T e c h n i c a l M a n u a l

FuGENE® HD Transfection Reagent

INSTRUCTIONS FOR USE OF PRODUCT: FuGENE® HD.

FuGENE® HD Transfection Reagent

1. Product Description

A. Description

FuGENE® HD Transfection Reagent is a proprietary, nonliposomal, low toxicity formulation designed for high efficiency delivery of DNA into a broad range of cell lines.
The use of FuGENE® HD does not require culture medium change or serum removal during transfection experiment. Active FuGENE® HD/DNA complex can be formed in different widely used cell culture media, buffers, or water. FuGENE® HD effectively transfects cells growing in chemically define media and does not contain any components derived from human or animal sources.
The list of cells successfully transfected with FuGENE® HD reagent can be found at www.FugeneHD.com

Product	**Size**	**Cat.#**
FuGENE® HD Transfection Reagent	1ml	
	5 × 1ml	

One 1ml vial of FuGENE HD transfection reagent contains sufficient material to transfect over 3,000 wells in 96-well cell culture plates at a 3:1 FuGENE® HD Transfection Reagent:DNA ratio (0.3µl reagent:100ng DNA per well). This is equivalent to over 150 wells in 6-well plate or 35mm tissue-culture dishes. The actual number of transfections will depend on particular reagent:DNA ratio, transfection volume and cell type.

B. Storage and Handling

FuGENE® HD Transfection Reagent is very stable and can be stored at room temperature. Storage in refrigerator at 4°C is also suitable if it is more convenient for your particular laboratory setting.
After each use close lid tightly to avoid evaporation.
The reagent is stable through the expiration date printed on the label when stored under these conditions.

DO NOT store FuGENE® HD at temperature below 0°C. That can cause precipitation of components and negatively alter results. If reagent has been accidentally kept in a freezer at -20°C, briefly warm it to 37°C to dissolve precipitate and then cool down to room temperature.

Before transfection, always allow FuGENE® HD Transfection Reagent to reach room temperature and briefly mix it by vortex for one second or by inverting vial several times.
Do not aliquot FuGENE® HD Transfection Reagent or transfer it to plastic vials. Chemical residues in plastic can drastically decrease the activity of the reagent.
Always pipette FuGENE® HD Transfection Reagent directly into serum-free medium. Do not allow undiluted FuGENE® HD Transfection Reagent to contact plastic walls of tube or culture plate during complex preparation.
Do not use siliconized pipette tips or tubes.

For some experiments, FuGENE® HD Transfection Reagent can be diluted in complex formation medium prior to combining it with DNA solution, however it should be used within first 30 minunes after dilution.

C. Additionally Required Equipment and Reagents

Additional equipment and reagents required for performing transfection experiments using FuGENE® HD Transfection Reagent, but not provided, include:

General Laboratory Equipment
- standard cell culture equipment (*e.g.*, biohazard hoods, incubators, microscope)
- standard pipetters and micropipetters
- vortex mixer

Plasmid DNA (experiment specific)
- purified plasmid stock solution (0.1 – 2.0 mg/ml) in sterile TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer or sterile water

For Verification of Vector Function
- assay appropriate for transfected gene
- G-418* or Hygromycin B* (optional; for stable transfection experiments)

For Transfection-Complex Formation
- Opti-MEM I Reduced Serum Medium, water, or serum-free medium
- sterile polystyrene tubes or round-bottom 96-well plates for complex formation
- FuGENE® HD Transfection Reagent storage box can be used as rack to hold reagent tube and other materials during transfection experiment

Cells
- for the best transfection result plate cells from high confluence culture (90-100%)
- at the time of transfection cells should be in log phase at 70-80% confluence
- method to quantify cell number to reproducibly plate the same number of cells

2. General Outline of Transfection Experiment

A. Transfection reagent to DNA ratio

For initial experiment transfect cells that are grown at 70-80% confluence in 96-well culture plate. However, for the best results with majority cell types, the cells for the experiment should be plated from high (90-100%) confluence culture. Use 0.1µg plasmid DNA per each well and six ratios of FuGENE® HD Transfection Reagent to DNA in a range of 2:1 to 4.5:1 (2:1, 2.5:1, 3:1, 3.5:1, 4:1 and 4.5:1). These FuGENE® HD Transfection Reagent:DNA ratios work very well for the most cell types. For further improvement of transfection efficiency refer to "Transfection Optimization" section of this manual.

