Users Guide: FuGENE® 6 Transfection Reagent

For the transient and stable transfection of animal cells.

For life science research only

Cat. No F6-1000 1ml

Cat. No F6-5000 5ml (5 x 1ml)

1. What this Product Does

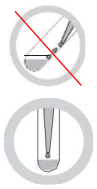
Formulation

FuGENE® 6 Transfection Reagent is a proprietary blend of lipids and other components supplied in 80% ethanol, 0.1um sterile-filtered, and packaged in glassvials.

Storage and stability

FuGENE® 6 Reagent is shipped at room temperature. FuGENE® 6 Transfection Reagent is stabilized for extended storage at +2 to +8°C through the expiration date printed on the label (four years from the date of manufacture) when very tightly closed. Always bring to room temperature and mix FuGENE® 6 Transfection Reagent prior to use. Always mix FuGENE® 6 Transfection Reagent prior to use (vortex for one second or use inversion).

Special Handling



Do not aliquot FuGENE® 6 Reagent from the original glass vials. Chemical residues in plastic vials can significantly decrease the biological activity of the reagent. Minimize the contact of undiluted FuGENE® 6 Reagent with plastic surfaces. Always dilute the reagent by pipetting directly into serum- free medium. Do not allow the FuGENE® 6 Reagent to contact the plastic walls of the tube containing the serum-free medium during the dilution step.

Note: FuGENE® 6 Transfection Reagent remains fully functional even after repeated vial openings (at least six times over a three-month period) as long as the vials are tightly recapped and stored at +2 to +8°C between uses.

1.1 Product overview

Number of transfection experiments

Using standard experimental conditions, one milliliter of FuGENE® 6 Reagent transfects over 3,300 wells of a 96-well plate at the recommended 3:1 ratio. This is equivalent to over 600 transfections in 24-well plates.

Quality control

Functional analysis

Six microliters of FuGENE® 6 Transfection Reagent is combined with 2 µg of reporter-gene vector DNA, and used to transfect COS-7, HEK293, and HeLa cells (in a monolayer [50–80% confluent]) in the presence of 10% fetal bovine serum (FBS). Following transfection, the percentage of transfected cells is analyzed. Typically, 50–70% of COS-7 cells express reporter-gene protein.

Cytotoxicity analysis

COS-7, HEK293, and HeLa cells that are continuously exposed to FuGENE® 6 Reagent for 24 hours, with or without DNA, in the presence of serum, and without a change of medium, are >90% viable by flow-cytometric analysis based on propidium-iodide staining.

1.2 Background Information

Application:

FuGENE® 6 Transfection Reagent is a multi-component reagent that forms a complex with DNA, then transports it into animal cells. Benefits of FuGENE® 6 Reagent include:

- High transfection efficiency in many common cell types, including HeLa, NIH 3T3, COS-1, COS-7, and CHO-K1. For a list of >600 cell types that have been successfully transfected with FuGENE® 6 Reagent, visit www.fugene.com
- Demonstrates virtually no cytotoxicity, allowing you to work with fewer cells, and eliminates the requirement to change media after the addition of transfection complex
- Suitable for transient and stable-transfection
- Functions exceptionally well in the presence or absence of serum; eliminates the need to change media
- Requires minimal optimization.

Required amount of FuGENE® 6 Reagent

For initial optimization experiments, transfect a monolayer of cells that is 50–80% confluent in a 96-well culture dish, using 2:1, 3:1, 4:1, and 6:1 ratios of FuGENE® 6 Transfection Reagent (µl) to DNA (µg), respectively. For most cell types, these FuGENE® 6 Reagent:DNA ratios provide excellent transfection levels.

Note: Subsequent optimization may further increase efficiency in your particular application. Hold the amount of DNA constant while increasing the volume of FuGENE® 6 Reagent. Transfection efficiency is greatly reduced with excess DNA. When the amount of DNA exceeds a ratio of 3:2 (volume FuGENE® [µl]:mass DNA [µg]), transfection may fail completely. For more information, visit www.fugene.com.

Plasmid DNA

It is critical to accurately determine the plasmid DNA concentration using 260-nm absorption. DNA content must be determined by 260-nm absorption (estimates of DNA content based on the intensity of gel bands are not sufficiently accurate). Determine the DNA purity using a 260 nm/280 nm ratio; the ratio should be 1.8. Prepare the plasmid DNA solution in sterile TE buffer or water at concentration of 0.2ug/ul to 2.0ug/ul.

