up and down to disrupt cell clumps.**

   If pipetted too vigorously, the cells will become damaged. Ensure that pipetting does not create foam.
9. **Proceed as required (e.g., with passaging, freezing, nucleic acid isolation, etc).**

Passaging cells

Many adherent cell cultures will cease proliferating once they become confluent (i.e., when they completely cover the surface of cell culture vessel), and some will die if they are left in a confluent state for too long. Adherent cell cultures therefore need to be routinely passaged, that is, once the cells are confluent, a fraction of the cells need to be transferred to a new cell culture vessel. Suspension cells will exhaust their culture medium very quickly once the cell density becomes too high, so these cultures similarly require regular passaging.

IMPORTANT: Although regular passaging is necessary to maintain animal cell cultures, the procedure is relatively stressful for adherent cells as they must be trypsinized. We do not recommend passaging adherent cell cultures more than once every 48 hours.

1. **Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium containing serum.**

   The volume of medium used to resuspend the cells depends on the split ratio required (see step 2) and the size of the cell culture vessel. If too small a volume is used, it may be difficult to accurately pipet the desired volume to the new culture vessel. Conversely, if too large a volume is used, the culture vessel may be too full following transfer of the cells.

   Note: Removal of trypsin may sometimes be necessary following harvesting of adherent cells, especially for primary and sensitive cell types. Centrifuge the cells at 200 x *g* for 5 min, carefully aspirate the supernatant, and resuspend in an appropriate volume of prewarmed medium containing serum.

2. **Transfer an appropriate volume of the resuspended cells to a fresh cell culture vessel containing prewarmed growth medium. Swirl the vessel gently to mix the cells with the medium.**

   IMPORTANT: Thoroughly mix the cells in the cell culture vessel to ensure even distribution of the cells throughout the vessel.

   IMPORTANT: Some cell types will not survive if too few cells are transferred into the new cell culture vessel. We do not recommend high split ratios for primary cells, sensitive cell types, or senescent cultures.

   For adherent cells, we recommend adding enough cells so that the culture takes approximately one week to reach confluence again. This minimizes the number of times the cells are trypsinized as well as the handling time required to maintain the culture.

   When determining how many cells to transfer to the new cell culture vessel, it can be helpful to think in terms of how many cell divisions will be required for the culture to reach confluence again. For example, if half the cells are transferred, then it will take the culture one cell division to reach confluency again; if a quarter of the cells are transferred then it will take 2 cell divisions, and so on. If a culture divides once every 30 h or so, then in one week it will undergo approximately 5 cell divisions. A split ratio of 1:32 ($1:2^5$) should therefore be appropriate for the cells to reach confluency in about one week. In step 1, resuspend the cells in 8 ml medium, and transfer 0.25 ml to the new cell culture vessel.

3. **Incubate cells under their usual growth conditions.**

Cell counting using a hemocytometer

It is often necessary to count cells, for example, when plating cells for transfection experiments. One method for counting cells is to use a hemocytometer. A hemocytometer contains two chambers (Figure 25, page 45). Each chamber is ruled into nine major squares (volume of 0.1 mm^3 or 1 x 10^{-4} ml each). Cell concentration is determined by counting the number of cells within a defined area of known depth (volume).

1. **Clean the surface of the hemocytometer with 70% ethanol or another suitable disinfectant, taking care not to scratch the surface of the central area. Dry with lens paper.**
2. **Clean the coverslip, wet the edges very slightly, lay it over the groves and central area of the hemocytometer and gently press down.**

   It is important that the coverslip is properly attached to obtain the correct chamber depth. The appearance of Newton's rings (bright and dark rings caused by interference in the air between the coverslip and the glass surface of the hemocytometer) will confirm that the coverslip is attached properly.

3. **Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium. At least 10^6 cells/ml are required for accurate counting.**

   It may be necessary to centrifuge cells and resuspend in a smaller volume to obtain the desired cell concentration for counting. For adherent cells, it is important to produce a single-cell suspension after trypsinizing. Cell clumping will make counting difficult and inaccurate.

4. **Mix the cell suspension sample thoroughly. Using a pipet, immediately transfer 20 µl to the edge of one side of the coverslip to fill one chamber of the hemocytometer. Repeat for the second chamber.**

   Note: The cell distribution should be homogeneous in both chambers.

   The cell suspension is drawn under the coverslip and into the chamber by capillary action. The cell suspension should just fill the chamber. Blot off any surplus fluid without disturbing the sample underneath the coverslip.

5. **Transfer the slide to the microscope and view a large square ruled by three lines using a 10x objective and 10x ocular. Count the total number of cells in 5 of the 9 major squares.**

   Count cells that overlap the top and left border of squares but not those overlapping bottom and right borders (Figure 25). This prevents counting overlapping cells twice. If the cell density is too high, the cell suspension should be diluted, noting the dilution factor.

6. **Repeat the counting for the second chamber to give a total of 10 squares.**

7. **Add the number of cells counted in all 10 squares together to give the number of cells in 1 x 10^{-3} ml. Multiply by 1000 to give the number of cells/ml.**

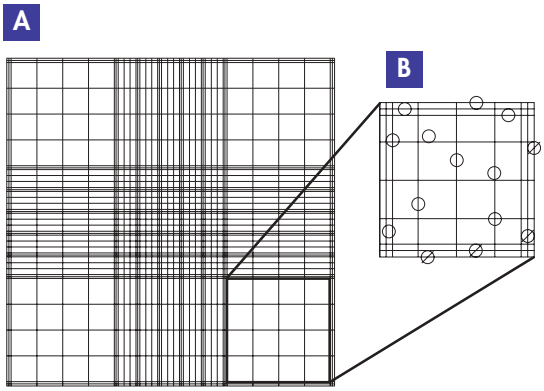


Figure 25. *A One chamber of a hemocytometer slide under 10x objective and 10x ocular. The chamber is divided into 9 major squares. B Detailed view of one of the 9 major squares. Only cells that overlap the top and left borders of squares should be counted to avoid overestimating the cell concentration. O: cells that should be counted; Ø: cells that should be ignored.*

   IMPORTANT: If the original cell suspension was diluted for counting, multiply by the dilution factor to obtain the number of cells/ml.

8. **Clean the hemocytometer and coverslip by rinsing with 70% ethanol and then with distilled water. Dry with lens paper.**

Viability staining

Trypan blue staining provides a method for distinguishing between viable (i.e., capable of growth) and nonviable cells in a culture. This staining method is based on "dye exclusion": cells with intact membranes exclude (i.e., do not take up) the dye and are considered viable.

1. **Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium to give a cell density of at least 10^6 cells/ml.**

2. **Add 0.5 ml 0.4% (w/v) trypan blue (see "Reagents and Solutions", page 48) and 0.3 ml PBS or HBSS (see "Reagents and Solutions", page 48) to 0.1 ml of the cell suspension, mix thoroughly, and let stand for 1–2 min.**

Alternatively, add 0.4 ml trypan blue directly to 0.4 ml of cells in growth medium.

At least 10^6 cells/ml are required for accurate counting.

3. **Count the stained and unstained cells using a hemocytometer. Blue-stained cells are non-viable and unstained cells are viable.**

   No. of viable cells/Total no. of cells = % viability.

