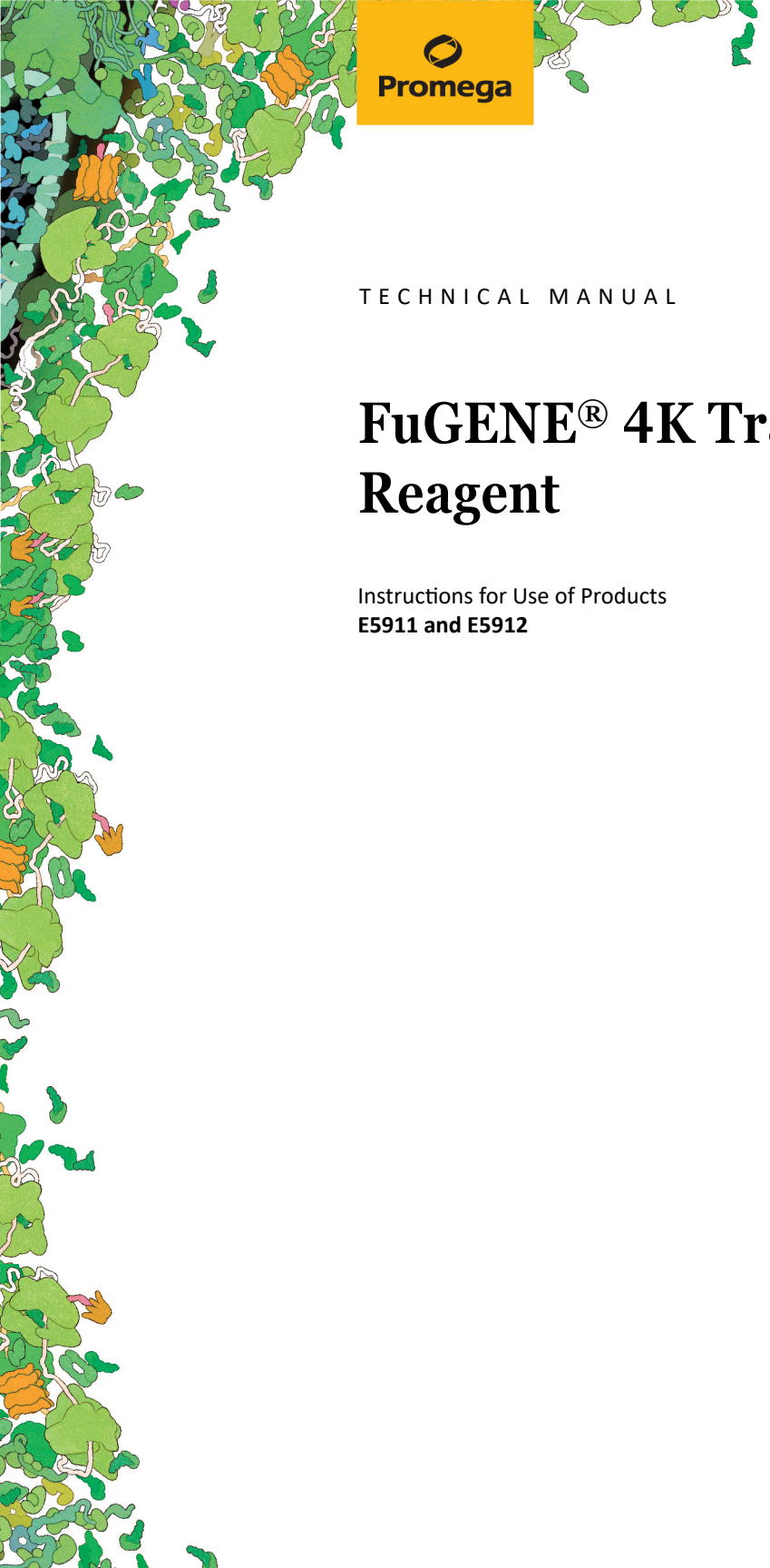

TECHNICAL MANUAL

FuGENE® 4K Transfection Reagent

Instructions for Use of Products
E5911 and E5912

5/22
TM694

FuGENE® 4K Transfection Reagent

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

Promega

1. Description

FuGENE® 4K Transfection Reagent is a multi-component, nonliposomal reagent designed to transfect DNA into a wide variety of mammalian cell lines with high efficiency and low toxicity, eliminating the need to change medium after introducing the reagent-DNA complex. The FuGENE® 4K Transfection Reagent functions in the presence or absence of serum and is suitable for transient and stable transfection.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
FuGENE® 4K Transfection Reagent	**1ml**	**E5911**
	5 × 1ml	**E5912**

The 1ml size contains sufficient reagent to transfect 333µg of DNA. This is equivalent to more than 3,300 wells in 96-well plates at a 3:1 FuGENE® 4K Transfection Reagent:DNA ratio (0.3µl reagent:100ng DNA per well). The actual number of transfections will vary with reagent:DNA ratio, transfection volume and cell type.

