3D Transfection System

Protocol for MIR 5800, 5804, 5805, 5806, 5820

Mirus The Transfection Experts

INTRODUCTION

The 3D Transfection System containing the *Trans*IT®-3D Transfection Reagent and alvetex® 12-well tissue culture plates enables DNA delivery to cells grown in 3-dimensional (3D) cell culture. *Trans*IT-3D Transfection Reagent is a broad-spectrum transfection reagent optimized for plasmid DNA delivery into cells grown in 3D matrices. Alvetex tissue culture plates consist of a three-dimensional polystyrene scaffold that provides a 3D environment which cells can migrate, proliferate and differentiate. Additional information on alvetex 3D tissue culture plates can be found at www.reinnervate.com/products.

3D culture methods are generally categorized as solid structural scaffolds or hydrogels of varying composition. *Trans*IT-3D Transfection Reagent can also be used for transfecting cells in hydrogel matrices. Further optimization is required due to an array of available gel compositions and cell culture conditions. Please contact Mirus Bio Technical Support @ 1-888-530-0801 or email techsupport@mirusbio.com for further details.

SPECIFICATIONS

Storage	Store *Trans*IT-3D Reagent tightly capped at –20°C. ***Before each use***, warm to room temperature and vortex gently. Store alvetex® plates at room temperature.
Product Guarantee	6 months from the date of purchase, when properly stored and handled.

Warm *Trans*IT-3D to room temperature and vortex gently before each use.

MATERIALS

Materials Supplied

3D Transfection System consists of the following components.

Product No.	Quantity
MIR 5820	1 × 0.4 ml TransIT®-3D (MIR 5804)
	2 × 12-well alvetex® plates (AVP002, Reinnervate)

*Trans*IT-3D Transfection Reagent available separately in ***one*** of the following formats.

Product No.	Quantity
MIR 5804	1 × 0.4 ml
MIR 5800	1 × 1.0 ml
MIR 5805	5 × 1.0 ml
MIR 5806	10 × 1.0 ml

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Materials required, but not supplied

- Cultured cells
- Appropriate scaffold or hydrogel for 3D cell culture
- Appropriate cell culture medium
- Purified plasmid DNA
- Serum-free medium (e.g. Opti-MEM® I Reduced-Serum Medium)
- Sterile tube for transfection complex preparation
- Micropipets
- Reporter assay as required

BEFORE YOU START:

Important Tips for Optimal Plasmid DNA Transfection

Optimize reaction conditions for each cell type to ensure best transfection results. The suggestions below yield high efficiency transfection using *Trans*IT-3D Transfection Reagent. **Table 1** presents suggested starting conditions depending on culture vessel size.

- **Cell density at transfection.** The optimal cell density at transfection will depend on the type of 3D scaffold, size of the cell and experimental objective. For solid scaffold matrices, such as alvetex, the distribution and adaptation of the cells into the 3D scaffold will depend on the matrix composition, pore size, cell density and cell type. The optimal cell density at transfection must be determined empirically to ensure maximum transfection efficiency.
- **DNA purity.** Use highly purified, sterile, and contaminant-free DNA for transfection. Plasmid DNA preps that are endotoxin-free and have $A_{260/280}$ absorbance ratio of 1.8–2.0 are desirable. DNA prepared using miniprep kits is not recommended as it might contain high levels of endotoxin. We recommend using MiraCLEAN® Endotoxin Removal Kit (MIR 5900) to remove any traces of endotoxin from your DNA preparation.

Do not use DNA prepared using miniprep kits for transfection.

- **Ratio of *Trans*IT-3D Reagent to DNA.** Determine the best *Trans*IT-3D Reagent:DNA ratio for each cell type. Start with 3 µl of *Trans*IT-3D Reagent per 1 µg of DNA. Vary the concentration of *Trans*IT-3D Reagent from 1–5 µl per 1 µg DNA to find the optimal ratio. **Table 1** provides recommended starting conditions based on cell culture vessel size.
- **Complex formation conditions.** Prepare *Trans*IT-3D Reagent:DNA complexes in serum-free growth medium. Mirus recommends Opti-MEM® I Reduced-Serum Medium.
- **Cell culture conditions.** Culture cells in the appropriate medium. The *Trans*IT-3D Reagent yields the highest efficiency when transfections are performed in complete growth medium without a post-transfection medium change. There is no need to perform a medium change to remove the transfection complexes.