Cell freezing

1. **Check that cells are healthy, not contaminated, and have the correct morphological characteristics.**
2. **Change the medium 24 h before freezing the cells.**

   Adherent and suspension cell cultures should not be at a high density for freezing. We recommend freezing cells when they are in the logarithmic growth phase.
3. **Adherent cultures: harvest the cells by trypsinization, resuspend in medium containing serum, centrifuge at 200 x *g* for 5 min, and then resuspend cells in freezing medium (see "Reagents and Solutions", page 48) at a density of 3–5 x 10^6 cells/ml.**

   Suspension cultures: centrifuge the cells at 200 x *g* for 5 min, and resuspend in freezing medium (see "Reagents and Solutions", page 48) at a density of 5–10 x 10^6 cells/ml.

   IMPORTANT: Freezing medium containing DMSO is hazardous and should be handled with caution.
4. **Transfer 1 ml of the cell suspension (approximately 3–5 x 10^6 adherent cells or 5–10 x 10^6 suspension cells) into each freezing vial. Label vials with the name of cell line, date, passage number, and growth medium.**

   It may also be useful to note the cell density in the freezing vials before storing. This enables determination of the cell density that provides optimal recovery after thawing.
5. **Place freezing vials in racks and transfer to a polystyrene box (with walls approximately 15 mm thick) lined with cotton wool. Store box in a –80°C freezer overnight.**

   It is important that cells are frozen at a rate of 1°C/min. A controlled-rate freezing device can be used instead of the polystyrene box and cotton wool method.
6. **The next day, quickly transfer the vials to a liquid nitrogen chamber, making sure that the vials do not begin to thaw.**

Selection of stable transfectants

Important notes before starting

- Check that the cell line used can produce colonies from isolated cells. Some cell lines can only grow if the cells are in contact with one another. In such cases, adapted or conditioned medium may be beneficial.
- Choose an appropriate selectable marker (see reference 4 for commonly used selectable markers).
- Select a transfection procedure suitable for your cell type. See "Plasmid DNA transfection technology", pages 15–19, for further information.
- Determine suitable selection conditions for your cell type as follows:

  Split a confluent dish of cells at approximately 1:5 to 1:10 (depending on cell density post-transfection and cell type) into medium containing various concentrations of the antibiotic; incubate for 10 days replacing selective medium every 4 days (or as needed), and then examine the dishes for viable cells by staining with trypan blue. Plot the number of viable cells versus antibiotic concentration to establish a dose-response curve (kill curve). Establishing a dose-response curve in this way determines the most appropriate concentration of antibiotic to use for selection of stable

transfectants. It is important to note that the optimal concentration of antibiotic varies with cell type.

Procedure

1. **Using the selected transfection method, cotransfect the cells with a 5:1 molar ratio of plasmid containing the gene of interest to plasmid containing a selectable-marker gene.**

   IMPORTANT: Perform replicate transfections for each treatment in case the transfection fails or the cultures become contaminated. Include control cotransfections (control plasmid plus selectable-marker plasmid). If colonies are obtained from cells transfected with the control plasmid but not from cells transfected with plasmid containing the gene of interest, this indicates that the latter may be toxic. Alternatively, promoter interference may reduce the expression of the gene of interest to a level below the detection limit of the gene expression assay.

2. **After transfection, allow the cells to divide twice under nonselective conditions. If using a plasmid encoding a selection tag, follow the manufacturer's instructions to preselect transfected cells using magnetic beads, and allow the cells to divide twice under nonselective conditions.**

3. **Seed the cells at low density in selective medium. Set up at least 5 dishes from each transfected dish to maximize the number colonies that can be isolated and expanded. Replace the selective medium every 4 days (or as needed). After 10 to 12 days, inspect the plates for antibiotic-resistant colonies.**

   Both adherent and suspension cells should be seeded at low density for antibiotic selection. Adherent cells should cover less than 50% of the culture vessel surface area at the time selection is applied. If cells are too dense, large clumps of cells, rather than individual cells, may detach from the culture vessel. This may reduce the efficiency of the selection process. Suspension cells can be selected in soft agar or in 96-well plates for single-cell cloning. It is important to note that high suspension cell densities require frequent medium changes that may deplete critical soluble growth factors, thereby reducing cell viability and the efficiency of the system.

4. **Select and isolate large (500–1000 cells) healthy colonies.**

   Colonies from monolayer cultures can be isolated either by using cloning rings (cylinders) or using sterile tooth-picks (see references 1 and 4). For isolation of suspension clones, see reference 1.

5. **To ensure that cells are stably transfected, we recommend continuing the selection process. A single cell from a resistant colony can be transferred into the wells of 96-well plates to confirm that it can yield an antibiotic-resistant colony.**

   Only one cell should be present per well after transfer. If required, adapted or conditioned medium should be used when continuing the selection process. For monolayer clones derived from single cells in individual wells of multiwell plates, the colonies can be isolated simply by harvesting all the cells in an individual well (i.e., subcloning).

Appendix B: Reagents and Solutions

1x PBS (phosphate-buffered saline)

137 mM NaCl

2.7 mM KCl

4.3 mM Na_2HPO_4

1.47 mM KH_2PO_4

The pH should be 7.4 without adjustment. Store at room temperature.

1x HBSS (Hank's balanced salt solution)

5 mM KCl

0.3 mM KH_2PO_4

138 mM NaCl

4 mM $NaHCO_3$

0.3 mM Na_2HPO_4

5.6 mM D-glucose

The pH should be 7.4 without adjustment. Store at room temperature.

1x trypsin-EDTA solution

0.05% (w/v) trypsin

0.53 mM EDTA

Dissolve trypsin and EDTA in a calcium- and magnesium-free salt solution such as 1x PBS or 1x HBSS. Store 1x trypsin-EDTA solution at –20°C. Small aliquots can be stored at 2–8°C for 1–2 weeks.

Work quickly when using trypsin during cell culture, since trypsin degrades and enzymatic activity declines at 37°C.

Freezing medium

Growth medium (RPMI, DMEM, etc.) containing 10–20% FBS and 5–20% glycerol or DMSO. Store at –20°C. Most suspension cells are frozen in freezing medium containing DMSO.

0.4% trypan blue

Dissolve 0.4 g trypan blue in 100 ml 1x PBS or 1x HBSS. Store at room temperature.

Appendix C: Promoters for Gene Expression

The choice of promoter is critical for efficient expression of genes cloned into DNA vectors. There are two general types of promoters — constitutive and inducible. These are discussed in more detail below.

Constitutive promoters

Constitutive promoters are promoters that function all the time; genes cloned into a vector with a constitutive promoter will therefore be expressed continuously. Commercially available and widely used constitutive promoters for gene expression in mammalian cells include the cytomegalovirus (CMV) and simian virus 40 (SV40) promoters and derivatives, and the pMC1 and phosphoglycerate (PGK) promoters. Promoters are also available that allow gene expression in certain cell types only.