Storage Conditions: Store FuGENE® 4K Transfection Reagent at +2°C to +10°C. Close lid tightly after use. Do not freeze or store below 0°C; components may precipitate. If reagent has been frozen accidentally, briefly warm at 37°C to dissolve any precipitate.

Formulation and Packaging: FuGENE® 4K Transfection Reagent is a 100% synthetic, proprietary mixture of lipids and other components in 70% ethanol. The transfection reagent is sterile filtered and supplied in sterile glass vials. The FuGENE® 4K Transfection Reagent does not contain any ingredients of human or animal origin.

Special Handling: Equilibrate FuGENE® 4K Transfection Reagent to room temperature, and mix briefly by inverting or vortexing prior to use. No precipitate should be visible. Use a standard 24-well tissue culture plate to serve as a rack for FuGENE® 4K Transfection Reagent. Always dilute FuGENE® 4K Transfection Reagent directly into medium, avoiding direct contact of the undiluted reagent with the plastic tube. Do **not** use siliconized pipette tips or tubes. Do **not** dispense FuGENE® 4K Transfection Reagent into aliquots from the original glass vials. FuGENE® 4K Transfection Reagent also can be prediluted into transfection medium but should be immediately added to the DNA solution to form the transfection complex.

3. General Considerations

Successful transfection involves optimizing the FuGENE® 4K Transfection Reagent-to-DNA ratio, amount of DNA used, complexing time, cells and medium used, etc. For a detailed optimization protocol, see Section 4.E. Plasmids with reporter gene functions can be used to monitor transfection efficiencies. An ideal reporter gene is unique to the cell, can be expressed from plasmid DNA and assayed conveniently. Generally, reporter gene assays are performed 1–2 days after transfection. Reporter genes and assays for luciferase, green fluorescent protein (hMGFP) and β-galactosidase, as well as reagents for covalent protein labeling (HaloTag® protein) are available at:
www.promega.com

3.A. Ratio of Transfection Reagent to DNA

For successful transfection of DNA into cultured cells, the FuGENE® 4K Transfection Reagent-to-DNA ratio must be optimized (see Section 4.E). Ratios of 3:1 to 5.5:1 of FuGENE® 4K Transfection Reagent (µl) to DNA (µg), respectively, work well with many cell lines. However, ratios outside of this range may be optimal for other cell types or applications.

3.B. DNA

Plasmid DNA for transfections should be free of protein, RNA and chemical contamination (A_{260}/A_{280} ratio of 1.7–1.9). The PureYield™ Plasmid Purification Systems will provide DNA of sufficient quality for most cell systems. Prepare purified DNA in sterile water or TE buffer at a final concentration of 0.2–2mg/ml. The plasmid DNA concentration must be accurately determined by absorbance at 260nm. The optimal amount of DNA to use in the transfection will vary widely, depending upon the type of DNA and target cell line used. For adherent cells, we recommend initially testing 100ng of DNA per well in a 96-well plate format at a FuGENE® 4K Transfection Reagent-to-DNA ratio of 4:1 and 3:1. Increasing the amount of DNA does not necessarily result in higher transfection efficiencies.

3.C. Time

The time required to form the FuGENE® 4K Transfection Reagent-DNA complex is 5–15 minutes at 22°C. Incubate transfected cells for 24–72 hours before assaying to allow time to express the transfected DNA.

3.D. Serum

Transfection protocols often require serum-free conditions for optimal performance because serum can interfere with many commercially available transfection reagents. FuGENE® 4K Transfection Reagent can be used in transfection protocols in the presence of serum for transfection of cell types that require continuous exposure to serum, such as primary cell cultures. However, the reagent-DNA complexes need to be formed in the absence of serum.

3.E. Cell Culture Conditions

Antibiotics can be used during the culture of cell lines. However, the presence of antibiotics during transfection may adversely affect transfection efficiency and the overall health of transfected cells. We do not recommend using antibiotics in the transfection medium unless previously tested in the cell type being transfected. To minimize intra- and inter-experimental variance in transfection efficiency, use cells that are proliferating well (preferably in log-growth phase), have been passaged regularly, and were plated at a consistent density to achieve 50–90% confluency on the day of transfection.

3.F. Stable Transfection

FuGENE® 4K Transfection Reagent can be used to produce stable transfectants. However, we recommend optimizing transfection conditions using transient transfection studies prior to applying selective pressure to generate stable transfectants.

