*Trans*IT-TKO® Transfection Reagent

Product	Quantity*	Product No.
*Trans*IT-TKO® Transfection Reagent	0.4 ml	MIR 2154
	1 ml	MIR 2150
	5 ml (5 x 1 ml)	MIR 2155
	10 ml (10 x 1 ml)	MIR 2156

*Each milliliter of *Trans*IT-TKO Reagent (MIR 2150) is sufficient quantity to perform up to 1000 transfections in 24-well plates, depending on the specific cell type.

1.0 DESCRIPTION

1.1 General Information

Cellular uptake of long double stranded RNA (dsRNA) has been shown to induce RNA interference in a diverse group of organisms as well as insect cells in culture. RNA interference leads to the inhibition of protein expression by utilizing sequence-specific, dsRNA-mediated destruction of the target messenger RNA (mRNA). Attempts to induce RNA interference using long dsRNA in mammalian cell lines have been met with limited success, due in part to the induction of the interferon response, which results in a general inhibition of protein synthesis. It has been shown that when short RNA duplexes are introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA can be realized without inducing an interferon response. These short dsRNA, referred to as small interfering RNAs (siRNA), act catalytically at sub-molar ratios to cleave greater than 95% of the target mRNA in the cell. The RNA interference effect can be long-lasting and may be detectable after many cell divisions. These properties make siRNA extremely effective at inhibiting target gene expression once introduced into the cell. [1,2,3]

Mirus Bio Corporation, in recognition of these significant findings, has developed *Trans*IT-TKO Transfection Reagent, which enables highly efficient siRNA transfection with significantly reduced levels of cell damage as compared to cationic-liposome based transfection reagents. Transfections are most effective when carried out in complete growth media, with no media change or serum addition required. TransIT-TKO Reagent efficiently delivers siRNA to a wide range of cell lines, promoting highly effective inhibition of target gene expression. These unique features make *Trans*IT-TKO Transfection Reagent ideal for siRNA-mediated gene silencing studies. The *Trans*IT-TKO Transfection Reagent is a complement to Mirus' *Trans*IT-siQUEST® Transfection Reagent, (see Related Products, Section 5.0). Each unique formulation provides a distinct transfection profile for high efficiency, broad-spectrum siRNA delivery.

1.2 Cell Lines Successfully Tested by Mirus Corporation

A549, BNL CL.2, BHK-21, C2C12, C6, CHO-K1, COS-7, Daoy, DB-TRG-05MG, DI-TNC1, DU 145, HEK 293, HeLa, Hepa1c1c7, HepG2, Jurkat, Keratinocytes (NIKS), MCF-7, Neuro-2a, NIH 3T3, PC-3, Primary Mouse Hepatocytes, RAW 264.7, SK-N-MC, THP-1, Vero

1.3 Cell Lines Successfully Tested by Other Laboratories

A549, Alpha 2, CHO-K1, ECR-293, H1299, HEK 293, HeLa, HeLa SS6, Jaws II, MDA-MB-231, NIH 3T3, rat fibrosarcoma cJ4, U2OS, U87

1.4 Specifications

Concentration: 2.5 mg/ml in 100% ethanol

Storage: Store tightly capped at 4˚C; DO NOT FREEZE. Warm to room temperature and gently vortex before each use to dissolve any precipitate that may have formed.

Stability: 1 year when stored properly

2.0 PROCEDURE

2.1 Transfection Optimization

The key to successful transfection is careful optimization of reaction conditions for each individual cell type. The transfection protocols described in Sections 2.2 and 2.3 should result in efficient transfection of most cell types; however, to ensure optimal results consider the following variables:

A. Media conditions – The *Trans*IT-TKO Reagent yields improved transfection efficiencies when transfections are performed in complete growth medium (instead of serum-free medium) without a post-transfection media change.

B. Cell density (% confluence) at the time of transfection – The recommended confluence for most cell types at transfection is 60-80% at the time of transfection (3×10^4 to 1.2×10^5 cells per well of a 24-well plate, depending on cell size and characteristics). If this confluence does not produce optimal results, test cell densities outside of the recommended range.