  For alvetex plates: A medium change is recommended at least 48-hours post-transfection but may be required earlier depending on cell density. Wait at least 4 hour post-transfection to change the media.

Do not use serum or antibiotics in the media during transfection complex formation.

- **Presence of antibiotics.** Antibiotics will inhibit transfection complex formation and therefore should be excluded during the complex formation step. Transfection complexes can be added to cells grown in complete culture medium containing serum and low levels of antibiotics (0.1–1X final concentration of penicillin/streptomycin mixture).
- **Post-transfection incubation time.** Determine the best incubation time post-transfection for each cell type. The optimal incubation time is generally 24–72 hours but will vary depending on the goal of the experiment and the nature of the plasmid used.

PLASMID DNA TRANSFECTION PROTOCOL

The following procedure describes how to perform plasmid DNA transfections using *Trans*IT-3D Transfection Reagent in alvetex 12-well plates and may be adapted for use with other 3D culture scaffolds. The surface areas of other culture vessels including other alvetex product formats may be different and transfections must be scaled accordingly. Appropriately increase or decrease the amounts of serum free medium, *Trans*IT-3D Reagent, DNA and complete culture medium based on the surface area of the culture vessel. Please refer to **Table 1** below.

Transient plasmid DNA transfection protocol per well of a 12-well plate using solid matrix scaffolds

Table 1. Recommended starting conditions for DNA transfections using *Trans*IT-3D Transfection Reagent with alvetex® 12-well and 6-well scaffolds

Culture vessel	12-well plate	6-well plate
Surface area	3.8 cm^2	9.6 cm^2
Growth medium during transfection *	1.0 ml	2.5 ml
Total maintenance growth medium	3.5 ml	8.75 ml
Serum-free medium	100 µl	250 µl
DNA (1µg/µl stock)	1 µl	2.5 µl
*Trans*IT-3D Reagent	3 µl	7.5 µl

*For alvetex 12-well and 6-well scaffolds, we recommend performing the transfections in 1.0 ml and 2.5 ml of complete growth media, respectively. Add the remaining complete growth medium 4 hours post-transfection.

All volumes given are per well for a given culture vessel.

If small volumes of *Trans*IT-3D need to be pipetted, dilute the reagent in 80% ethanol before each use to avoid pipetting errors. ***Do not*** store diluted *Trans*IT-3D Reagent.

A. Prepare the alvetex scaffold and plate cells

1. Prepare the alvetex 3D matrix scaffold for use (e.g. ethanol and/or PBS washes). For activation of alvetex wells, please refer to the enclosed alvetex Product Information Leaflet.
2. Approximately 2 – 7 days before transfection, plate cells in 3.5 ml complete growth medium per well in an alvetex 12-well plate. For most cell types, optimal transfection results are obtained when cells are plated 48 hours prior to transfection.
3. Plate cells at a density of 2–6 × 10^5 cells/well depending on the cell type. Add quantity of cells in 1 ml per well.
4. Add 2.5 ml of complete medium per well after 4 hours for a total of 3.5 ml.
5. Change the media every 48 hours to ensure sufficient nutrients for cell growth.

Note: Due to the high density of cells within the alvetex scaffold, it is recommended to change the cell culture medium every 48 hours to ensure sufficient nutrients for growth.

Divide cultured cells 2 – 7 days before transfection to ensure adaption of the cells to the 3D environment. For most cell types, optimal transfection results are obtained when the cells are plated 48 hours prior to transfection.