4. Recommended Protocol

Figure 1 provides an overview of the transfection procedure. We recommend using a 96-well plate format to optimize transfection conditions for a particular cell type.

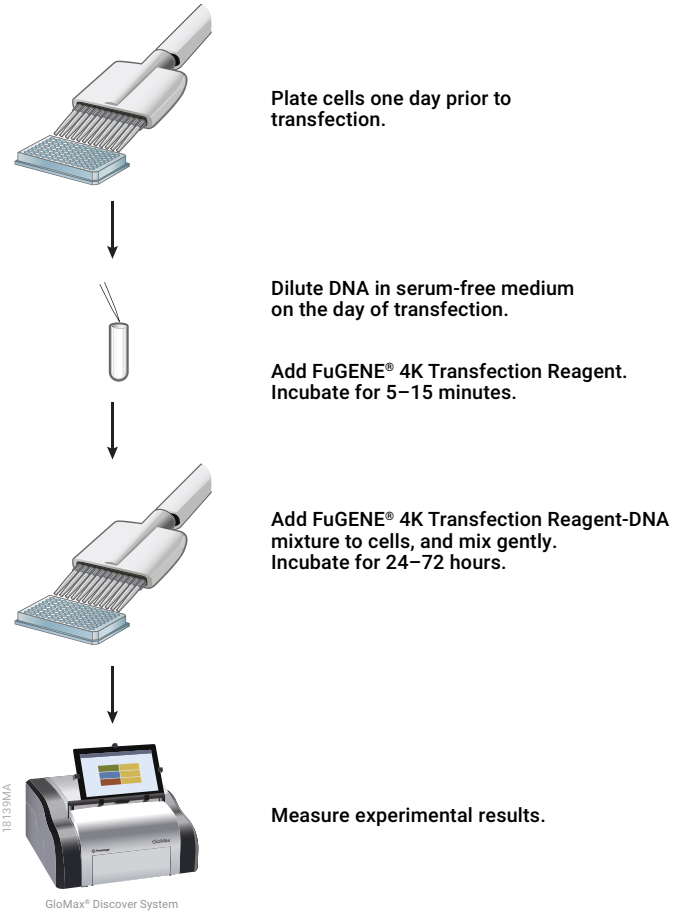

Figure 1. Overview of adherent cell transfection protocol for a 96-well plate.

Materials to Be Supplied by the User

- cell culture medium with serum appropriate for the cell type being transfected
- serum-free cell culture medium for complex formation (Opti-MEM® I reduced-serum medium, standard medium or sterile water can be used.)
- 96-well or other culture plates
- 24-well culture plate to serve as a rack for FuGENE® 4K Transfection Reagent
- plasmid DNA solution (0.2–2.0µg/µl) in sterile TE (Tris EDTA) buffer or sterile water

4.A. Plating Cells

Plate adherent cells one day before transfection so that cells are 50–90% confluent on the day of transfection. Suspension cells can be plated the day of transfection. As a general guideline, plate $1–3 \times 10^4$ adherent cells or $2.5–5 \times 10^4$ suspension cells in 100µl per well of a 96-well plate. Adjust cell numbers proportionately for different size plates (see Table 2). To prepare cells, collect enough cells to complete the transfection experiment, and centrifuge for 5 minutes at $300 \times g$ in a swinging-bucket rotor. Suspend the cell pellet to an appropriate concentration in medium, then plate.

Table 2. Area of Culture Plates for Cell Growth.

Plate Size	Growth Area (cm^2)[1]	Relative Area[2]
96-well	0.32	1X
24-well	1.88	5X
12-well	3.83	10X
6-well	9.4	30X
35mm	8.0	25X
60mm	21	65X
100mm	55	170X

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

[2]Relative area is expressed as a factor of the total growth area of the 96-well plate recommended for optimization studies. To determine the proper adherent cell plating density, multiply $1–3 \times 10^4$ cells by this factor.

4.B. Preparing the FuGENE® 4K Transfection Reagent

1. Before use, equilibrate the vial of FuGENE® 4K Transfection Reagent and diluent to room temperature.
2. Mix FuGENE® 4K Transfection Reagent by inverting or vortexing briefly. No precipitate should be visible. If reagent has been frozen accidentally and precipitate is observed, briefly warm at 37°C to dissolve precipitate and then cool to room temperature.

4.C. General Transfection Protocol

We strongly recommend that you optimize transfection conditions for each cell line. If you have optimized transfection parameters as described in Section 4.E, use the empirically determined conditions for your experimental transfections. If you choose not to optimize transfection parameters, use the general conditions recommended below.