A strong constitutive promoter (i.e., a promoter that causes high levels of gene expression) is not always the best choice for production of stable transfectants, as strong overexpression of the transfected gene product may have toxic effects on the cells. A weak promoter is sometimes better. Alternatively, an inducible promoter can be used to obtain stable transfectants.

Inducible promoters

The use of a plasmid DNA vector with an inducible promoter allows controlled, reversible expression of a gene of interest in transfected cells. This is particularly useful for selecting stable transfectants when the product of the transfected gene is toxic to the cell, or for precisely analyzing the effect of expression of the transfected gene.

Inducible promoters generally require the presence of a specific molecule (e.g., a metal ion, metabolite, or hormone) in order to function — in the absence of the molecule, the promoter is inactive and gene of interest is not expressed, while in the presence of the molecule, the promoter is "switched on" and the gene is expressed. Some inducible promoters function in the opposite manner, that is, gene expression is induced in the absence of a specific molecule.

Various inducible promoters are available for controlled gene expression in mammalian cells. Each has associated advantages and disadvantages, including ease of set up; pleiotropic; nonspecific effects or toxicity of the inducing agent or treatment; high levels of background; uninduced expression; and/or slow clearance of the inducing agent from the cells.

Natural promoter systems

Relatively simple systems use natural inducible promoters with their natural inducing agent. These systems include metallothionine (MT) promoters, which can be induced by addition of heavy metal ions such as Zn^{2+} to the medium, and heat-shock gene promoters, which can be induced by shifting cells to a higher temperature. While these systems are comparatively easy to work with (see below), they have the disadvantage that the inducing agent can induce endogenous promoters, and, especially in the case of heavy metal ions, is toxic to cells. Thus, these inducing agents affect cell behavior, making it difficult to assess the effect of expression of the gene of interest.

Steroid hormone-inducible systems

Steroid hormone-inducible systems utilize *cis*- and *trans*-acting elements derived from different species to control gene expression. These systems use a fusion protein comprising the activation domain of a transcription factor and the hormone-binding

domain of a hormone receptor. The fusion protein also contains a binding domain for a specific yeast DNA sequence, which is present in the inducible promoter. Cells are transfected with the gene for the fusion protein and the gene of interest under the control of the inducible promoter. In the absence of the inducing hormone, the conformation of the fusion protein is such that its DNA binding domain is unable to bind to the inducible promoter, and the gene of interest is not expressed. Following addition of the inducing hormone to the cells, the fusion protein binds to the hormone and undergoes an conformational change, allowing it to bind to the promoter. Once bound to the promoter, the activation domain drives expression of the gene of interest. Since the inducible promoter contains a yeast regulatory element, the fusion protein should not affect expression of any endogenous genes.

One problem with hormone-inducible systems is that the inducing hormone can activate endogenous genes, making it difficult to assess the effect of expression of the gene of interest. This can be circumvented through the use of systems that respond to either synthetic hormones or hormones from other species (e.g., ecdysone from insects).

While more sophisticated than natural promoter systems, steroid hormone-inducible systems are more complicated in that cells must be transfected with two vectors (either sequentially or by cotransfection), one encoding the fusion protein, and one encoding the gene of interest. Expression of the fusion protein in the transfected cells must be fully characterized before the effect of the gene of interest can be assessed. Thus, considerable time is often required for setting up the system.

Tetracycline-regulated systems

The general principle of tetracycline-regulated systems is the same as that of steroid hormone-inducible systems, except that expression of the gene of interest occurs in the absence of tetracycline. Tetracycline-regulated systems utilize components of the *E. coli* tetracycline-resistance operon (33, 2). Gene expression is mediated through constitutive expression of a fusion protein (called the tet transactivator protein, or tPA) comprising domains from the tet repressor protein and the transcriptional activation domain of the VP16 protein of herpes simplex virus. Cells are transfected with plasmids encoding the tPA gene and the gene of interest under the control of a inducible promoter containing the tet resistance operator sequence. In the presence of tetracyline, tPA does not bind to the operator sequence, so the gene of interest is not expressed. In the absence of tetracycline, the conformation of tPA changes such that it does bind to the operator sequence, and the gene of interest is expressed.

Advantages of tetracycline-regulated systems are that gene expression can be more tightly controlled than by other inducible promoter systems and that the presence of tetracycline does not cause cytotoxic effects as only a low level is required to prevent expression of the gene of interest. In addition, systems are available that allow expression of the gene of interest in the presence of tetracycline, making the system extremely versatile. The tetracycline-regulated system also has the potential to allow tissue-specific gene expression by placing tPA under the control of a tissue-specific promoter. One drawback of the system is that constitutive expression of tPA may be cytotoxic. This can be overcome by using system where tPA expression is autoregulated. Many companies offer vectors for tetracycline-regulated gene expression. Single vector expression systems (i.e., tPA and the gene of interest in the same plasmid) have also been developed.

Appendix D: Genetic Reporter Systems

Genetic reporter systems, or reporter genes, are useful tools for analysis of gene regulation by *cis*-acting sequences, such as promoters and enhancers, and *trans*-acting factors, such as transcription factors. The presumed *cis*-acting sequence(s) from the gene of interest is cloned with the coding sequence of an unrelated reporter gene whose expression can easily be monitored (2, 3). Analysis of reporter gene expression in transfected cells allows indirect assessment of how the regulatory sequence(s) influences gene expression.

Reporter genes are also useful in serving as controls. For example, transfection efficiencies between different experiments can be standardized by comparing the expression of a reporter gene that is used in all experiments.

In choosing a suitable reporter system, several considerations should be taken into account:

- The reporter gene should be absent from the cells used in the study or easily distinguished from the native form of the gene.
- The assay for the reporter gene product should be quick, easy, sensitive, and inexpensive.
- A broad linear detection range is important to enable detection of both small and large changes in reporter gene expression.
- The presence of the reporter gene should not affect the physiology of the cells being used.

Further information on genetic reporter systems, including protocols for reporter-system assays, can be obtained from current molecular biology manuals (2, 3).

Chloramphenicol acetyltransferase

The prokaryotic enzyme chloramphenicol acetyl-transferase (CAT) is commonly used as a reporter. This enzyme catalyzes the transfer of acetyl groups from acetyl-coenzyme A to chloramphenicol. In the common CAT assay, cell lysates prepared from transfected cells are incubated with ^{14}C-labeled chloramphenicol. The resulting acetylated and unacetylated forms of chloramphenicol are separated by thin-layer chromatography. A qualitative estimate of CAT activity can be obtained simply by exposing the plates to X-ray film. For quantitative analysis, the separated bands can be scraped from the thin-layer plate and the levels of radioactivity measured in a scintillation counter. Currently, a CAT ELISA is also frequently used. In this assay the total expression of the CAT is measured by immunological detection, in contrast to the classic CAT assay described above, which measures enzyme activity.

Firefly luciferase

Firefly (*Photinus pyralis*) luciferase was introduced as a reporter of gene expression in 1986 (34). Firefly luciferase catalyzes a bioluminescent reaction involving the substrate luciferin, ATP, Mg^{2+}, and molecular oxygen. When these components are mixed with cell lysates containing luciferase, a flash of light is emitted, which decays rapidly. Light signals are detected using a luminometer or a liquid scintillation counter. The light emission is proportional to the luciferase activity of the sample, thereby providing an indirect measurement of the expression level. The reliability and sensitivity of the original firefly luciferase assay has been enhanced by including coenzyme A in the reaction mixture. Due to the favorable reaction of luciferase with luciferyl-CoA, a longer light signal is produced, which increases luminescence intensity and the sensitivity of the assay.