Lower cell densities may be necessary when post-transfection incubation times are greater than 48 hours. If lower cell densities are plated, ensure that the levels of *Trans*IT-TKO Reagent and siRNA are titrated accordingly. Alternatively, trypsinize and replate cells 24 hours post-transfection to accommodate longer incubation times. Determine the optimal cell density for each cell type in order to maximize knockdown efficiency. Maintain this density in future experiments for reproducibility.

C. siRNA concentration – siRNA used for transfection should be highly pure, sterile, and the correct sequence. As a starting point, use 25 nM siRNA (final concentration in well). Depending on the type of experiment, the optimal final siRNA concentration for transfection is typically within the range of 10 to 50 nM.

D. *Trans*IT-TKO Reagent – As a starting point, test three levels of *Trans*IT-TKO Reagent, such as 1 µl, 2.5 µl, and 4 µl per well of a 24- well plate, using 25 nM siRNA (final concentration in the well). The optimal *Trans*IT-TKO Reagent volume can be determined by titrating the reagent within the ranges listed in Table 1. Each cell line responds differently to a given transfection reagent. It is important to test different conditions to identify those that produce the highest knockdown efficiency with the lowest cellular toxicity .

E. Transfection incubation time – The optimal incubation time can be determined empirically by testing a range from 24-72 hours post-transfection, depending on the stability of the target mRNA and its encoded protein. When quantifying knockdown efficiencies at the mRNA level, assaying at 24 hours post-transfection is often sufficient. When quantifying knockdown efficiencies at the protein level, longer post-transfection incubations may be necessary if the target protein has a long cellular half-life and therefore degrades slowly.

F. Proper Controls – Consider the following controls to properly assess knockdown efficiencies:

1. Serum-free media alone
2. Serum-free media + *Trans*IT-TKO Reagent
3. Serum-free media + *Trans*IT-TKO Reagent + a non-specific siRNA

Table 1. Recommended titration ranges for *Trans*IT-TKO Transfection Reagent transfectio

Culture Vessel	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate	10 cm dish
Surface Area*	0.35 cm^2	1.0 cm^2	1.9 cm^2	3.8 cm^2	9.6 cm^2	59 cm^2
Serum-free Media	9 µl	25 µl	50 µl	100 µl	250 µl	1500 µl
*Trans*IT-TKO Transfection Reagent**	0.18-0.74 µl	0.5-2 µl	1-4 µl	2-8 µl	5-20 µl	30-120 µl
1 µM stock siRNA (10-50 nM final concentration in well)	0.5-2.6 µl	1.5-7.5 µl	3-15 µl	6-30 µl	15-75 µl	90-450 µl
Complete Growth Media	44 µl	125 µl	250 µl	500 µl	1250 µl	7500 µl

*Surface areas are based on Greiner tissue culture plates and Falcon 10 cm dishes.

**At a given well size, test low, medium and high levels of *Trans*IT-TKO Transfection Reagent to determine optimal knockdown efficiency and lowest cellular toxicity.

NOTE: All volumes in Table 1 are per well of the indicated size.
To dilute siRNA, use the manufacturer's recommended buffer. Alternatively, use 100 mM NaCl in 50 mM Tris, pH 7.5, made with RNase-free water. Do not use water alone to dilute siRNA, as this may result in denaturation of the siRNA.

NOTE: The protocols below in Sections 2.2 and 2.3 are recommended for performing transfections with the *Trans*IT-TKO Transfection Reagent in 24-well plates. When performing transfections in different sized wells, the amount of siRNA, serum-free media, *Trans*IT-TKO Reagent, and culture medium should be scaled up or down in proportion to the surface area of the dish (see Table 1). Proper scaling of the total volume of culture medium is important to ensure optimal *Trans*IT-TKO Reagent and final siRNA concentrations.

2.2 siRNA Transfection in 24-well Plates

A1. Cell Plating

1. Approximately 24 hours prior to transfection, plate cells at an appropriate cell density to obtain ~60-80% confluence the following day (3×10^4 to 1.2×10^5 cells per well of a 24-well plate, depending on cell size and characteristics).[a] Plate adherent cells in 500 µl of complete growth media per well.
2. Incubate the cells overnight.[b]

A2. Cell Plating for Suspension Cells

1. Immediately prior to transfection, plate cells in 0.25 ml of complete growth media per well, at an appropriate density to obtain ~60-80% confluence at time of transfection ($3\text{-}5 \times 10^5$ cells per well). Alternatively, plate cells the day prior to transfection to obtain 60-80% confluence at the time of transfection ($1.5\text{-}2.5 \times 10^5$ cells per well).