Note: The following protocol is a guideline for transfecting approximately 10–50 wells, depending on the volume of FuGENE® 4K Transfection Reagent/DNA mixture used. For additional wells, scale volumes accordingly.

1. The total volume of serum-free medium, DNA and FuGENE® 4K Transfection Reagent to add per well of a 96-well plate is 2–10µl (Table 3). To a sterile tube or U- or V-bottom plate, add 90–98µl of medium prewarmed to room temperature so that the final volume after adding the DNA is 100µl. Add 2µg of plasmid DNA (0.2–1µg/µl) to the medium, and mix by vortexing or pipetting. For a 4:1 FuGENE® 4K Transfection Reagent-to-DNA ratio, add 8µl of FuGENE® 4K Transfection Reagent, and mix immediately.

Add FuGENE® 4K Transfection Reagent directly to medium. Do not allow undiluted FuGENE® 4K Transfection Reagent to contact the sides of the tube or U- or V-bottom plate.

The FuGENE® 4K Reagent-DNA complex must be prepared in medium without serum, even if the cells are transfected in the presence of serum.

Table 3. Total Volume of Medium, DNA and FuGENE® 4K Transfection Reagent for 96-Well Plates at a 4:1 FuGENE® 4K Transfection Reagent-to-DNA Ratio.

Plate Size	Total Transfection Volume (Per Well)[1]	Amount of DNA (Per Well)[1]	Amount of FuGENE® 4K Reagent (Per Well)[1]
96-well	2–10µl	0.04–0.2µg	0.16–0.8µl

[1]See Tables 2 and 4 for information on scaling up for larger wells or plates. See Table 5 for FuGENE® 4K Transfection Reagent volumes at different reagent-to-DNA ratios.

2. Incubate the FuGENE® 4K Transfection Reagent-DNA mixture for 5–15 minutes at room temperature.

   Note: We suggest starting with a 15-minute incubation. However, the optimal time may be 5–45 minutes, depending on the cell line used. Optimize the incubation time for best performance.

3. Add 2–10µl of the FuGENE® 4K Transfection Reagent-DNA mixture per well to a 96-well plate containing 100µl of cells in growth medium. We suggest 5µl of mixture as a starting point. Add the transfection complex to the cells in a drop-wise manner. Ensure complete mixing and even distribution over the entire surface by pipetting, using a plate shaker or rocking flasks. Return cells to the incubator for 24–72 hours.

   Note: The total growth medium volume may vary, depending on well format and standard laboratory practice.

4. Measure transfection efficiency using an assay appropriate for the reporter gene. For transient transfection, cells are typically assayed 24–72 hours after transfection.

Table 4. Guidelines for Preparing FuGENE® 4K Transfection Reagent-DNA Complex for Various Culture Vessel Sizes. The starting volume and DNA ratio to add to the different culture vessels is based upon preparing a 100µl transfection complex as described in Section 4.C. For best results, prepare a 100µl complex at different ratios and add varying amounts of each ratio when optimizing transfection.

Culture Vessel	Total Medium Volume	Suggested Seeding Density				Suggested Starting Volume of the 100µl Transfection Complex Dispensed to Each Well (µl)	Final Transfection Reagent (µl) Dispensed into Each Well after Adding the 100µl Transfection Complex	
		Cells/Well (adherent cells[1])		Cells/Well (small or suspension cells[2])			4:1 ratio	3:1 ratio
		low	high	low	high			
96-well plate (1 well)	0.1ml	10,000	30,000	25,000	50,000	5	0.4	0.3
24-well plate (1 well)	0.5ml	50,000	150,000	125,000	250,000	25	2.0	1.5
12-well plate (1 well)	1.0ml	100,000	300,000	250,000	500,000	50	4.0	3.0
35mm dish or six-well plate (1 well)	2.0ml	200,000	600,000	500,000	1,000,000	100	8.0	6.0
60mm dish	5.0ml	500,000	1,500,000	1,250,000	2,500,000	250[3]	20.0	15.0
10cm dish	15ml	1,500,000	4,500,000	2,500,000	5,000,000	500[3]	40.0	30.0

[1]Suggested seeding density for adherent cells = 30,000–70,000 cells per cm^2

[2]Suggested seeding density for suspension cells = 250,000–500,000 cells per ml

[3]Scale up total volume for larger vessels.

Table 4 lists suggested seeding densities that are dependent on the medium used, passage level, laboratory practices and selected cell line. Only use log-phase cultures to subculture for the transfection experiments, and seed cultures at the proper density for the transfection experiment. Observe cultures and plate them so that the monolayer is 50–90% confluent at the time of transfection. The confluency must be determined empirically. If you desire cells that are less confluent at the time of assay (24–72 hours after transfection), try seeding cells so that they are <50% on the day of transfection and use less transfection complex.