Simultaneous expression and measurement of two different luciferase reporter enzymes (experimental and control) within a single system can be achieved

by cotransfecting plasmids encoding firefly luciferase (experimental reporter) and Renilla (*Renilla reniformis*) luciferase (control reporter). The activities of firefly luciferase and Renilla luciferase are measured sequentially from a single sample. This system controls inherent assay-to-assay variability that can undermine experimental accuracy, such as differences in the number and health of cultured cells, and the efficiencies of cell transfection and lysis. It is also suitable for analyzing two separate events within the same system.

β-Galactosidase

The prokaryotic enzyme β-galactosidase can be assayed colorimetrically using the substrate *o*-nitro-phenyl-β-D-galactopyranoside (ONPG). The hydrolysis of ONPG by β-galactosidase yields a yellow-colored product, *o*-nitrophenol, which can be measured photometrically. Expression of β-galactosidase is frequently used as an internal control to normalize the variability of other reporter assays, in particular CAT and luciferase assays.

Human growth hormone (hGH)

The assay for human growth hormone is based on immunological detection of hGH secreted by transfected cells. Specific ^{125}I-labeled antibodies against hGH are used and results are monitored in a scintillation counter. Currently, a sandwich ELISA is also often used, which involves an antibody-coated ELISA plate. The hGH protein binds to the antibody on the plate, a digoxygenated antibody binds to hGH, and a secondary antibody coupled to alkaline phosphatase is used for detection.

Green fluorescent protein (GFP)

Green fluorescent protein, originally isolated from the jellyfish *Aequorea victoria* (2), has the ability to absorb blue light and emit green light. Therefore, GFP requires no additional proteins, substrates, or cofactors to emit light, unlike other bioluminescent reporters. This unique protein can be expressed in mammalian cells, and protein expression can be visually monitored in living cells. Although the system provides a convenient way to detect protein expression without a specific assay, quantitative analysis is limited. This reporter gene system is best suited for in situ detection of gene expression, such as localization studies of fusion proteins within cells. Recent advances have led to the development of GFP variants with "human" codon usage for more efficient translation in mammalian cells, and with sequence alterations that intensify the fluorescence 10–30 fold. In addition, their shifted emission wavelengths enable simultaneous examination of the expression of two or more genes in the same cell population.

Appendix E: Guidelines for RNA Interference (RNAi) Experiments

RNA interference (RNAi) is a technique that allows post-transcriptional gene silencing. Historically, RNAi has been used as a tool for functional genomics research in *C. elegans* and *D. melanogaster*. Initial attempts to use RNAi in mammalian cells were unsuccessful and some researchers were led to believe gene-specific RNAi was not possible in mammalian systems. However, as RNAi became better understood in other organisms, it was demonstrated that RNAi could be used to target and silence a specific gene in cultured mammalian cells.

Elbashir et al. (29) were the first to demonstrate that gene-specific repression can be mediated by double-stranded short interfering RNA (siRNA) in HeLa, COS-7, NIH/3T3, and 293 cells. This report was soon followed by a paper demonstrating specific suppression of target genes in mouse embryonic fibroblasts (MEF), HeLa, and 293 cells (30).

The findings that RNA-mediated gene silencing occurs in a wide variety of organisms and appears to have been conserved throughout evolution strongly suggests RNAi can be used in virtually any cell line and on a variety of targets. The specific pathways and mechanism of RNAi in mammalian cells are currently under investigation.

siRNA design

A number of factors need to be considered for the design of an siRNA that will effectively silence gene expression. The guidelines and protocol below are adapted from the Tuschl laboratory web site (**www.mpibpc.gwdg.de/abteilungen/100/105/sirna_u.html**). The guidelines do not take into account mRNA secondary structure. At present it does not appear that mRNA secondary structure has a significant impact on gene silencing. Please note that RNAi is a relatively new field. The guidelines below reflect current knowledge about RNAi, but this may change as the field develops further.

Further information is available online at **www.mpibpc.gwdg.de/abteilungen/100/105/sirna_u.html**.

siRNA structure

Initial studies of mammalian RNAi suggest that the most efficient gene-silencing effect is achieved by using a double-stranded siRNA having a 19-nucleotide complementary region and a 2-nucleotide 3' overhang at each end. siRNA duplexes with TT 3' overhangs are preferred for RNAi. Alternatively, siRNA duplexes can be synthesized with either UU or dTdT overhangs, both of which appear to work equally well. Oligonucleotides with a dTdT overhang are often cheaper to synthesize and probably more resistant to nucleases.

Target sequence

In gene-specific RNAi, the open reading frame segment of the mRNA is targeted. The search for an appropriate target sequence should begin 50–100 nucleotides downstream of the start codon. To avoid interference from mRNA-regulatory proteins, sequences in the 5' or 3' untranslated region or near the start codon should not be targeted.

Initial searches should look for sequences in the open reading frame of the target gene with the pattern AA(N_{19})TT. Once such a sequence has been found, the percentage of guanosine and cytosine residues should be determined. An ideal sequence will have a GC content of about 50%. However, it

is possible to use sequences having between 30 and 70% GC. Sequences with GC contents outside this range are not recommended. If sequences matching these criteria are not found, sequences with the pattern AA(N_{21}) should be considered. If an appropriate AA(N_{21}) sequence is not found, sequences with the pattern CA(N_{21}) should be considered. Since it appears that the extreme 3' nucleotide of the antisense strand is not involved in specificity and that the sense strand does not appear to be involved in target recognition, the sequence of the sense and antisense strand can be synthesized as 5' (N_{19})TT. Examples of siRNA target sequences and the resulting siRNA are shown in Table 12.

Once an appropriate sequence with a suitable GC content is found, it is a good idea to run a BLAST® search (**www.ncbi.nlm.nih.gov/BLAST/**) of the sequence to help ensure that only a single gene is targeted.