B. Complex Formation (perform this procedure immediately prior to transfection)

1. In a sterile, plastic tube, add 50 µl (see Table 1) of serum-free medium[c] .
2. Add 1 to 4 µl (test three different levels, such as 1 µl, 2.5 µl, and 4 µl per well of a 24-well plate; see Table 1) of the *Trans*IT-TKO Transfection Reagent directly into the serum-free media. Mix thoroughly by pipetting or vortexing.
3. Incubate at room temperature for 5-20 minutes.
4. Add 10-50 nM siRNA, (the starting recommendation is 25 nM, final concentration in the well) to the diluted *Trans*IT-TKO Reagent. Mix by gentle pipetting.
5. Incubate at room temperature for 5-20 minutes.

C. Cell Preparation for Transfections in Complete Growth Medium

NOTE: The *Trans*IT-TKO Reagent yields improved transfection efficiencies when the transfections are performed in complete growth medium (instead of serum-free medium).

1. For adherent cell types, adjust the volume in the well to 250 µl of complete growth media by removing ~250 µl (half) of the original plating media. This media reduction requires the use of less siRNA to obtain the recommended 25 nM (final concentration in the well).
2. Add the *Trans*IT-TKO Reagent/siRNA complex mixture prepared in step B dropwise to the cells. Gently rock the dish back and forth and from side to side to distribute the complexes evenly. Do not swirl the plate.
3. Incubate for 24-72 hours[b]
   NOTE: The above incubation is designed for transfections performed with no media change. If a media change is necessary to remove the transfection complexes, incubate the cells for 24 hours, replace the original medium with fresh complete growth medium, and incubate for an additional 24-48 hours.[b]
4. Assay for knockdown of target gene expression.

2.3 Simultaneous Transfection of Plasmid DNA and siRNA in 24-well Plates

If the transfection of both plasmid DNA and siRNA is required, follow the procedure below.

A. Cell Plating

1. Approximately 24 hours prior to transfection, plate cells at an appropriate cell density to obtain ~60-80% confluence the following day (3×10^4 to 1.2×10^5 cells per well of a 24-well plate, depending on cell size and characteristics).[a] Plate adherent cells in 500 µl of complete growth media per well.
2. Incubate the cells overnight.[b]

B. Complex Formation

NOTE: Mirus offers several reagents for plasmid transfection, including cell line specific reagents. To choose the appropriate reagent, please see Related Products, our website (www.mirusbio.com) or contact Technical Support at techsupport@mirusbio.com or 888-530-0801. The following protocol recommends *Trans*IT®-LT1 Transfection Reagent, Mirus' broad spectrum transfection reagent, which efficiently delivers DNA into many cell types.

1. In a sterile, plastic tube, add 50 µl of serum free media[c].
2. Add *Trans*IT-LT1 Transfection Reagent (1-2 µl per well for 24 well plates; scale up or down for different size plates) directly to the serum-free media. Mix thoroughly by pipetting or vortexing.
3. Incubate at room temperature for 5-20 minutes.
4. Add plasmid DNA (0.5 µg per well for 24 well plates; titration may be necessary) to the diluted *Trans*IT-LT1 Reagent. Mix by gentle pipetting.
   NOTE: If transfecting more than one plasmid, mix the plasmids together in a microcentrifuge tube and incubate for 5-10 minutes at room temperature before adding to the diluted *Trans*IT-LT1 Reagent.
5. Incubate at room temperature for 5-20 minutes.
6. In the same complexing tube, add the *Trans*IT-TKO Reagent (1-4 µl per well for 24 well plates). Mix by gentle pipetting.
7. Incubate at room temperature for 5-20 minutes.
8. Add siRNA (10-50 nM final concentration in the well) to the diluted complex mixture. Mix by gentle pipetting.
9. Incubate at room temperature for 5-20 minutes.

C. Cell Preparation

NOTE: The *Trans*IT-TKO Reagent yields improved transfection efficiencies when transfections are performed in complete growth medium (instead of serum-free medium).