4.D. Protocol for Stable Transfection

The goal of stable transfection is to isolate and propagate individual clones containing transfected DNA. Therefore, it is necessary to distinguish nontransfected cells from those that have taken up the exogenous DNA. This screening can be accomplished by drug selection when an appropriate resistance marker is included in the transfected DNA.

Typically, cells are maintained in nonselective medium for 1–2 days post-transfection, then plated in selection medium (medium containing the appropriate compound). The use of selection medium is continued for 2–3 weeks, with frequent changes of medium to eliminate dead cells and debris until distinct colonies can be visualized. Individual colonies then are trypsinized and transferred to flasks for further propagation or to multiwell plates for limiting dilution cloning in the presence of selective medium.

Several drug selection markers are commonly used for long-term transfection studies. For example, cells transfected with recombinant vectors containing the bacterial gene for aminoglycoside (e.g., neomycin) phosphotransferase can be selected for stable transformation in the presence of the antibiotic G-418. Similarly, expression of the gene for hygromycin B phosphotransferase from the transfected vector will confer resistance to the drug hygromycin B. Find vectors that confer resistance to G-418, hygromycin B, puromycin or blasticidin at: **www.promega.com**

Before using a particular drug for selection purposes, determine the amount of drug necessary to kill the cells you will be using. This may vary from one cell type to another. Construct a kill curve using varying concentrations of the drug to determine the amount needed to select resistant clones. The optimal drug concentration is generally the amount that induces cell death in >90% of nontransfected cells within 5–7 days.

For stable transfections, cells should be transfected with a plasmid containing a drug-resistance gene using the transfection protocols outlined in Sections 4.C and 4.E.

Optional: As a selection-drug-negative control, transfect cells using DNA that does not contain the drug-resistance gene.

1. Forty-eight hours post-transfection, harvest adherent cells and plate at several different dilutions (e.g., 1:2, 1:5, 1:10) in selective medium.
2. For the next 14 days, replace the selective medium every 3 to 4 days.
3. During the second week, monitor cells for distinct colonies of surviving cells. Complete cell death should occur in cultures transfected with the negative control plasmid.
4. Transfer individual clones by standard techniques (e.g., using cloning cylinders or limiting dilution cloning) to 96-well plates, and continue to maintain cultures in selective medium. **Note:** If single clones are not required, pools of stable transfectants can be maintained and frozen.

4.E. Transfection Optimization

We recommend optimizing transfection conditions for each cell line using standard growth conditions in 96-well plates. For this initial optimization, use 40–200ng of DNA per well at various FuGENE® 4K Transfection Reagent-to-DNA ratios (Table 5).

Table 5. Optimization Protocol Using Varying Ratios of FuGENE® 4K Transfection Reagent to DNA.

	Ratio of FuGENE® 4K Transfection Reagent to DNA					
	5.5:1	**5:1**	**4.5:1**	**4:1**	**3.5:1**	**3:1**
DNA amount	2µg	2µg	2µg	2µg	2µg	2µg
Volume of FuGENE® 4K Transfection Reagent[1]	11µl	10µl	9µl	8µl	7µl	6µl
Medium to a final volume of[1]	100µl	100µl	100µl	100µl	100µl	100µl

[1]The volumes were calculated for 20 wells (5µl/well) of a 96-well plate for each ratio.

The volume of DNA and FuGENE® 4K Transfection Reagent complex added should be optimized. We recommend testing 2–10µl per well, but other volumes may be optimal, depending on the transfection parameters.

We also recommend including the following controls:

- **Cells control:** Medium alone (e.g., 5µl of medium)
- **DNA control:** DNA without FuGENE® 4K Transfection Reagent (e.g., 100ng DNA in a 5µl volume)
- **Reagent control:** FuGENE® 4K Transfection Reagent without DNA (e.g., 0.4µl of reagent in a 5µl volume)

1. For a 96-well plate, the total volume of medium and cells per well prior to transfection should be 100µl. Calculate the total amount of complex needed for each transfection condition (Table 2). In a sterile polystyrene tube or a U- or V-bottom plate, combine the indicated amount of medium (prewarmed to room temperature) and plasmid DNA. Add the indicated amount of FuGENE® 4K Transfection Reagent, and vortex immediately or mix by pipetting.

   Add FuGENE® 4K Transfection Reagent directly to medium; do not allow undiluted FuGENE® 4K Transfection Reagent to contact the sides of the tube or plate. If desired, FuGENE® 4K Transfection Reagent also can be diluted in transfection medium immediately prior to mixing with diluted DNA.