Protocol for identification of an RNAi target sequence

1. **Start your sequence search 50–100 bases downstream from the start codon.**
2. **Search the coding strand for a 23-nucleotide sequence of AA(N_{19})TT. If a suitable sequence is not found, search for AA(N_{21}) or CA(N_{21}) sequences.**

   In the latter cases, the sequence of the sense and antisense siRNA strands can still be synthesized as 5'(N_{19})TT. This is possible because the sense strand does not appear to be responsible for target recognition and the nucleotide at the 3'-most position of the antisense strand does not appear to contribute to specificity.
3. **Determine the percentage GC content of the 21-base sequence that will form the body of the siRNA.**

   Ideally, the GC content should be ~50%, but GC contents between 30 and 70% are acceptable. If the sequence does not fall within this range, we suggest searching the sequence again as described in step 2.
4. **Subject the 21-base sequence to a BLAST search against EST libraries to ensure that only one gene is targeted (the complement is automatically searched as well.) If multiple genes are homologous to the 21-base sequence, search for a new sequence as described in step 2.**

Table 12. Examples of siRNA target sequences and the resulting siRNA

Target/siRNA	Sequence
AA (N_{19}) TT target sequence (vimentin; reference 29)	
Targeted region (coding DNA)	5' AACTACATCGACAAGGTGCGCTT 3'
Targeted mRNA	5' AACUACAUCGACAAGGUGCGCUU 3'
siRNA	5' CUACAUCGACAAGGUGCGCdTdT 3' 3' dTdTGAUGUAGCUGUUCCACGCG 5'
AA (N_{21}) target sequence (lamin A/C; reference 29)	
Targeted region (coding DNA)	5' AACTGGACTTCCAGAAGAACATC 3'
Targeted mRNA	5' AACUGGACUUCCAGAAGAACAUC 3'
siRNA	5' CUGGACUUCCAGAAGAACAdTdT 3' 3' dTdTGACCUGAAGGUCUUCUUGU 5'

Annealing of RNA oligonucleotides to create an siRNA duplex

Many companies that produce RNA oligonucleotides will synthesize and anneal RNAs to create an siRNA ready for use in RNAi. However, if your oligonucleotide supplier does not perform this service or if you prefer to anneal your own RNA, this can be accomplished by following the protocol below.

When working with RNA oligonucleotides, be certain that all solutions and equipment are RNase-free. See "Prevention of RNase contamination", pages 27–28, for information on handling RNA.

1. **Determine the concentration of the oligonucleotides from their absorbance at 260 nm. An absorbance of 1 unit at 260 nm corresponds to 20–30 µg of oligonucleotide per ml.**

   Note: This relation is only valid for measurements made at neutral pH. Therefore, if it is necessary to dilute the oligonucleotide samples for measurement, this should be done using a low-salt buffer with neutral pH (e.g., 10 mM Tris·Cl, pH 7.0). Readings should be between 0.15 and 1.0 to ensure significance.
2. **Incubate oligonucleotides (20 µM each) in 50–100 µl annealing buffer (100 mM potassium acetate; 30 mM HEPES·KOH; 2 mM magnesium acetate, pH 7.4) at 90°C for 1 min.**
3. **Centrifuge the tube for 15 s.**
4. **Incubate at 37°C for 1 h.**

   The final concentration of the duplex is 20 µM (approximately 0.25 µg/µl)*. The siRNA is ready to use in RNAi experiments and can be stored at –20°C and undergo multiple freeze–thaw cycles.

** Calculated for an siRNA made of two 21-nt RNA oligos and using a general value of 660 g/mole for the molecular weight of each base pair. Please note that the actual concentration in µg/µl will depend on the base composition of each individual siRNA.*

RNAi and TransMessenger Transfection Reagent

Using an experimental approach and siRNA sequences similar to those used by Elbashir et al. (29), scientists at QIAGEN have demonstrated that TransMessenger Reagent can be used to effectively deliver siRNA into cultured cells (35). Western blot analysis showed that expression of the targeted genes was repressed in transfected cells (see Figure 19, page 26; references 29, 35). Please bear in mind that for a specific cell line and target gene, optimization of the transfection conditions may be required to achieve the highest level of gene silencing. See "Optimization of RNA transfection", page 29, for further information.

Appendix F: References

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References citing QIAGEN Transfection Reagents

The following references are a selection from the QIAGEN online reference database. Find the full, up-to-date list at **www.qiagen.com/literature**.

Cotransfection

Ghosh, T.K., Packham, E.A., Bonser, A.J., Robinson, T.E., Cross, S.J., Brook, J.D. (2001) Characterization of the TBX binding site and analysis of mutations that cause Holt-Oram syndrome. Hum. Mol. Genet. ***10****, 1983.*
Transient cotransfection of 3 different plasmids in rat cardiomyocyte-cell–line H9c2 cells using PolyFect Transfection Reagent.

Holmqvist, M.H. et al. (2001) Kinetic Modulation of Kv4-mediated A-current by arachidonic acid is dependent on potassium channel interacting proteins. J. Neurosci. ***21****, 4154.*
Human embryonic kidney (HEK) 293T cells were cotransfected using PolyFect Transfection Reagent. Cells were lysed 48 h after transfection and treated in immunoprecipitation reactions using anti-Kv4.2 antibody.

Pieri, I., et al. (1999) Regulation of the murine NMDA-receptor-subunit NR2C Promoter by Sp1 and fushi tarazu factor 1 (FTZ-F1) homologues. Eur. J. Neurosci. ***11****, 1.*
SuperFect Transfection Reagent was used to cotransfect NIH/3T3 cells with a reporter gene vector, a coactivating vector, or the control vector pRC/CMV to study promoter activities.

Shimohate, T. et al. (2000) Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. Nature Gen. ***26****, 29.*
COS-7 cells seeded onto an 8-well chamber slide were transfected with plasmid DNA (0.25 µg each) using SuperFect Transfection Reagent. The nuclear extracts were immunoprecipitated, followed by Western blot analysis. Cotransfections of COS-7 cells using SuperFect Transfection Reagent were also performed.

Stable Transfection

Hauck, C.R., Hsia, D.A., and Schlaepfer, D.D. (2000) Focal adhesion kinase facilitates platelet-derived growth factor-BB-stimulated ERK2 activation required for chemotaxis migration of vascular smooth muscle cells. J. Biol. Chem. ***275,*** *41092.*
Rat aortic SMCs (ATCC, CRL-2018) were grown in DMEM, 10% calf serum containing sodium pyruvate, penicillin, and streptomycin (SMC medium). SMCs were transfected in 10 cm dishes (migration assay) or on collagen-coated glass coverslips in 24-well plates (wound healing assay) using Effectene Transfection Reagent. Transfection efficiency (~15%) was determined by FACS analysis on GFP-transfected cells. For selection of stable cell lines, hygromycin-resistant populations of cells were single-cell–sorted by FACS and expanded, and clones were analyzed by blotting for expression of FRNK.

Wu, L., et al. (2001) MAT1-modulated CAK activity regulates cell cycle G1 Exit. Mol. Cell. Biol. ***21****, 260.*
Saos-2 cells were cultured in RPMI 1640 containing 15% FBS. pcDNA3/AsMat (MAT1-AS), a pcDNA3 vector containing a 462 bp antisense MAT1 fragment, was transfected into Saos-2 cells using Effectene Transfection Reagent. The G418 concentration for selection of stable clones was determined by a 7 day lethal-dose test. After 48 h of posttransfection incubation, Saos-2 cells next were selected for 7 days with 0.6 mg of G418 per ml. About 20 single stable clones were picked up from pcDNA3/AsMat (MAT1-AS)-transfected Saos-2 cells and expanded for detection of the MAT1 expression phenotype.

Erbay, E. and Chen, J. (2001) The mammalian target of rapamycin regulates c2c12 myogenesis via a kinase-independent mechanism. J. Biol. Chem. ***276****, 36079.*
C2C12 mouse myoblasts were stably transfected with various mTOR cDNAs that were constructed in pCDNA3, using PolyFect Transfection Reagent. Cells were selected in G418-containing medium and induced to differentiate in the presence or absence of rapamycin.