1. For adherent cell types, adjust the volume in the well to 250 µl of complete growth media by removing ~250 µl (half) of the original plating media. This media reduction requires the use of less siRNA to obtain the recommended 25 nM (final concentration in the well).
2. Add *Trans*IT-LT1 Reagent/pDNA/*Trans*IT-TKO Reagent/siRNA complex mixture prepared in Step B dropwise to the cells. Gently rock the dish back and forth and from side to side to distribute the complexes evenly. Do not swirl the plate.
3. Incubate for 24-72 hours.[b]
   NOTE: The above incubation is designed for transfections performed with no media change. If a media change is necessary to remove the transfection complexes, incubate the cells for 24 hours, replace the original medium with fresh complete growth medium, and incubate for an additional 24-48 hours.[b]
4. Assay for knockdown of target gene expression.

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[a] Since the optimal cell density (confluence) for efficient transfection can vary between cell types, this should be determined for each cell type. Maintain the optimal seeding protocol for each cell type between experiments.

[b] Standard incubation conditions for mammalian cells are 37˚C in 5% CO_2. Other cell types, such as insect cells, require different temperatures and CO_2 concentrations. Use conditions appropriate for the cell type of interest.

[c] The *Trans*IT- Reagent/nucleic acid complex may not form properly if the complex formation medium contains serum, resulting in poor transfection efficiencies. Any serum-free media can be used for complex formation, provided it does not contain polyanions such as dextran sulfate and heparin.

3.0 TROUBLESHOOTING

Low Transfection Efficiency

- **Suboptimal *Trans*IT-TKO Reagent**
  As a starting point, test three levels of *Trans*IT-TKO Reagent, such as 1 µl, 2.5 µl, and 4 µl per well of a 24-well plate, using 25 nM siRNA (final concentration in the well). It may be necessary to titrate outside of this range depending on the cell type. Use the volume of reagent that gives the highest knockdown efficiency with the lowest cellular toxicity for future transfections.

- **Suboptimal siRNA concentration**

Determine the optimal siRNA concentration by titrating from 10 nM to 50 nM (final concentration in the well). We recommend starting with 25 nM siRNA (final concentration in the well). In some instances higher concentrations of siRNA such as 100 or 200 nM may be necessary to achieve sufficient knockdown of the gene of interest.

- **Low Transfection Efficiency**
  Follow transfection protocol steps carefully. To assess transfection efficiency of siRNA, use Mirus' Label IT® siRNA Tracker Kits (see Related Products, Section 5.0).
- **Denatured siRNA**
  To dilute siRNA, use the manufacturer's recommended buffer, or 100 mM NaCl, 50 mM Tris, pH 7.5 in RNase-free water. Do not use water as this can denature the siRNA.
- **Incorrect siRNA Sequence**
  Ensure that the sequence of the siRNA is correct for the gene of interest. More than one sequence may need to be tested for optimal knockdown efficiency.
- **Poor quality of transfecting siRNA**
  Avoid siRNA degradation by using RNase-free handling procedures and plastic ware. For high quality siRNA design and manufacture, Mirus recommends Dharmacon Research, Inc. (dharmacon.com). Degradation of siRNA can be detected on acrylamide gels.
- **Cell density (% confluence) not optimal at time of transfection**
  The recommended cell density for most cell types at the time of transfection is 60-80% confluence. Determine the optimal cell density, which may be outside the recommended range, for each cell type in order to maximize transfection efficiency. Maintain the optimal density in future experiments for reproducibility.
- **Fetal calf serum present during TransIT-TKO Reagent/siRNA complex formation**
  Use serum-free medium during the complex formation steps.
- **Inhibitor present during transfection**
  The presence of polyanions, such as dextran sulfate or heparin, can inhibit transfection. Use transfection medium that does not contain these polyanions. If polyanions are necessary for the health of the cells, the transfection media can be replaced with the optimal cell growth media 24 hours post-transfection.
- **Proper controls were not included**
  1. Serum-free media alone
  2. Serum-free media + *Trans*IT-TKO Reagent
  3. Serum-free media + *Trans*IT-TKO Reagent + a non-specific siRNA

  To verify efficient transfection and knockdown, use *Trans*IT-TKO Reagent to deliver a siRNA targeted against a ubiquitous gene, such as GAPDH or Lamin A/C, followed by western blotting or target mRNA quantification.