2. Allow the FuGENE® 4K Transfection Reagent-DNA complex to incubate at room temperature for 5–15 minutes. While 15 minutes is a good initial incubation time, transfection efficiency can be further optimized by testing different incubation times between 5–45 minutes. Incubations longer than 30 minutes can result in decreased transfection efficiency.

4.E. Transfection Optimization (continued)

3. Briefly vortex or mix the FuGENE® 4K Transfection Reagent-DNA complex. Add 2–10µl of complex per well to a 96-well plate containing 100µl of cells in growth medium (see Figure 2 for an example plate layout). Mix by pipetting or using a plate shaker for 10–30 seconds. Return plates to the incubator. For many reporter systems (luciferase, CAT, β-galactosidase, etc.) a 24- to 48-hour incubation is sufficient.

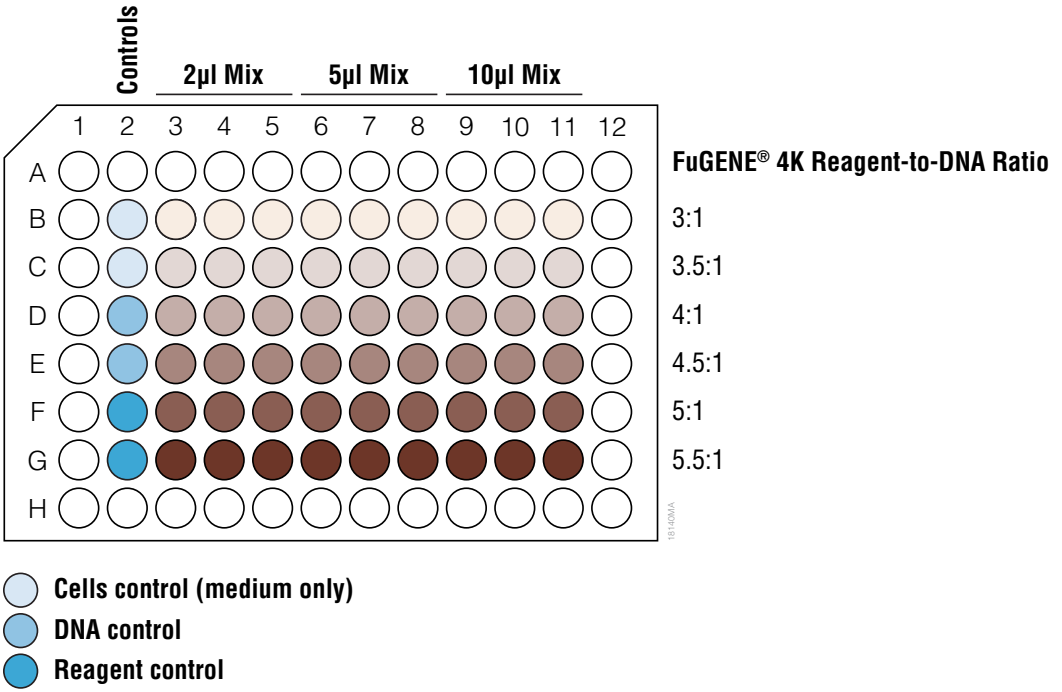

Figure 2. Plate layout for transfection optimization.

4. Check the transfection efficiency using an assay appropriate for the reporter system. For multiplexing a reporter assay with cell viability or toxicity measurement, see Section 4.F.

5. If a FuGENE® 4K Reagent-to-DNA ratio of 5.5:1 yields the highest transfection efficiency, reoptimize with a matrix of higher ratios (e.g., 5.5:1 to 12:1). Likewise, if a 3:1 ratio yields the highest transfection efficiency, test lower ratios (e.g., 1.5:1 to 3:1).

6. Additional optimization parameters include:
    - **Number of cells plated:** Plating more cells will overcome negative growth effects from excess transfection complex. For cells with special growth characteristics, do not use this as the first parameter for optimization.
    - **Incubation time for transfection complex formation:** Vary the length of incubation time for transfection complex formation. Add complex to cells at different time points after combining reagent and DNA (e.g., 5, 15 and 30 minutes).
    - **Working with sensitive cells:** Reduce the time of exposure to transfection complex (2–3 hours maximum) and replace the medium. Alternatively, use lower reagent-to-DNA ratios, allow complex to form for a longer period of time, and add less of the complex.

Notes:

a. The optimal ratio of transfection reagent to DNA and the optimal total amount of complex may vary with cell line, cell density, assay day and gene expressed.

b. After performing the optimization experiment where several ratios were tested, select a ratio in the middle of the plateau for future experiments.