Teixeira, L.A., Fricke, C.H., Bonorino, C.B., Bogo, M.R. and Nardi, N.B. (2001) An efficient gene transfer system for a hematopoietic cell line using transient and stable vectors. J. Biotechnol. ***88****, 159.*
In this work the authors describe efficient EGFP gene transfer into the K562 human hematopoietic cell line using SuperFect Transfection Reagent. Episomal plasmid vectors were used for both transient and stable gene expression. FACS analysis of transient transfection efficiency showed that 98.1% of the viable cells (85% of the total sample) expressed EGFP 24 h after transfection. FACS analysis for stable transfection showed that 99.2% of the viable K562 cells (92% of the total sample) were positive 24 h after transfection. FACS data confirmed the stable maintenance and efficient expression of the transfected gene 10 weeks post-transfection.

Brightbill, H.D. et al. (1999) Host Defense Mechanisms Triggered by Microbial Lipoproteins Through Toll-Like Receptors. Science ***285****, 732.*
Mouse macrophage cell line RAW 264.7 was transfected with IL-12 and iNOS promoter constructs using SuperFect Transfection Reagent at a ratio of 1:3, in order to study CAT induction. In addition, SuperFect Transfection Reagent was used for stable and transient transfection of HEK 293 cells.

Transfection of Oligonucleotides

Bolz, S-S. et al. (2000). Antisense oligonucleotides against cytochrome P450 2C8 attenuate EDHF-mediated Ca(2+) changes and dilation in isolated resistance arteries. FASEB Journal ***14****, 255.*

SuperFect Transfection Reagent was used to transfect isolated resistance arteries from Syrian Hamster gracilis muscle with antisense oligonucleotide to determine if CYP2C8/9 is an EDHF synthase. The authors describe a novel vessel-culture technique for organ culture.

Helin, V., Gottikh, M., Mishal, Z., Subra, F., Malvy, C., and Lavignon, M. (1999) Cell cycle-dependent distribution and specific inhibitory effect of vectorized antisense oligonucleotides in cell culture. Biochem. Pharmacol. ***568,*** *95.*

SuperFect Transfection Reagent was used to transfect HeLa, NIH/3T3, and CEM-4 cells with antisense phosphodiester oligodeoxynucleotides (ODNs). Uptake efficiency was measured by flow cytometry and confocal microscopy analysis. SuperFect Transfection Reagent was also used to cotransfect HeLa cells with pEGFP-N1 and pCMVb-Gal to study the biological activity of ODNs.

Lanz, R.B. et al. (1999) A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. Cell ***97****, 17.*

SuperFect Transfection Reagent was used to cotransfect HeLa, COS, and T-47D cells with various hormone receptors (progesterone, glucocorticoid, androgen, estrogen, thyroid, retinoic acid, and peroxisome proliferator-activated) and either luciferase or CAT reporter plasmids in order to analyze receptor activation by the appropriate ligand. In addition, HeLa cells transfected with an antisense oligonucleotide directed against the steroid hormone receptor activator (SRA) demonstrated inhibition of PR activation.

Beutel, G. et al. (1999) Delivery of antisense oligonucleotides into C6 glioblastoma cells using Effectene Transfection Reagent. QIAGEN News 1999 No. 5, 1.

Effectene Transfection Reagent was used to transfect fluorescently labeled CD44s antisense oligodeoxynucleotide (ODN) into C6 glioblastoma cells. The fluorescence intensity of ODNs was 10–40 times higher in cells transfected using Effectene Reagent than in control cells transfected without Effectene Reagent. Furthermore, Effectene Reagent displayed no significant cytotoxicity.

Cotransfection of RNA and DNA

Kohrer, C., Xie, L., Kellerer, S., Varshney, U., and RajBhandary, U.L (2001) Import of amber and ochre suppressor tRNAs into mammalian cells: A general approach to site-specific insertion of amino acid analogues into proteins. Proc. Nat. Acad. Sci. USA ***98****, 14310.*

Effectene Transfection Reagent was used to cotransfect suppressor tRNA and reporter plasmid DNA into COS-1 cells. Suppression of amber and ochre termination codons in the reporter gene was measured using CAT assays of cell extracts. Both aminoacylated and non-aminoacylated tRNAs were transfected and shown to be active in the suppression of termination codons. Other methods used for transfection included electroporation, DEAE-dextran, calcium phosphate, Lipofectamine, and DMRIE-C. CAT activity was highest by a >25-fold factor in extracts of cells co-transfected using Effectene.

RNA interference

Dennig, J. and Konrad, J. (2002) TransMessenger Transfection Reagent enables targeted gene silencing in HeLa-S3 cells through RNA interference. QIAGEN News 2002 No. 2, 7.

Tranfection of short duplex dsRNA using TransMessenger Transfection Reagent allowed suppression of gene expression in cultured mammalian cells.

Special Applications

A4573 and KP-EWS-Y1 cells

Maruyama-Tabata, H. et al. (2000) Effective suicide gene therapy in vivo by EBV-based plasmid vector coupled with polyamidoamine dendrimer. Gene Therapy ***7****, 53.*

Combination of an Epstein-Barr virus based plasmid vector with a polyamidoamine dendrimer (SuperFect Transfection Reagent) for in vitro transfection of A4573 and KP-EWS-Y1 cells. Intratumoral injection of the same complexes into tumors generated from inoculation of A4573 Ewing's sarcoma cells into severe combined immunodeficiency (SCID) mice was performed to assess the possible applicability of this method to in vivo suicide gene therapy.

Plasmodium falciparum

Mamoun, C.B., Troung, R., Gluzman, I., Akopyants, N.S., Oksman, A., Goldberg, D.E. (1999) Transfer of genes into Plasmodium falciparum *by polyamidoamine dendrimers. Molecular and Biochemical Parasitology* ***103****, 117.*

Transient and stable transfection of intraerythrocytic *P. falciparum* with Superfect Transfection Reagent using GFP and pSSN vectors.

Rabbit and human corneas

Hudde, T., et al. (1999). Activated polyamidoamine dendrimers, a non-viral vector for gene transfer to the corneal endothelium. Gene Therapy ***6****, 939.*

Investigation of activated polyamidoamine dendrimers' transfection efficiency in rabbit and human corneas in ex vivo culture. Plasmids containing *lacZ* or *TNFR-Ig* genes were efficiently transfected using activated dendrimers (e.g., SuperFect Transfection Reagent).

Cell microarrays

Ziauddin, J., and Sabatini, D. (2001) Microarrays of Cells expressing defined cDNAs. Nature ***411****, 107.*

HEK 293T cells were transfected using Effectene Transfection Reagent. The authors demonstrate an alternative to protein microarrays for the identification of drug targets and an expression cloning system for the discovery of gene products that alter cellular physiology. By screening transfected cell microarrays expressing 192 different cDNAs, the authors identified proteins involved in tyrosine kinase signalling, apoptosis, and cell adhesion. The authors have also tested other cell lines such as HeLa, A549 and obtained similar results but with efficiencies of 30–50% of those obtained with 293 cells.