Cell-culture conditions

Minimize both intra- and interexperimental variance in transfection efficiency by using cells that are regularly passaged, proliferating well *(best when in a log-growth phase)*, and plated at a consistent density.

Other media additives

In some cell types, antimicrobial agents (*e.g.*, antibiotics and fungicides) that are commonly included in cell-culture media may adversely affect the transfection efficiency of FuGENE® 6 Transfection Reagent. Up to a 25% decrease in efficiency has been observed. If possible, exclude additives for initial experiments. Once high-efficiency conditions have been established, these components can be added back while monitoring your transfection results.

Verification of vector function

Optimize transfection conditions with a known positive-control reporter- gene construct prior to transfecting cells with a new vector construct:

- Determine transfection efficiency with a reporter-gene assay (such as GFP, CAT, β-Gal, Luciferase, SEAP, or hGH)
- Sequence across the flanking vector insert regions to verify the integrity of your new construct.

Incubation Time of cells after Transfection

Incubate the cells for 24–72 hours. The length of incubation depends upon the transfected vector construct, the cell type being transfected, and the type of protein being expressed. After this incubation period, measure protein expression using an assay that is appropriate for your system.

1.3 Procedures and Required Materials

Before you begin

Additional required reagents and supplies

1.) Sterile, serum-free culture medium without additives or supplements (optional: add 12.5 mM HEPES buffer to serum-free medium)
2.) Plasmid DNA solution (between 0.2 µg/µl and 2.0 µg/µl) in sterile TE (Tris/EDTA) buffer or sterile water.

To prevent spillage, you can use a 24-well plate as a test tube rack for the FuGENE® 6 Transfection Reagent.

Preparation of cells for transfection

Adherent cells

One day before the transfection experiment, trypsinize, adjust the cell concentration, and plate the cells in the chosen cell-culture vessel. For most cell types, plating 2-5 x 10^5 cells in a 35-mm culture dish in 2 ml of medium (or a six-well plate) overnight will achieve the desired density of 50–80% confluency. If using culture plates of a different size, adjust the seeding density proportionally (Table 1).

Suspension cells

Use freshly passaged cells at a concentration 2.5-5 x 10^5 (2 ml in a 35-mm culture dish or six-well plate). Determine the cell number based on your needs and the cell type to be transfected.

Preparation of FuGENE® 6 Reagent:DNA complex and transfection of cells

Adherent and suspension cells in a 35-mm culture dish

For initial optimization, use FuGENE® 6 Reagent:DNA ratios of 2:1, 3:1, 4:1, and 6:1 (µl, for FuGENE® 6 Reagent, and µg for DNA, respectively). The preparation of the complex for a single well of a six-well plate, or a 35-mm culture dish, is described below. These ratios will function very well for commonly used adherent cells and suspension cells.

Important: The FuGENE® 6 Reagent:DNA complex must be prepared in medium that does not contain serum, even if the cells are transfected in the presence of serum.

Note: For additional optimization tips please visit the Documents & Manuals Section at: www.fugene.com or contact us directly at contact@fugene.com

Ratio overview

Preparation of a complex that is sufficient for a 35-mm culture dish, one well of a six-well plate, or twenty 96-well plate wells, at 4 different ratios:

Tube Label	Medium Final (ul)	FuGENE® 6 Reagent (ul)	DNA (ug)	Comments
2:1	100	4	2	Add the entire volume to each well of six well plate/35mm, or 5ul to each well of 96-well plate. Additional suggested volumes for different vessels are included in Table 1
3:1	100	6	2	
4:1	100	8	2	
6:1	100	12	2	

2.) How to use this product

1.) Dilute FuGENE 6 Reagent with serum-free medium (without antibiotics or fungicides):

• Label four small sterile tubes: “2:1, 3:1,” “4:1,” and “6:1.” Pipet pre-warmed serum-free medium into the tubes so final volume after addition of FuGENE 6® Reagent (in step 1) & DNA (in step 2) is 100ul total volume.
• Pipet the FuGENE® 6 Reagent directly into the medium without allowing contact with the walls of the plastic tube:4 µl into 1st tube, 6ul into 2nd tube, 8ul into 3rd tube, and 12ul into final tube
• Vortex for one second or flick the tube to mix. Incubate for 5 minutes at room temperature.

