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TARGEFECT-HUVEC Transfection Protocols

Transfections per kit: 600 transfections (24 well), 300 transfections (12 well), 150 transfections (6-well)

Efficient gene transfer into primary endothelial cells is an important prerequisite for studying regulation of gene expression in vascular tissue. Here we present a simple set of protocols using the Targefect-HUVEC transfection kit to transfect HUVECs, HMVECs, and different types of primary endothelial cells. The kit consists of Targefect F-2, a non-lipid cationic transfection reagent with low toxicity along with two enhancer formulations- Virofect ™ and a Peptide Enhancer. Virofect enhances gene transfer by using adenoviral receptors on the cell surface to enhance intracellular delivery of transfection complexes. Once the transfection complex is internalized, Virofect helps the transfection complex escape degradation in the lysosome and enhances the both duration and efficiency of transgene expression. The Peptide Enhancer increases efficiency of transgene expression by escorting genes to the nucleus. Using the protocols mentioned here researchers have been able to achieve 80-95% transfection efficiencies in HUVECs and efficiencies ranging from 40-80% in different types of primary endothelial cells (see attached figures showing data from other lab groups and a list of product citations).

I. STORAGE

Store the transfection reagents at the following temperatures immediately upon arrival. The reagents are stable for 1 year.

Reagent	Volume	Temp.
Targefect-HUVEC (Targefect F2)	600 µl	4°C
Peptide Enhancer (PE)	600 µl	4°C
Virofect	600 µl	-20°C

Mix each reagent prior to use by inverting 10 times.

The Targefect-HUVEC endothelial cell transfection kit contains 3 components. Optimal transfection protocols are provided in the kit to carry out successful transfection of the following endothelial cells with high efficiency/viability.

Cell Types	Transfection Efficiency (%)
HUVECs (human umbilical vein endothelial cells)	70-95
Human dermal microvascular endothelial cells	40
Human lung microvascular endothelial cells	70-90
Human aortic endothelial cells	60
Human coronary artery endothelial cells	65
Human pulmonary artery endothelial cells	50
Human brain microvascular endothelial cells	
Bovine aortic endothelial cells	70
Bovine coronary artery endothelial cells	60
Porcine aortic endothelial cells	60
Rat aortic endothelial cells	60
Rat brain microvascular endothelial cells	

II. PREPARATION OF CELLS

Plate Endothelial Cells at a density of 25,000-30,000 cells per cm^2 in the Antibiotics-Free Growth Medium. Transfect cells when cell density reaches 70-80% confluency. Preferred media: MCDB131 from VEC technologies, Media 199 plus supplements and 20% fetal calf serum or EBM from Cambrex plus supplements and 10% fetal calf serum. Cells grown in these media appear healthier and give higher transfection efficiency.

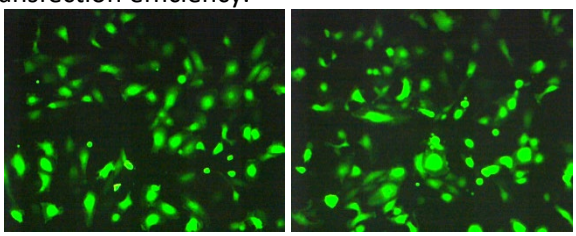

Figure 1: Transfection of HUVECs with a GFP expression vector using the Targefect F2 reagent plus Virofect enhancer (both components of the Targefect-HUVEC kit): 6 µg of DNA was complexed with 12 µl of Targefect-HUVEC and 25 µl of Virofect in 0.5 ml of high glucose DMEM, and then incubated at 37°C for 20 mins to form transfection complexes. 0.5 ml of transfection complexes were added to 2 ml of fresh EBM (Cambrex), 10% FCS (GIBCO) and supplements (CAMBREX) in one 60cm dish of HUVECs. The media the next day with 3 ml of complete media Transfection efficiency approx. 80%. **Data courtesy of Dr. Michael Potente, Department of Cardiology, University of Frankfurt, Germany.**

Figure 2: Transfection of human lung microvascular endothelial cells with a green fluorescent protein expression vector using Targefect-HUVEC and the Peptide Enhancer. Confocal images of cells transfected with a GFP-expression vector and counter-stained with rhodamine-phalloidin (actin stain). **Data Courtesy of Dr. Steve Duffy and J. Murphy, UT, Southwester Medical Ctr., Dallas, TX.**

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PROTOCOL 1: (PREFERRED PROTOCOL) Transfect Endothelial Cells with Targefect-HUVEC and Peptide Enhancer

III. FORMATION OF TRANSFECTION COCKTAIL

A. Preparation of DNA (Step 1)

1. Dilute the plasmid DNA with Transfection Medium serum-free high glucose DMEM or OptiMEM1) as shown in Table 1.
2. Mix thoroughly by flicking 10 times.