4.F. Multiplexing Reporter and Cell Health Assays

The optimal transfection protocol will give the highest transgene expression with the lowest possible toxicity. In general, a 96-well plate provides a sufficient number of sample wells to perform optimization and provides a relatively easy format for performing cell-based assays. To accurately establish optimal conditions, we find it practical to use a multiplex assay to measure cell viability and reporter gene activity from a single sample in a single well. The procedure to do this using the ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay (Cat.# E7110) is as follows:

1. Follow the instructions in Section 4.E to create a plate with titrations and replicates of different transfection conditions. Use a plasmid DNA encoding a constitutive reporter gene such as the pGL4.13[*luc2*/SV40] Vector (Cat.# E6681), pGL4.50[*luc2*/CMV/Hygro] Vector (Cat.#E1310) or pGL4.51[*luc2*/CMV/Neo] Vector (Cat.#E1320).
2. Incubate cells for 24–48 hours.
3. Add 20µl of CellTiter-Fluor™ Reagent (prepared as 10µl of substrate in 2ml of Assay Buffer) to all wells, and mix briefly by orbital shaking (300–500rpm for approximately 30 seconds). Incubate for at least 30 minutes at 37°C.

    Note: Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate longer than 3 hours, and be sure to shield plates from ambient light.
4. Measure resulting fluorescence (380–400nm$_{Ex}$/505nm$_{Em}$) using a fluorometer or multimode plate reader, such as the GloMax® Discover System (Cat.# GM3000).

    Note: You may need to adjust instrument gain (applied photomultiplier tube energy).
5. Add an equal volume of ONE-Glo™ Luciferase Assay Reagent to the volume of the mixture already in each well (100–120µl per well), incubate for 3 minutes at room temperature and then measure luminescence using a luminometer or multimode plate reader, such as the GloMax® Discover System.
6. Determine the condition that provides the highest luciferase activity and cell viability.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low transfection efficiency	Poor-quality DNA or insufficient quantity. • Verify the amount, purity and sequence of nucleic acid. • Perform a control transfection experiment with a commercially available transfection-grade plasmid preparation. • Chemical contaminants may be in the plasmid preparation. Avoid phosphate buffers until you have tested them in your system. • Endotoxins are reported to be cytotoxic to some very sensitive cell lines.
	Insufficient number of cells. Use adherent cells that are at least 80% confluent. Low cell density results in fewer cells available to take up transfection complex, and excess complex may be cytotoxic. Fewer cells also yield less protein.
	Too many cells or cells post-log phase. When confluent cultures are subcultured, or cells are plated at too high a density, the cells fail to divide in the culture being transfected. This results in suboptimal expression.
	Suboptimal FuGENE® 4K Transfection Reagent-to-DNA ratio, complex incubation time, total amount of transfection complex added or cell density. Optimize the FuGENE® 4K Transfection Reagent-to-DNA ratio, complex incubation time, amount of complex added to cells and cell density as described in Section 4.E.
	FuGENE® 4K Transfection Reagent was dispensed into aliquots. Check that FuGENE® 4K Transfection Reagent is stored in the original container. If the reagent was dispensed into plastic containers, the reagent can be inactivated. Make sure the reagent is immediately mixed with the diluted DNA either by vortexing or pipetting 10–15 times.

Symptoms	Causes and Comments
Low transfection efficiency (continued)	FuGENE® 4K Transfection Reagent came into contact with plastic or was inadequately mixed. Repeat transfection, carefully pipetting FuGENE® 4K Transfection Reagent directly into the serum-free medium, carefully avoiding the sides of the container while adding the transfection reagent to the diluted DNA. If the FuGENE® 4K Transfection Reagent is pipetted too gently, the reagent may layer on top of the medium, thus making contact with the plastic.
	Transfection complex was formed in serum-containing medium. Check original bottle of medium used for complex formation. Repeat experiment using new bottle of medium without any additives (e.g., serum, antibiotics, growth enhancers, heparin, dextran sulfate, etc.). Try forming the complex in phosphate-buffered saline (PBS) or plain Dulbecco's Modified Eagle Medium (DMEM).
	Medium and medium components may interfere with transfection. • Different media and medium components may influence the level of transfection efficiency and subsequent growth of the transfected cells, as well as recombinant protein expression. Some lots of sera have been reported to interfere with optimal transfection. • Quality and/or lot-to-lot differences that affect transfection experiments have been noted in both sera and media. Check that the medium and/or serum is from the same lot that worked previously. Try new lots or a different vendor.
	Cell culture may be contaminated with mycoplasma. Cultures contaminated with mycoplasma have decreased transfection efficacy. Determine if culture is contaminated with mycoplasma via commercially available mycoplasma contamination assay.