2.) Add DNA to diluted FuGENE® 6 Reagent from Step 1:

Add 2 µg of plasmid DNA into each of the tubes so that you now have a final volume of 100ul in each tube (medium, FuGENE, & DNA)

3.) Mix and incubate the complex:

Tap the tube or vortex for one second to mix the contents. Incubate the transfection reagent:DNA complex for a minimum of 15 minutes at room temperature. Continued incubation for up to 45 minutes (for some cell lines up to two hours) can increase transfection efficiency

4.) Add complex to the cells:

Remove culture vessel from the incubator. Removal of growth medium is not necessary. Add the transfection reagent:DNA complex to the cells in a drop-wise manner. (see Table 1 for details on the amount of complex to add to each specific vessel size). Swirl the wells or flasks to ensure distribution over the entire plate surface.

5.) Return the cells to the incubator until the assay for gene expression is to be performed.

Once the FuGENE® 6 Reagent:DNA complex has been added to the cells, there is no need to remove and replace with fresh medium (as is necessary with some other transfection reagents). In our experience, the continous exposure of most common laboratory cell types (COS-1, CHO-K1, HEK-293, HeLa) to the reagent:DNA complex until performance of the gene-expression assay (24–48 hours later) does not affect the viability. If you desire to use serum-free medium during the transfection procedure (Step 4), then replace the medium with serum-containing medium 3–8 hours after transfection. If you observe more than 10% cell death, refer to troubleshooting section page 7.

Notes:

- For stable transfection experiments, the complex-containing medium should be left unchanged until the cells need to be passaged and treated with selection antibiotics
- To prepare transfection complexes for larger experiments or parallel experiments, proportionally increase the quantity of all components according to the total surface area of the cell-culture vessel being used (Table 1)
- For ease of use when transfecting small volumes, as in 96 well plates. Prepare 100ul of complex and add 2-10ul (typically 5 ul) to each well
- As with any experiment, include appropriate controls. Prepare wells with cells that remain non-transfected, cells with transfection reagent alone, and cells with DNA alone.
- The optimal ratio of transfection reagent:DNA and the optimal total amount of complex may vary with cell line, cell density, day of assay, and gene expressed.
- After performing the optimization experiment where several ratios were tested, select a ratio in the middle of the plateau for future experiments.

Optimization Parameters:

Parameter to be optimized	Procedure
FuGENE® 6 Transfection Reagent to DNA Ratio	Form the transfection complex at several rations: 2:1, 3:1, 4:1, & 6:1 (Fugene ul : 2ug DNA)
Amount of transfection complex to add to cells	Try adding 200%, 150%, 75%, 50%, and 35% of the amount of 100ul transfection comlex suggested in Table 1
Number of cells plated	Plating more cells will overcome negative growth effects of excess transfection complex. For cells with special growth characterists, do not use this as the first parameter for optimization
Incubation time for transfection complex to form	Vary the length of incubation time for transfection-complex formation. Add complex to cells at different timepoints after combining reagent and DNA (0, 10, 25, 30, up to 45 minutes)
Special tips for sensitive cells	Reduce the time of exposure to transfection complex (2-3 hours max.), then replace the medium. Or, use lower ratios and allow complex to form for longer period of time, then add lower amounts of complex.

Table 1: Guidelines for Preparing FuGENE® 6 Transfection Reagent:DNA Complex for Various Culture Vessel Sizes

The starting volume and mass to add to the different culture vessels is based upon preparing a 100-µl transfection complex as described in previous sections. For best results, prepare a 100-µl complex at different ratios and add varying amounts of each ratio when optimizing. The amounts below are based on the 100-µl complex as prepared in sections 2.3 and 2.4.