B. Plasmid DNA

It is crucial for successful transfection to use properly quantified concentration of plasmid DNA. We recommend determining DNA concentration by 260nm adsorption method. DNA should also be pure and free of protein, RNA, salts and other contaminants. Determine DNA

purity using a 260nm/280nm adsorption ratio; it should be close to 1.8. Prepare the plasmid DNA stock solution in sterile TE (Tris/EDTA) buffer or sterile water at a concentration between 0.5mg/ml and 2.0 mg/ml.

C. Complex Formation Time

Optimal complex formation time for the FuGENE® HD Transfection Reagent is between 0 minutes and 15 minutes. Different cell types may require different complex incubation time for maximal transfection efficiency.

D. Serum, antibiotics and other media additives

Unlike many transfection protocols for commercially available transfection reagents that require serum-free condition for successful transfection, the FuGENE® HD Transfection Reagent can be used in the presence of serum (up to 100%). Thus, no media change is necessary after combining transfection complex with cell culture.

The use of some antimicrobial or fungicidal agents in cell culture may negatively affect transfection efficiency. If possible, first try to achieve high-efficiency transfection conditions without use of these additives. Once satisfactory results achieved the agents can be added back to the media.

Some chemically define media containing heparin or dextran sulphate will suppress transfection and should not be used.

3. Recommended transfection protocol

For initial transfection experiment and transfection optimization we suggest using 96-well plate format. The amount of DNA and the FuGENE® HD Transfection Reagent in protocol described bellow calculated to be sufficient to transfect cells in 20 wells of 96-well plate at standard conditions.

1. The day before transfection plate adherent cells in the amount that by the time of transfection will grow to approximately 80% confluence. For most common cell types plating 10^4 cells per well in 100µl of medium in 96-well flat bottom culture plate overnight will achieve proper density. For cell lines with special characteristics low or higher amount of cell per well can be used.

Cell growing in suspension can be plated just before transfection with density between $2x10^4$ and $8x10^4$ per well in 100 µl of medium for 96-well plate format. Density of $5x10^4$ cells per well is a good starting point.

2. Allow FuGENE® HD Transfection Reagent, DNA, and diluent reach room temperature. Mix FuGENE® HD Transfection Reagent by vortex for 1 second or by inverting vial for several times.

3. Dilute DNA with Opti-MEM I reduced serum medium, serum-free cell growth medium or sterile water to a concentration of 0.02mg/ml (2µg of DNA in 100 µl of diluent).

For insect cells use sterile water. For cells not compatible with Opti-MEM I medium use cell-specific growth medium without serum, or sterile water. Some cells will produce higher transfection results with Opti-MEM I, some with sterile water as diluents; try both for your particular experiment.

4. Place 100µl of DNA solution into each of six wells of round bottom 96-well tissue culture plate. Label the wells for following FuGENE® HD Transfection Reagent:DNA ratios: 2:1, 2.5:1, 3:1, 3.5:1, 4:1 and 4.5:1.
Alternatively, polystyrene tubes can be used for complex formation. Do not use polyethylene or polypropylene tubes.

5. To form transfection complex add FuGENE® HD Transfection Reagent to the wells containing DNA solution. Pipet the FuGENE® HD Transfection Reagent (4, 5, 6, 7, 8 and 9 µl correspondingly) directly into the medium containing DNA, avoiding contact with the plastic walls of the wells or tubes.