5. Troubleshooting (continued)

Symptoms	Causes and Comments
Inconsistent results	Ratio or amount of transfection complex is at the edge of performance plateau. Perform initial optimization experiments to determine the ratios, amount of complex to be added and length of time for complex formation. In our experience, we have found the plateau to be relatively broad. We recommend that future experiments be performed with ratios, incubation times and complex amounts that were in the middle of the plateau. If conditions are selected at the edge of the plateau, very small procedural differences may cause large differences in the resulting protein expression. Increase consistency by shifting parameters from the edge of the plateau to the middle of the plateau.
	Transfection complex formation affected by timing, amounts and ratio. Formation of the complex involves a multifaceted interaction between the transfection reagent and DNA as well as biological parameters. Differences in any of the components or techniques may result in inconsistencies. If results do not meet your expectations, then repeat the optimization experiment selecting areas near the plateau found in previous experiments. For current experiments, determine if you should use a different ratio, length of time or amount of complex for more consistent transfection results.
	Poorly cultured cells. For consistent results, cells must be properly maintained. Cells change with passage level, passage conditions, medium and serum. For some cell lines, these changes have little to no effect on transfection experiments, but for other cell lines, these changes have profound effects. Each cell type may have different optimal transfection conditions. Optimal values for a single cell type may also change slightly with vector construct and type of protein expressed.
Signs of cytotoxicity	Transfected protein is cytotoxic if produced at high levels. Reduced viability or slow growth rates may be the result of high protein expression levels because the cell metabolic resources are directed toward production of the heterologous protein. The expressed protein may also be toxic to the cell at the level expressed.

Symptoms	Causes and Comments
Signs of cytotoxicity (continued)	To analyze cytotoxicity, prepare experimental controls as described below. Prepare extra control wells containing: • Cells that are not transfected • Cells treated with DNA alone (e.g., without FuGENE® 4K Transfection Reagent) • Cells treated with FuGENE® 4K Transfection Reagent alone (no DNA added) • Cells transfected with a non-toxic or secreted protein. Compare experimental transfected cells to cells in the control wells (described above). Consider repeating the experiment with a secreted reporter gene such as secreted embryonic alkaline phosphatase (SEAP), human growth hormone (hGH) or a standard β-galactosidase control vector. Cells expressing SEAP should show little to no evidence of cytotoxicity.
	Too much transfection complex for cell number. Increase the number of cells plated, decrease the total amount of complex added to the cells or both. Try different ratios and allow the complexes to form for different time intervals. Add different amounts of complex; for example, make the complex as usual but add 75%, 50% or 25% of the usual amounts to each well. See Section 4.E for optimizing transfection conditions.
	Cell culture may be contaminated with mycoplasma. Cultures contaminated with mycoplasma have decreased transfection efficacy. Determine if culture is contaminated with mycoplasma via commercially available mycoplasma contamination assay.
	Cells may not be healthy. Assess physiological state of cells and the incubation conditions (e.g., check the incubator CO_2, humidity, and temperature levels). Observe cells prior to each passage for morphology and absence of contaminants. Make sure cells do not overgrow. Routinely passage cells prior to reaching confluency. Make sure that culture medium and additives are within expiration date and have been stored properly.
	Diluent is toxic to the cells. DMEM is toxic to some insect cell lines. For these cells, prepare the transfection complex in sterile water. Also try forming the complex in the medium in which the cells are growing, providing that the medium does not contain serum, heparin or dextran sulfate.
	Plasmid preparation contaminated with endotoxin. Endotoxin is cytotoxic to sensitive cell lines.

5. Troubleshooting (continued)

Symptoms	Causes and Comments
Signs of cytotoxicity (continued)	High protein expression levels. High expression levels of certain intracellular proteins (e.g., green fluorescent protein [GFP]) may be cytotoxic to some cell types. Cell proliferation, toxicity and cell death may be monitored using such assays as the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570), CellTiter-Fluor™ Cell Viability Assay (Cat.# G6080) or CellTox™ Green Cytotoxicity Assay (Cat.# G8741).
	Medium and medium components may interfere with transfection. • Different media and medium components may influence the level of transfection efficiency and subsequent growth of the transfected cells, as well as recombinant protein expression. Some lots of sera have been reported to interfere with optimal transfection. • Quality and/or lot-to-lot differences that affect transfection experiments have been noted in both sera and media. Check that the medium and/or serum is from the same lot that worked previously. Try new lots or a different vendor.

[a]**FuGENE® 4K is sold with a Limited Use Label License (LULL).** You shall use this Product in accordance with all applicable laws, rules and regulations. You may not attempt to reverse engineer this Product. If you are not willing to accept these conditions, the distributor from whom the Product was purchased will accept the return of the unopened Product and provide you with a full refund. However, in the event this Product is opened, you agree to be bound by the conditions of this limited use statement.