B. Preparation of transfection Complex (Step 2)

1. Mix Targefect by inverting tube 10 times.
2. Add Targefect to diluted DNA as shown in Table 1.
3. Mix thoroughly by gently flicking 10 times.
4. Mix Peptide Enhancer (PE) by inverting tube 10 times.
5. Add PE to the Targefect-DNA mixture as shown in Table 1.
6. Mix thoroughly by gently flicking 10 times.
7. Incubate the Transfection Complex at 37°C for 25 minutes.

IV. TRANSFECTION OF CELLS

A. Addition of Transfection Complex (Step 3)

1. Aspirate off antibiotics-Free Growth Media from cell culture.
2. Add the appropriate amount of Transfection Complex to each well as shown in Table 2 by gently pipetting the Transfection mix along the side of the well so as not to disrupt the cells. Incubate cells with the Transfection mix in 37°C, 5% CO_2 humidified incubator for 2 hours.

B. Replacement of Transfection Complex with Antibiotic-Free Growth Medium (Step4)

1. Aspirate off the Transfection Complex from cells.
2. Add Antibiotics-Free Growth Medium to the transfected cells as shown in Table 2.
3. Incubate the transfected cells in 37°C, 5% CO_2 humidified incubator for 24 hours.
4. Change to Endothelial Cell Growth Medium and assay.

Table 1: Formation of Transfection Complex

	STEP 1: Preparation of DNA			STEP 2: Preparation of Transfection Complex					
Tissue Culture Plate	**DNA (µg)**	**Transfection Medium (µl) DMEM**	***Gently* Flick 10X**	**ADD Targefect (µl)**	**Flick 10X**	**ADD PE (µl)**	***Gently* Flick 10X**	**37°C for 25 mins**	**Total Transfection Complex (µl)**
96-well	0.06	60		0.25		0.75			61
24-well	0.2	200		1.0		3.0			204
12-well	0.4	400		2.0		6.0			408
6-well	1.0	1000		5.0		15.0			1020

Table 2: Transfection of Endothelial Cells with Targefect-PE Transfection Complex

	Step 3: Addition of Transfection Complex			STEP 4 Replacement of Transfection Complex with Antibiotic-Free Growth Medium		
Tissue Culture Plate	**Aspirate off Antibiotic-Free Growth Medium**	**ADD Transfection Complex (µl)**	**37°C 5% CO_2 for 1-2 hrs**	**Aspirate off Transfection Complex**	**ADD Antibiotic-Free Growth Medium (µl)**	**37°C 5% CO_2 for 24 hrs**
96-well		61			100	
24-well		204			500	
12-well		408			1000	
6-well		1020			2000	

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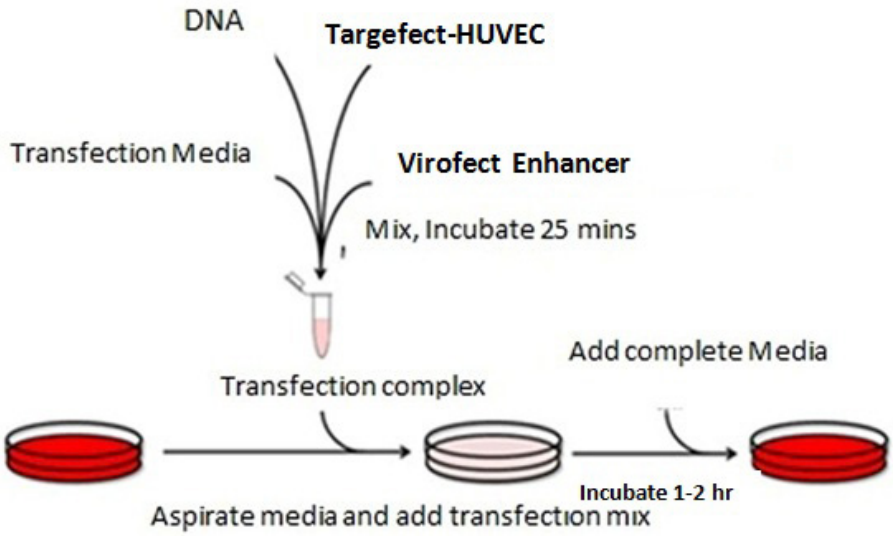
PROTOCOL 2: Transfect Endothelial Cells with Targefect-HUVEC and Virofect Enhancer