6. Mix and incubate the transfection complex. Pipeting complex for 7-10 times is the best way to mix it in the wells of the 96-well plate. If complex to be formed in tubes, it can be mixed by tapping or inverting the tube for several times. (Do not vortex complex as it will result in decreased activity).
Incubate transfection reagent:DNA complex for 5 minutes at room temperature. For many cell types incubation is not required, on the other hand for some cell types longer incubation will produce better results, however, incubation for longer than 15 minutes usually will not improve transfection efficiency.

7. Add transfection complex to the cells. There is no need to remove medium before addition of the FuGENE® HD Transfection Reagent:DNA complex. For 96-well plate format add 5µl of transfection complex per well containing cells in 100µl growth medium. Pipet medium for

7-10 times to facilitate uniform distribution of transfection complex over the cells. Alternatively, place 96-well plate on plate shaker for 5-10 seconds. After transfection complex has been added to the cells, removal or replacement of growth medium is not necessary.
8. Incubate cells and evaluate transfection results. After transfection, incubate cells for 18-72 hours and measure your particular protein expression using appropriate detection method.

A. Cotransfection experiments.
For cotransfection experiments calculate the FuGENE® HD Transfection Reagent:DNA ratio based on total amount of DNA to be delivered. The amount of complex that has to be added to cells also should be based on total combined amount of DNA in the system and equal to the amount of DNA normally used for complex formation with a single plasmid.

B. Stable transfection experiments.
FuGENE® HD Transfection Reagent can be used to obtain stably transfected cells. For stable transfection experiments carry out all transfection steps according to standard protocol with cell growth medium without selective pressure. After transfection, once cells reach point when they need to be passaged, growth medium with selective agent can be used.

C. Transfection experiments in cell culture vessels other than 96-well cell culture plate.
Transfection protocol can be scaled up from 96-well plate format. In general, the amount of transfection complex added to the cells have to be proportionally increased according to the total volume of cell culture growth medium in particular experiment setting. Follow Table 1 suggestions for most common cell culture vessels.

Table 1

Culture Vessel	Surface area (cm^2)	Total volume of medium (ml)	Suggested amount of The transfection complex to add to each well (µl)	Total amount of DNA for each well (µg)	Total volume of FuGENE® HD Transfection Reagent (µl) at ratio 3:1
96-well plate (1 well)	0.3	0.1	5	0.1	0.3
24-well plate (1 well)	1.9	0.5	25	0.5	1.5
12-well plate (1 well)	3.8	1.0	50	1.0	3.0
35-mm dish/ 6-well plate (1 well)	8/9.4	2	100	2.0	6.0
60-mm dish	21	5	250	5.0	15.0
10-cm dish	55	10	500	10.0	30.0
T-25 flask	25	6	300	6.0	18.0
T-75 flask	75	20	900	18.0	54.0

4. Optimization of transfection parameters.

Even if you may achieve good transfection results with FuGENE® HD Transfection Reagent from the first experimental attempt we strongly recommend to optimize transfection conditions for each cell line. The two most important optimization parameters are ratio of transfection reagent to DNA (µl of reagent to µg of DNA) and amount of DNA in transfection complex added to the cells (µg). For optimization we suggest using 96-well plate format as it allows modifying several parameters in a single experiment.

The Transfection reagent:DNA ratios mentioned in previous section should work well for the most cell types, however if in your particular situation the maximum of transfection efficiency is located at the end of the chosen ratio range, amount of reagent have to be adjusted correspondingly. For instance: if maximum transfection was achieved at 4.5:1 ratio, we recommend to move reagent:DNA ratio 3 steps higher to the range from 3.5:1 to 6:1, and amount of reagent added to the 0.2µg of DNA in this experiment should be 7, 8, 9, 10, 11 and 12µl respectively (Table 2).