**Suggested seeding density for adherent cells = 30,000–100,000 cells per cm^2

**Suggested seeding density for suspension cells = 250,000–500,000 cells per ml

Culture vessel	Total volume of medium	Suggested seeding density				Suggested starting amount of the 100-µl transfection complex to add to each well (µl)*	Final Transfection Reagent(µl) in each well following addition of suggested amount of 100-µl transfection complex	
		Cells/well Adherent cells		Cells/well Small or suspension cells			Utilizing 3:1 ratio	Utilizing 4:1 ratio
		low	high	low	high			
96-well plate (1 well)	0.1mL	10,000	30,000	25,000	50,000	5	0.3	0.4
24-well plate (1 well)	0.5mL	50,000	150,000	125,000	500,000	25	1.5	2.0
12-well plate (1 well)	1.0mL	100,000	300,000	375,000	750,000	50	3.0	4.0
35-mm dish or 6-well plate (1 well)	2.0mL	200,000	600,000	500,000	1,000,000	100	6.0	8.0
60-mm dish	5.0mL	500,000	1,500,000	1,250,000	2,500,000	250[1]	15.0	20.0
10-cm dish	10mL	1,500,000	4,500,000	2,500,000	5,000,000	500[1]	30.0	40.0

[1] Scale up total volume for larger vessels.

These are suggested seeding densities and are media, passage level, laboratory, and cell-line dependent. It is critical that log phase cultures are selected for subculture for the transfection experiments, and that cultures are seeded at the proper density for the transfection experiment. Observe cultures and plate them so that the monolayer is 50-80% confluent at the time of transfection. This must be determined empirically.

1. Troubleshooting

Observation	Possible cause	Recommendation
Low transfection efficiency	Poor quality or Insufficient quantity of nucleic acids	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 50% confluent. Low cell density results in fewer cells available to take up transfection complex, and excess complex may be cytotoxic; in addition, fewer cells 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® 6 Transfection Reagent:DNA ratio, complex incubation time, total amount of transfection complex added, or cell density	Optimize the FuGENE® 6 Transfection Reagent:DNA ratio, complex incubation time, amount of complex added to cells, and cell density, according to the following procedure: **Day before transfection:** Prepare two 96-well plates of cells at high and low seeding densities (see Table 1 for suggestions). **Day of transfection:** • Form 200 µl of transfection complex at ratios of 2:1, 2.5:1, 3:1. 3.5:1, and 4:1 following the users guide and double the amount of all reagents. • As soon as the complexes are combined and mixed, add 10, 5, or 2.5 µl of each complex to one of 3 columns of cells in each 96-well plate (*i.e.*, columns 2, 3, and 4). Leave all outer wells empty as controls. • Continue to incubate the complexes at room temperature. After an additional 10–15 minutes, add 10, 5, or 2.5 µl of each complex to the next 3 columns (5, 6, and 7) of cells in each 96-well plate. • Continue to incubate the complexes at room temperature. After an additional 10–15 minutes, add 10, 5, or 2.5 µl of each complex to the next 3 columns (8, 9, and 10) of cells in each 96-well plate. • Assay the plates 1–2 days later. Select the ratio, amount of complex, and time of transfection-complex incubation that resulted in optimal expression. • If optimal transfection occurs at the higher ratios, repeat this process using ratios of 3:1, 3.5:1, 4:1, 5:1, 6:1, and 7:1. Add 5, 10, and 15 µl of complex. See section 2.6, Optimization of FuGENE® 6Transfection Reagent:DNA ratio, for more information and visit *www.fugene.com*
	FuGENE® 6 Transfection Reagent was aliquoted	Check that FuGENE® 6 Transfection Reagent is stored in the original container. If the reagent was aliquoted into plastic containers, there is a high chance of inactivation. Make sure the reagent is immediately mixed with the dilute DNA either by vortexing or pipetting up to 10–15 times.
	FuGENE® 6 Transfection Reagent came into contact with plastic or was inadequately mixed	Repeat transfection, carefully pipetting FuGENE® 6 Transfection Reagent directly into the serum-free medium, being careful not to touch the sides of the container while adding the FuGENE® 6 Transfection Reagent to the diluted DNA. If the FuGENE® HD Transfection Reagent is added too gently, it may layer on top of the medium, thus making contact with the plastic.
	Transfection complex was formed in serum containing media	Check original bottle of medium used for complex formation. Repeat experiment using new bottle of medium that does not contain any additives *(e.g.,* serum, antibiotics, growth enhancers, heparin, dextran sulfate, etc) Try forming the complex in PBS or plain DMEM
	Media and media components	Different media and media components may influence the level of transfection efficiency and subsequent growth of the transfected cells, as well as expression of the recombinant protein. 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.
	Culture may be contaminated with mycoplasma	Cultures contaminated with mycoplasma have been shown to have decreased transfection efficacy. Determine if culture is contaminated with mycoplasma; use the Mycoplasma Detection Kit* or Mycoplasma PCR ELISA* to assess contamination.
Inconsistent results	Ratio or amount of transfection complex is at the edge of performance plateau	Initial experiments should be completed to determine the ratios, amount of complex to be added, and length of time for complex formation for optimal performance. In our experience, we have found the plateau to be relatively broad. We recommend that future experiments be performed with ratios, incubation time, and amounts of complex 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. Increased consistency may be achieved by shifting parameters away from the edge of the plateau to the middle of the plateau.
	Transfection complex formation: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. *Extensive testing of the FuGENE® 6 Transfection Reagent is performed on two cell lines: one easy to transfect and one very difficult to transfect. All reagent lots must pass this rigorous testing before we make it available to you. However, we cannot test all cell lines, media, sera, and vectors; in your laboratory, you may find slight differences in the optimal ratio, amount of complex, or time for complex formation for some lots of FuGENE® 6 Transfection Reagent.*
	Cells	For consistent results, cells must be properly maintained. Cells change with passage level, passage conditions, media, and sera. 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 a different optimal transfection condition. Optimal values for a single cell type may also change slightly with vector construct and type of protein expressed.