Transfection complexes with Virofect are added directly to the culture media (with serum) covering the cells

General considerations: Use early passage endothelial cells, avoid using collagen coated dishes as these may lower transfection efficiency. **Culture media we recommend is Media 199 with 20% serum or EBM (Cambrex) 10% FCS (Gibco) & supplements (Cambrex). We recommend using HUVEC media supplement edx with serum (10% serum concentration) if possible. If you wish to use serum-free media to culture HUVECs, then transfection complexes should be aspirated after two hours of incubation with cells.**

Preparation of the complexes and transfection procedure:
Since reagents sometimes freeze during shipping, we recommend gently mixing the Targefect-F2 solution once upon receipt. The Targefect-F-2 reagent should be stored at 4°C. Do not vortex the Targefect-HUVEC reagent. The Peptide Enhancer can be stored at 4°C. The Virofect Enhancer should be stored at -20°C or -70°C.

TABLE 2

Tube #	High Glucose DMEM (Serum free)	DNA	Targefect HUVEC	Virofect Enhancer Reagent
1	1 ml	1 µg	2 µl	4 µl
2	0.6 ml	6 µg	12 µl	25 µl

Add DMEM first. Add DNA, mix well by flicking the tube about 12 times to create a vortexing action. Add Targefect next, mix well again by flicking the tube. Incubate the tubes at 37°C for 25 minutes to form the transfection complexes.

Condition 1 above is for transfecting endothelial cells with transfection complexes in the absence of serum or any HUVEC culture media. It is very important that you try this condition as it works very well for all types of endothelial cells. At the end of the 25 minute incubation period, aspirate off the culture medium covering the cells, and add 0.2 ml of transfection complex per well of a 24-well dish. Incubate 2 hours at 37°C. Aspirate off

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transfection complex and add 0.5 ml of complete media (with 10% serum. Incubate overnight, and replace with fresh medium the next day. Assay at 36-48 hours post transfection.

Condition 2 (complexes formed in tube 2) is for transfecting cells in the presence of serum according to our fast protocol.-250 µl of transfection complex is added to 1 ml of complete media (with serum) per well of a six-well dish. The dish is swirled to enable mixing of the transfection complex with the cell culture medium and the cells are incubated at 37°C overnight and assayed for gene expression 36-48 hours post transfection.
Add 0.5 ml of transfection complex to 2 ml of complete media with serum for one 60 mm dish. For transfecting cells in a 12-well dish add 0.125 ml transfection complex to 0.5 ml of complete media.
Swirl the dish to gently mix transfection complexes with the cell culture media. Incubate overnight. Replace media the next day. Assay at 24-48 hours after transfection.

Citations for transfection of DNA and transfection of siRNA into endothelial cells using Targefect-HUVEC components

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Protocol for siRNA delivery using the Targefect-HUVEC kit

(You can also use these protocols to deliver microRNA)

The Targefect-HUVEC kit contains three components , Targefect-HUVEC (same as Targefect-F2), Peptide Enhancer and Virofect. Please find below two protocols for siRNA delivery one using the peptide enhancer and the second using the Virofect enhancer, Where ever the protocol mentions Targefect-F-2 you should use the Targefect-HUVEC component of your kit

Set up cells to be transfected so that they are about 70% confluent at the time of the experiment.

Prepare transfection complexes as follows:

Use clear plastic tubes for complex formation. If kept frozen thaw the reagents and mix well before use (do not vortex)

Tube #	OptiMEM1	dsRNA	Targefect-HUVEC	Peptide Enhancer
1	1 ml	75 pmols	5 ul	15 ul
2	1 ml	200 pmols	5 ul	15 ul

Make aditions as follows: Add Optimem 1 first, then add dsRNA, mix well by flicking the tube about 12 times to create a vortexing action. Add Targefect-HUVEC next, mix well again by flicking the tube and then add the Peptide enhancer, mix well again. Incubate the tubes at 37^{o}C for 25 minutes to form the transfection complexes.

Wash cells to be transfected twice with Opitmem 1. Aspirate the second wash completely. Add 1 ml of the transfection mix for 1 well for a 6-well dish (or for a 35 mm dish). Prepare 250 ul of transfection complex per well of a 12-well dish,

150 ul per well of a 24-well dish or 50 ul if using a 96-well plate Incubate the transfection complexes with the cells at 37^{o}C for 2 hrs. Add Complete media with serum (2 ml for a 35 mmm dish or one well of a 6-well plate, 1ml/well for a 12-well dish and 0.5 ml/well for a 24-well dish. Incubate overnight. Replace the media with fresh complete media the next morning and assay at 24-72 hours post-transfection.