Primary cells

Bone marrow cells

Boden, S.D., et al. (1998) Lumbar spine fusion by local gene therapy with a cDNA encoding a novel osteoinductive protein (LMP-1). Spine ***23****, 2486.*

SuperFect Transfection Reagent was used to transfect bone marrow cells harvested from 4–5 week old rats. The transfected cells were applied to a bone matrix cancer and implanted during spine fusions of rats to test the local delivery of an osteoinductive protein. This local delivery allowed successful spine fusions.

Aortic smooth muscle cells

Rafty, L.A., and Khachigian, L.M. (1998) Zinc finger transcription factors mediate high constitutive platelet-derived growth factor–B expression in smooth muscle cells derived from aortae of newborn rats. J. Biol. Chem. ***273****, 5758.*

Cotransfection of primary smooth muscle cells from aortic cell wall of pups or adult rats with different plasmids using SuperFect Transfection Reagent. CAT assays of cell lysate and enzyme-linked immunosorbent assays for determination of soluble growth hormone from medium were performed.

Rabbit gastric parietal cells

Parente, J.A. Jr., Chen, X., Zhou, C., Petropoulos, A.C., and Chew, C.S. (1999) Isolation, cloning and characterization of a new mammalian coronin family member, coroninse, which is regulated within the protein kinase C signaling pathway. J. Biol. Chem. ***274****, 3017.*

Either Effectene Transfection Reagent or a liposome reagent was used to transfect primary parietal cells with pEGFP-pp66 fusion-construct DNA prepared using the EndoFree Plasmid Kit. The newly identified protein coroninse, appears to be highly expressed in secretory epithelia and may play an important protein kinase C-dependent role in regulating membrane/cytoskeletal rearrangements in epithelial cells.

Schwann cells

Khursigara, G., Orlinick, J.R., and Chao, M.V. (1999) Association of the p75 neurotrophin receptor with TRAF6. J. Biol. Chem. ***274****, 2597.*

Schwann cells isolated from sciatic nerves of rats were plated on poly-D-lysine-coated coverslips and cultured in Dulbecco's modified Eagle's medium containing 1% fetal calf serum and glial growth factor. Effectene Transfection Reagent was used to transfect the cells with 0.3 µg cDNA.

Cell lines

HUVEC cells

Imaizumi, T., et al. (2000) Expression of tumor necrosis factor-α in cultured human endothelial cells stimulated with lipopolysaccharide or interleukin-1 α. Arterioscler. Thromb. Vasc. Biol. (2000) ***20****, 410.*

SuperFect Transfection Reagent was used to transfect HUVEC cells with a full-length human TNF-α cDNA isolated from a cDNA library constructed from LPS-stimulated HUVEC cells and cloned into the pcDNA3 vector. TNF-α expression was measured to examine whether HUVEC cells are able to translate TNF-α mRNA. DNA was purified using a QIAGEN Plasmid Kit.

RIE-1 cells

Pruitt, K., Pestell, R.G., and Der, C.J. (2000) Ras inactivation of the retinoblastoma pathway by distinct mechanisms in NIH 3T3 fibroblast and RIE-1 epithelial cells. J. Biol. Chem. ***275****, 40916.*

For transient expression reporter assays, parental RIE-1 cells were transfected with SuperFect or Effectene Transfection Reagent. Briefly, RIE-1 cells were seeded the day before transfection and incubated with transfection complexes for 24 h. The growth medium was then replaced with DMEM supplemented with 0.2% fetal bovine serum. Cells were serum-starved for 24 h and harvested with 300 µl of luciferase lysis buffer, and 50 µl of lysate was analyzed using enhanced chemiluminescence reagents and a Monolight 2010 luminometer.

CEF cells

Kataoka, K., Yoshitomo-Nakagawa, K., Shioda, S., and Nishizawa, M. (2001) A set of Hox proteins interact with the Maf oncoprotein to Inhibit Its DNA binding, transactivation, and transforming activities. J. Biol. Chem. ***276****, 819.*

3 x10^5 CEF cells, grown on a 35 mm dish, were transfected with a total of 1 µg of plasmid (125 ng of luciferase reporter plasmid, 750 ng of expression plasmids, and 125 ng of pEF-Rluc plasmid) using 1 µl of SuperFect Transfection Reagent. For a focus formation assay, 2.5 µg of the recombinant retrovirus vector plasmid DNA was transfected into 1.2 x 10^6 CEF cells grown on a 60 mm dish using 12.5 µl of Effectene Transfection Reagent.

Mouse embryonic fibroblasts

Piu, F., Aronheim, A., Katz, S. and Karin, M. (2001) AP-1 repressor protein JDP-2: inhibition of UV-mediated apoptosis through p53 down-regulation. Mol. Cell. Biol. ***21****, 3012.*

Immortalized mouse embryonic fibroblasts were transiently transfected using SuperFect or PolyFect Transfection Reagent.

ECV304

Fowler, A.A., III, et al. (1999) Nitric oxide regulates interleukin-8 gene expression in activated endothelium by inhibiting NF-κB binding to DNA: effects on endothelial function. Biochem. Cell Biol. ***77****, 201.*

SuperFect Transfection Reagent was used to transfect the human endothelial cell line ECV304 with a pGL-3BF2 construct in order to examine the effects of NO (nitric oxide) on NF-κB activation. DNA was purified using the QIAfilter™ Plasmid Maxi Kit.

Raji B cells

Kiermer, V., et al. (1998) An interferon regulatory factor binding site in the U5 region of the bovine leukemia virus long terminal repeat stimulates tax-independent gene expression J. Virol. ***72****, 5526.*

SuperFect Transfection Reagent was used to transiently transfect 5 x10^6 human Raji B cells with 5 µg CAT reporter plasmid. 48 h post-transfection, cells were harvested, washed once with TNE and resuspended in Tris·Cl (pH 7.8). The cells were then lysed and subjected to CAT assays. Raji cells were grown in RPMI 1640-Glutamax I supplemented with 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin.

CMK11-5, Dami (megakaryoblastic leukemia), U937 (monocytic lymphoma), and HepG2

Fujimori, K., Kanaoka, Y., Sakaguchi, Y., and Urade, Y. (2000) Transcriptional activation of the human hematopoietic prostaglandin D synthase gene in megakaryoblastic cells. J. Biol. Chem. ***275****, 40511.*

The human cell lines CMK11-5, Dami (megakaryoblastic leukemia), U937 (monocytic lymphoma), and HepG2 (hepatoblastoma) were cultured at 37°C under 5% CO_2 in RPMI 1640 medium or in Dulbecco's modified Eagle's medium. Cells grown to ~70% confluence were cotransfected with each construct (1 µg) and pRL-SV40 (0.2 µg) carrying the *Renilla* luciferase gene under the control of the SV40 early promoter as the internal control. Transfection was achieved with Effectene Transfection Reagent. Cells were harvested 48 h after transfection, and cell extracts were prepared.

HT-29 cells

Ogier-Denis, E., Pattingre, S., El Benna, J., and Codogno, P. (2000) ERK1/2-dependent phosphorylation of Ga-interacting protein stimulates its GTPase accelerating activity and autophagy in human colon cancer cells. J. Biol. Chem. ***275****, 39090.*

Vectors encosing a His-tagged wild-type GAIP and a S151A mutant were introduced into exponentially growing HT-29 cells using Effectene Transfection Reagent. Cells were used 72 h after cell transfection.