Observation	Possible cause	Recommendation
Signs of cytotoxicity	Transfected protein is cytotoxic or is produced at high levels	Reduced viability or slow growth rates may be the result of high levels of protein expression, as the cell's metabolic resources are directed toward production of the heterologous protein. The expressed protein may also be toxic to the cell at the level expressed. To analyze cytotoxicity, prepare experimental controls as described below. Prepare extra control wells containing: a. Cells that are not transfected b. Cells treated with DNA alone *(e.g.,* without FuGENE® 6 Transfection Reagent) c. Cells treated with FuGENE® 6 Transfection Reagent alone (no DNA added) d. 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 SEAP, hGH, or a standard β-gal control vector. Cells expressing SEAP should show little to no evidence of cytotoxicity.
	Too much transfection complex for number of cells	Increase the number of cells plated, and/or decrease the total amount of complex added to the cells. 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 Suboptimal FuGENE® 6:DNA Ratio in "Low transfection efficiency" section of this table for details or optimization protocol.
	Culture may be contaminated with mycoplasma	Determine if culture is contaminated with mycoplasma; use commercially available Mycoplasma Detection Kit to assess contamination.
	Cells may not be healthy	Assess physiological state of cells and the incubation conditions (*e.g.,* check 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 media 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. You may 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 reported to be cytotoxic to sensitive cell lines.
	If above tests prove negative, FuGENE® 6 Transfection Reagent may not be optimal for your cells.	Try FuGENE® HD Transfection Reagent, visit www.fugene.com
	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 Apoptosis and Cell Proliferation assays.
	Media and media components	Test different media and optimize the level of each medium component for these cytotoxic effects. Although it is not usually necessary to remove the transfection complex following the transfection step, it may be necessary to feed your cells with fresh media for extended growth periods. This is particularly important if the transfected cells are allowed to continue to grow for 3–7 days to provide maximal protein expression.

Notice to Purchaser:

Purchaser represents and warrants that it will use FuGENE® 6 Transfection Reagent purely for research purposes. Transfected cells, materials produced, and any data derived from the use of FuGENE® 6 Transfection Reagent, may be used only for the internal research of Purchaser whether Purchaser is a “for-profit” or a “not-for-profit” organization. Under no circumstances may FuGENE® 6 Transfection Reagent be used by Purchaser or any third party for a commercial purpose unless Purchaser has negotiated a license for commercial use with Fugent, LLC (contact information: contact@fugene.com). For purposes of the foregoing sentence, “commercial purpose” shall mean use of FuGENE® 6 Transfection Reagent for profit or commercial gain. By using FuGENE® HD Transfection Reagent, Purchaser agrees to be bound by the above terms. If Purchaser wishes not to be bound by these terms, Purchaser agrees to return the FuGENE®6 Transfection Reagent to Fugent LLC. for a full refund.

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