When using fluorescently labeled dsRNA we have observed very efficient delivery into several cell types at 24 hours post transfection.

For testing for silencing of transiently transfected genes we recommend transfecting the siRNA 12 hours following transient transfection of the gene of interest and assaying for silencing of the transiently transfected gene 24 hours after transfection of the siRNA.

For testing silencing of endogenous genes we recommend analysis 48-72 hrs after transfection of the siRNA.

Protocol for siRNA delivery using Targefect-HUVEC(Targefect-F2) plus virofect

This protocol has been standardized in HUVECs but can be applied to other cell types or suspension cells. Please note that the Targefect-HUVEC component of the Targefect-HUVEC kit is the same as Targefect-F2. Data on siRNA delivery using Targefect F-2 plus Virofect has been kindly provided by Dr Michael Potente, Department of Cardiology, University of Frankfurt, Germany

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1. HUVECs used were from from Cell Systems
2. The culture media is EBM with 10% FCS and supplements (Single quotes)
3. HUVEC should be 80-85% confluent at the time of transfection
4. Be default we use 60 mm dishes for cell culture, therefore, the following protocol is designed for 60 mm wells

- Add 500 ul DMEM (Optimem works as well) first
- Add 18 ul of the desired siRNA (20 uM) and mix
- Add 12 ul of Targefect- F2 and mix
- Immediately add 25 ul of Virofect and mix
- Incubate for 20 min at 37°C
- In the meantime replace the culture media with 2 ml of fresh EBM 10% & suppl.
- Add siRNA complexes dropwise to the cells
- Change culture media the next day and assay for gene expression silencing after 24, 48 and 72 hours

Very recent data suggest that you can actually reduce the siRNA amount by half without losing any silencing effect. As you can see from the attached western blot, the silencing efficiency seems to greatly depends on the target sequence of the siRNA and not so much on the transfection method per se as all three methods gave comparable results. In our hands HUVECs work very well for the siRNA approach and we routinely get silencing efficiencies about 80 to 90% using different target genes. Nevertheless, the Virofect protocol is very easy to handle, suitable and time saving.

Protocol modifications for Co-delivery of DNA and siRNA:

Modifications suggested
To 800 ul of supernatant media covering cells in one well of a 6-well dish add 4 ul of Virofect. Incubate cells for 2 hrs. Then add 200 ul of the Transfection complex containing siRNA- Targefect F2 prepared as follows

Preparation of transfection complex
to 200 ul of DMEM add siRNA to a concentration of 25 nM and 2 ul of Targefect F'2. Incubate 20-25 min at 37 ° C . Add the transfection complex to cells pretreated with Virofect and incubate overnight.

If transfecting DNA plus siRNA then to 200 ul of DMEM add 1 ug DNA plus siRNA (add enough to give a 25 nM concentration) . Incubate at 37 ° C for 25 min and add to cells pretreated with virofect as above.

We always recommend trying two different concentrations of siRNA - the concentration mentioned above and another which is 4 times higher.

Note: You can also co-transfect DNA and siRNA using the Targefect-F2 reagent in combination with the peptide enhancer If transfecting DNA plus siRNA then to prepare transfection complex mix 200 ul of DMEM add 1 ug DNA plus siRNA (add enough to give a 25 nM concentration) with 2 ul of Targefect -F2 and 4 ul of Peptide enhancer and follow instructions as in the standard protocol for siRNA delivery using the Targefect siRNA

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Please read the protocols accompanying the products carefully.
We recommend contacting tech support at 619 562 1518 for helpful tips before starting the experiment if you are a new user

The protocols on our website need updating please do not use them. Follow the protocols that are supplied with the product

Please use antibiotic free / serum-free media for complex formation. **Regarding media for growing cells please refer to the section on "Preparation of cells" in the transfection protocol. If using complete media from Lonza or other reduced serum media for maintaining endothelial cells it is important to supplement the media with 10% serum.** It is really important to try both protocol 1 (using the peptide enhancer) and protocol 2 (using Virofect), and to test both the conditions outlined for protocol 2 (red fonts) as in our hands this works the best. However, there does seem to be a lab to lab variation wherein one protocol works better than the other. This results partly from differences in the culture media being used. Most labs prefer the peptide enhancer protocol