Podocytes

Schiffer, M., et al. (2001) Apoptosis in podocytes induced by tgf-b and smad7. J. Clin. Invest. ***108****, 807.*

Podocytes were transiently cotransfected with a pSmad7 expression construct or the empty vector pcDNA3 together with NF-B-Luc, and a β-galactosidase expression vector, pRSV-β-gal, using Effectene Transfection Reagent. Luciferase and β-galactosidase activities in cell lysates were measured and normalized for transfection efficiency. For indirect immunofluorescence assays, cotransfections were performed with pcDNA3 or pSmad7, together with pEGFP. Cells were maintained without IFN-gamma before and after transfection. Recombinant mouse TNF-α was applied to transfected cultures 36 h after transfection.

WiDr cells

Kitada, T., et al. (2000) The addition of bisecting N-acetylglucosamine residues to E-cadherin down-regulates the tyrosine phosphorylation of β–catenin. J. Biol. Chem. ***276****, 475.*

Mock and GnT-III transfectants of WiDr cells were transfected using Effectene Transfection Reagent according to the standard protocol. After 48 h, the tyrosine phosphorylation of β-catenin in these cells was investigated.

THP-1 cells

Maiti, D., Bhattacharyya, A., and Basu, J. (2000) Lipoarabinomannan from Mycobacterium tuberculosis promotes macrophage survival by phosphorylating Bad through a phosphatidylinositol 3-kinase/Akt pathway. J. Biol. Chem. ***276****, 329.*

THP-1 cells (derived from a patient with acute monocytic leukemia) are mature cells from the monocyte/macrophage lineage. These were obtained from the National Center for Cell Science (Pune, India). Transfections were carried out on adherent THP-1 cells (2 x 10^6 cells/well in six-well plates). Cells were transfected with 2 µg of plasmid (recombinants or empty vectors) using Effectene Transfection Reagent in RPMI with 10% fetal bovine serum.

Ordering Information

Product	Contents	Cat. No.
TransMessenger Transfection Reagent — for transfection of eukaryotic cells with RNA		
TransMessenger Transfection Reagent (0.5 ml)	For 60 transfections in 6-well plates or 80 transfections in 12-well plates	301525
PolyFect Transfection Reagent — for optimized transfection of COS-7, NIH/3T3, HeLa, 293, and CHO cells		
PolyFect Transfection Reagent (1 ml)	For 25-65 transfections in 60 mm dishes or 50-100 transfections in 6-well plates	301105
PolyFect Transfection Reagent (4 x 1 ml)	For 100-260 transfections in 60 mm dishes or 200-400 transfections in 6-well plates	301107
PolyFect Transfection Reagent (100 ml)	For 2500-6500 transfections in 60 mm dishes or 5000-10,000 transfections in 6-well plates	301108
Effectene Transfection Reagent — for transfection of a wide variety of cells, particularly effective for primary cells		
Effectene Transfection Reagent (1 ml)*	For 40 transfections in 60 mm dishes or 160 transfections in 12-well plates	301425
Effectene Transfection Reagent (4 x 1 ml)*	For 160 transfections in 60 mm dishes or 640 transfections in 12-well plates	301427
SuperFect Transfection Reagent — for high transfection efficiencies in a broad range of cell types		
SuperFect Transfection Reagent (1.2 ml)*	For 40 transfections in 60 mm dishes or 160 transfections in 12-well plates	301305
SuperFect Transfection Reagent (4 x 1.2 ml)*	For 160 transfections in 60 mm dishes or 640 transfections in 12-well plates	301307
Transfection Reagent Selector Kit — for assessment of Effectene Reagent and SuperFect Reagent		
Transfection Reagent Selector Kit†	0.3 ml SuperFect Transfection Reagent, 0.3 ml Effectene Transfection Reagent	301399

* *Bulk quantities available; please inquire.*

† *PolyFect Transfection Reagent is not included in the Transfection Reagent Selector Kit.*

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Related products		
Plasmid DNA isolation*		
EndoFree Plasmid Maxi Kit (10)	10 QIAGEN-tip 500, Reagents, 10 QIAfilter Maxi Cartridges, Endotoxin-free Buffers	12362
EndoFree Plasmid Mega Kit (5)	5 QIAGEN-tip 2500, Reagents, 5 QIAfilter Mega-Giga Cartridges, Endotoxin-free Buffers	12381
EndoFree Plasmid Giga Kit (5)	5 QIAGEN-tip 10000, Reagents, 5 QIAfilter Mega-Giga Cartridges, Endotoxin-free Buffers	12391
HiSpeed Plasmid Maxi Kit (10)	10 HiSpeed Maxi Tips, 10 QIAfilter Maxi Cartridges, 10 QIAprecipitator Maxi Modules plus Syringes, Reagents, Buffers	12662
QIAfilter Plasmid Maxi Kit (10)	10 QIAGEN-tip 500, Reagents, Buffers, 10 QIAfilter Maxi Cartridges	12262
QIAfilter Plasmid Mega Kit (5)	5 QIAGEN-tip 2500, Reagents, Buffers, 5 QIAfilter Mega-Giga Cartridges	12281
QIAfilter Plasmid Giga Kit (5)	5 QIAGEN-tip 10000, Reagents, Buffers, 5 QIAfilter Mega-Giga Cartridges	12291
QIAwell 96 Ultra Plasmid Kit (4)	For 4 x 96 ultrapure plasmid minipreps, 4 each: QIAfilter 96, QIAwell 96, and QIAprep 96 Plates; Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	16191
QIAprep 96 Turbo Miniprep Kit (4)	For 4 x 96 high-purity plasmid minipreps, 4 each: TurboFilter 96 and QIAprep 96 Plates; Flat-Bottom Blocks and Lids, Reagents, Buffers, Collection Microtubes (1.2 ml), Caps	27191
Oligonucleotide synthesis		
Oligonucleotide Synthesis Service†	Custom synthesis of high-quality oligos, longmers, and modified oligos	—
Oligonucleotide purification*		
QIAquick Nucleotide Removal Kit (50)	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28304

* *Other prep and kit sizes available; please inquire.*

† *Available from QIAGEN Operon; please visit www.operon.com or contact QIAGEN Technical Support for further information.*

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RNA isolation*		
RNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy Midi Kit (10)†	10 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-free Reagents and Buffers	75142
RNeasy Maxi Kit (12)†	12 RNeasy Maxi Spin Columns, Collection Tubes (50 ml), RNase-free Reagents and Buffers	75162
Oligotex mRNA Mini Kit (12)‡	For 12 mRNA minipreps: 200 µl Oligotex Suspension, Small Spin Columns, Collection Tubes (1.5 ml), RNase-free Reagents and Buffers	70022

* *Other kit sizes available; please inquire.*

† *Requires use of a centrifuge capable of attaining 3000–5000 x g equipped with a swing-out rotor for 15 ml (Midi) or 50 ml (Maxi) centrifuge tubes.*

‡ *Not available in Japan.*

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