B. Prepare *Trans*IT-3D Reagent:DNA complexes (Complex formation)

1. Warm *Trans*IT-3D Reagent to room temperature and vortex gently before using.
2. Place 100 µl of Opti-MEM® I Reduced-Serum Medium in a sterile tube.
3. Add 1.0 µg plasmid DNA.
4. Pipet gently to mix completely.
5. Add 3.0 µl *Trans*IT-3D Reagent to the diluted DNA mixture. Avoid any contact of the *Trans*IT-3D Reagent with the sides of the plastic tube.
6. Pipet gently to mix completely.
7. Incubate at room temperature for 15–30 minutes.

C. Distribute the complexes to cells in the alvetex wells

1. Remove media from the wells containing cells without disturbing the matrix, and add 1 ml of complete growth medium to each well.
2. Add the *Trans*IT-3D Reagent:DNA complexes (prepared in Step B) drop-wise to different areas of the wells.
3. Incubate for 4 hours at the temperature and CO_2 culture conditions recommended for your cell type.
4. Add an additional 2.5 ml of complete growth medium to each well.
5. Incubate for an additional 20–72 hours. It is not necessary to replace the complete growth medium with fresh medium unless harvest is longer than 48 hours post-transfection. If an earlier medium change is required for your experiments, please wait at least 4 hours post-transfection before changing the medium.
6. Harvest cells or supernatant and assay as required.

Note: If lysis is necessary in alvetex plates, cells can be lysed using at least 500ul of a lysis buffer containing 2% Triton X-100 or equivalent.

*Trans*IT-3D is a low-toxicity reagent. There is no need to change culture medium after transfection unless harvest is longer than 48 hours post-transfection. If required, perform a medium change at least 4 hours post-transfection.

Transient plasmid DNA transfection using hydrogel matrices

Due to the wide variety of hydrogels available for 3D cell culture, further protocol optimization is required for this format dependent on gel composition, cell type, culture conditions and application. For further experimental recommendations and suggestions, please contact Mirus Bio @ 1-888-530-0801 or email techsupport@mirusbio.com.

TROUBLESHOOTING GUIDE

Problem	Solution
LOW PLASMID DNA TRANSFECTION EFFICIENCY	
*Trans*IT-3D Reagent was not mixed properly	Warm *Trans*IT-3D to room temperature and vortex gently before each use.
Suboptimal *Trans*IT-3D Reagent:DNA ratio	Determine the best *Trans*IT-3D Reagent:DNA ratio for each cell type. Titrate the *Trans*IT-3D Reagent from 1–5 µl per 1 µg DNA. Refer to "Before You Start" on Page 2.
Suboptimal DNA concentration	Determine the DNA concentration accurately. Use plasmid DNA preps that have an $A_{260/280}$ absorbance ratio of 1.8–2.0. The optimal DNA concentration generally ranges between 1–3 µg/well of a 12-well plate. Start with 1.0 µg/well of a 12-well plate. Consider testing more or less DNA while scaling the amount of *Trans*IT-3D Transfection Reagent accordingly.
Low-quality plasmid DNA	Use highly purified, sterile, endotoxin and contaminant-free DNA for transfection. We recommend using Mirus Bio's MiraCLEAN Endotoxin Removal Kit (MIR 5900) for removal of endotoxin from your DNA preparation. Alternatively, use cesium chloride gradient or anion exchange purified DNA which contains levels of endotoxin that do not harm most cells. **Do not** use DNA prepared using miniprep kits as it might contain high levels of endotoxin.
Inhibitor present during transfection	Serum and antibiotics inhibit transfection complex formation. Prepare *Trans*IT-3D Reagent:DNA complexes in serum-free growth medium. We recommend Opti-MEM® I Reduced-Serum Medium. Once transfection complexes are formed, they can be added directly to cells cultured in complete growth medium containing serum and 0.1–1X antibiotics. Polyanions such as dextran sulfate or heparin can inhibit transfection. Use culture medium that does not contain these polyanions. If necessary, the transfection medium can be replaced with polyanion containing medium 24 hours post transfection.
Incorrect vector sequence	If you do not observe expression of your target insert, verify the sequence of the plasmid DNA.
Transfection incubation time	Determine the optimal transfection incubation time for each cell type and experiment. Test a range of incubation times (e.g.12–72 hours). The best incubation time is generally 24–48 hours.
Complexes are not able to penetrate 3D matrix	The penetration and adaptation of the cells into the 3D scaffold will depend on the composition and pore size, cell density and the cell type. For alvetex 3D plates, optimal transfection results are obtained when wells are plated 48 hours prior to transfection.
Proper experimental controls were not included	To verify efficient transfection, use TransIT-3D Reagent to deliver a positive control such as a luciferase, beta-galactosidase or green fluorescent protein (GFP) encoding plasmid. For reporter assays requiring cell lysis, cells can be lysed using at least 500ul of a lysis buffer containing 2% Triton X-100 or equivalent. For GFP visualization, the transfected cells can be visualized in alvetex plates using confocal microscopy.
Precipitate formation during transfection complex formation	During complex formation, scale all reagents according to Table 1 on page 3, including serum-free media, *Trans*IT-3D and plasmid DNA. Precipitation maybe observed when excess DNA is used during complex formation. This may negatively impact transfection efficiency. To avoid precipitation when using high concentrations of DNA, increase the volume of serum-free medium during complex formation by two-fold.