High Cellular Toxicity

- **Media change or addition may be necessary**
  If incubating for 48-72 hours, it may be necessary to change the complete media 24 hours post-transfection. Alternatively, add additional complete media 4-24 hours post-transfection.
- **Excessive amount of *Trans*IT-TKO Reagent**
  Reduce the amount of *Trans*IT-TKO Reagent in the transfection. See Table 1 for recommended starting concentrations. Use the volume of reagent that gives the highest knockdown efficiency with the lowest cellular toxicity for future transfections.
- **Cell density was too low at time of transfection**
  Grow cells to a higher cell density and repeat the transfection.
- ***Trans*IT-TKO Reagent/siRNA complex mixture and cells were not mixed thoroughly**
  Mix thoroughly to evenly distribute the complexes to all of the cells. Rocking the dish back and forth and from side to side is recommended. Do not swirl or rotate the dish, as this may result in uneven distribution.
- **Complexes were added to cells in serum-free media**
  *Trans*IT-TKO/siRNA complexes should be added to cells in complete media (serum-containing media) 5-20 minutes after complex formation. If these complexes are added to cells in serum-free media, cytotoxicity may be observed. If you must add the complexes to serum-free media, add complete media after 4 hours to minimize toxic effects.
- **Cell morphology has changed**

If the passage number of the cells is too high or too low they may be more sensitive. Maintain a similar passage number between experiments to ensure reproducibility.

- **Knockdown of an essential gene**
  If the siRNA is directed against a gene that is essential to the cell, cytotoxicity may be observed due to knockdown of the target gene.

For specific questions or concerns, please contact Mirus Technical Support at 888.530.0801 or techsupport@mirusbio.com.
For a list of citations using Mirus products, Frequently Asked Questions, and a Technical Report, please visit the Technical Resources section of our website at www.mirusbio.com.

4.0 REFERENCES

1. Elbashir, S.M. et al. (2001) Nature **411:** 494-498.
2. Caplen, N.J. et al (2001) Prot. Natl. Acad. Sci. **98:** 9742-9747.
3. Sharp, P.A. (2001) Genes and Development **15:** 485-490.

5.0 RELATED PRODUCTS

Additional RNA Interference Products*:
*Trans*IT-siQUEST® siRNA Transfection Reagent (Product # MIR 2110)
siXpress® PCR Vector Systems (Product # MIR 7300, 7301, 7302)
Label IT® siRNA Tracker Intracellular Localization Kit (Product # MIR 7200, 7201, 7202, 7203, 7204, 7205)
For DNA Tracking Studies:
Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit (Product # MIR 7010, 7011, 7012, 7013, 7014, 7015)
Additional Transfection Reagents:*
*Trans*IT®-LT1 Transfection Reagent (Product # MIR 2300)
*Trans*IT®-LT2 Transfection Reagent (Product # MIR 2400)
*Trans*IT®-Express Transfection Reagent (Product # MIR 2000)
*Trans*IT®-HeLaMONSTER® Transfection Kit (Product # MIR 2900)
*Trans*IT®-293 Transfection Reagent (Product # MIR 2700)
*Trans*IT-Neural® Transfection Reagent (Product # MIR 2140)
*Trans*IT®-Keratinocyte Transfection Reagent (Product # MIR 2800)
*Trans*IT®-CHO Transfection Kit (Product # MIR 2170)
*Trans*IT®-3T3 Transfection Kit (Product # MIR 2180)
*Trans*IT®-COS Transfection Kit (Product # MIR 2190)
*Trans*IT®-Insecta Transfection Reagent (Product # MIR 2200)
*Trans*IT®-Prostate Transfection Kit (Product # MIR 2130)
*Trans*IT®-Jurkat Transfection Reagent (Product # MIR 2120)
*Trans*IT®-Oligo Transfection Reagent (Product # MIR 2160)
For Determination of Gene Expression Efficiency:
Beta-Gal Staining Kit (Product # MIR 2600)
For Endotoxin Removal from DNA:*
MiraCLEAN® Endotoxin Removal Kit (Product # MIR 5900)

*These products are available in additional sizes.