Table 2

		Reagent:DNA ratio								
1ST experiment		2:1	2.5:1	3:1	3.5:1	4:1	4.5:1			
	Maximum transfection						X			
2nd experiment					3.5:1	4:1	4.5:1	5:1	5.5:1	6:1
	Maximum transfection							X		

To optimize the amount of DNA simply add various volumes of transfection complex to the cells. For 96-well plate format use 0.05 to 0.3 µg of DNA per well range, that's corresponds to 2.5 to 15µl of transfection complex per well. Consider achieved level of transfection as well as possible toxicity at high end of complex amount range for your particular cell type and experimental needs for determining the optimal amount of transfection complex.

There are several other optimization parameters you can use to achieve even higher levels of transfection. First, try different complex formation time. The optimum formation time may vary from 0 (no incubation needed) to up to 15-20 minutes. Add complex at different incubation time points to the cells to determine time optimal for your cell type.

We found out that for some cell types varying the concentration of DNA in complex preparation may further improve transfection. Try to vary DNA concentration in initial solution in a range from 0.04 to 0.01µg/µl (default concentration for standard protocol is 0.02 µg/µl). In this case the range of DNA solution volume for complex formation will be from 50µl to 200µl and the volume of complex added to the cells from 2.5 to 10µl respectively (Table 3).

Table 3

DNA concentration	0.01µg/µl	0.0125 µg/µl	0.02 µg/µl	0.04 µg/µl
Volume of DNA solution for complex formation	200µl	160 µl	100 µl	50 µl
Volume of the	10 µl	8 µl	5 µl	2.5 µl

complex added to the cells				

Finally, the cell density in the experiment can influence the transfection results. Try to change the amount of cells plated. Also plating some cells types for the experiment from a high confluence culture may drastically increase transfection efficiency.

For a list of conditions that were used to transfect various cell types, visit the FuGENE® HD web page at: www.fugenehd.com/reference-material/

5. 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 80% 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® HD Transfection Reagent:DNA ratio, complex incubation time, total amount of transfection complex added, or cell density	Optimize the FuGENE® HD Transfection Reagent:DNA ratio, complex incubation time, amount of complex added to cells, and cell density, according to the "Optimization Transfection Parameters" section of this manual.
	FuGENE® HD Transfection Reagent was aliquoted	Check that FuGENE® HD 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 diluted DNA either by pipetting up to 10 – 15 times or inverting the tube for several times.
	FuGENE® HD Transfection Reagent came into contact with plastic or was inadequately mixed	Repeat transfection, carefully pipetting FuGENE® HD Transfection Reagent directly into the serum-free medium, being careful not to touch the sides of the container while adding the FuGENE® HD 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 medium	Check original bottle of medium used for complex formation. Repeat experiment using new bottle of Opti-MEM that does not contain any additives (*e.g.,* serum, antibiotics, growth enhancers, heparin, dextran sulfate, etc.). Try forming the complex in sterile water 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.
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.
	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.
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. 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.
	Culture may be contaminated with mycoplasma	Determine if culture is contaminated with mycoplasma.
	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® HD Transfection Reagent may not be optimal for your cells.	Try FuGENE® 6 Transfection Reagent
	High protein expression levels	High expression levels of certain intracellular proteins (*e.g.,* Green Fluorescent Protein [GFP]) may be cytotoxic to some cell types.
	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.

6. Further reading

Functional Analysis of Transfection Reagents in the context of *bax*-induced apoptosis. (Cellular Analysis Application Note No. 3)

Transfection of androgen responsive luciferase reporter vectors with FuGENE® FuGENE®HD Transfection Reagent in LNCaP and 22Rv1. (Cancer Res. 2009 Apr 1;69(7):2941-9 Epub 2009 Mar 24.)

Stable transgene expression in human embryonic stem cells after simple chemical transfection. (Molecular Reproduction and Development. Volume 76, Issue 6, pages 580–586, June 2009)

FuGENE®HD Choice of a Transfection Reagent with Minimal Off-Target Effect as Analyzed by Microarray Transcriptional Profiling. (Nature Methods 3. Application Notes. published online 17 October 2006)

Rapid production of antigen-specific monoclonal antibodies from a variety of animals. (BMC Biology 2012, 10:80)