TROUBLESHOOTING GUIDE continued

Problem	Solution
HIGH CELLULAR TOXICITY	
Transfection complexes and cells not mixed thoroughly after complex addition	Add *Trans*IT-3D Reagent:DNA complexes drop-wise to different areas of the wells containing the cells.
Transfection complexes added to cells cultured in serum-free medium	Allow *Trans*IT-3D Reagent:DNA complexes to form in serum-free medium, then add these complexes to cells cultured in complete growth medium. The presence of serum in the growth medium improves transfection efficiency and reduces cytotoxicity. No culture medium change is required after the addition of transfection complexes to cells.
Endotoxin-contaminated plasmid DNA	Use highly purified, sterile, endotoxin and contaminant-free DNA for transfection. We recommend using Mirus Bio's MiraCLEAN Endotoxin Removal Kit (MIR 5900) for removal of any traces of endotoxin from your DNA preparation. Alternatively, use cesium chloride gradient or anion exchange purified DNA which contains levels of endotoxin that do not harm most cells. Do not use DNA prepared using miniprep kits as it might contain high levels of endotoxin.
Expressed target gene is toxic to cells	Compare toxicity levels against a cells alone control and cells transfected with an empty vector to assess the cytotoxic effects of the target protein being expressed. If lower levels of target gene expression are desired in your transfection experiments, consider reducing the amount of target plasmid. Maintain the optimal *Trans*IT-3D:DNA ratio by using carrier DNA such as an empty cloning vector.
Cell density not optimal at time of transfection	Determine the best cell density and 3D adaptation time for each cell type to maximize transfection efficiency. Use this cell density and incubation time in subsequent experiments to ensure reproducibility. For most cell types grown in alvetex® wells, a cell density of 2–6 × 10^5 cells/well and a 48 hour pre-transfection incubation time is recommended. Additionally, you can divide the culture at least 2-7 days before transfection to ensure adaptation to the 3D environment.
Cell morphology has changed	Mycoplasma contamination can alter cell morphology and affect transfection efficiency. Check your cells for Mycoplasma contamination. Use a fresh frozen stock of cells or use appropriate antibiotics to eliminate Mycoplasma. A high or low cell passage number can make cells more sensitive and refractory to transfection. Maintain a similar passage number between experiments to ensure reproducibility.

Technical Support | Toll-free (U.S.) 888.530.0801 | Direct: 608.441.2852 | techsupport@mirusbio.com | www.mirusbio.com

RELATED PRODUCTS

- *Label* IT Plasmid Delivery Controls
- *Label* IT Tracker Intracellular Nucleic Acid Localization Kits
- MiraCLEAN Endotoxin Removal Kits

For details on the above mentioned products, visit www.mirusbio.com or www.TheTransfectionExperts.com.

Contact Mirus Bio for additional information.

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