N-TER/siRNA nanoparticle mediated knockdown of gene expression

HUVEC Cells

Recommended assay conditions

As with any biological system, assay conditions must be optimized by the end-user. However, using the following transfection conditions, we routinely see a decrease in GAPDH gene expression of 75-80%, with cell viability ranging from 70-100%.

Cell density = 1.6 x 10^4 to 3.1 x 10^4 cells/cm^2
[siRNA]final =10 to 20 nM

Materials

Cell culture

- HUVEC Umbilical Vein Endothelial Cells, Pooled (Cambrex, CC-2519)
- EGM™-2 Endothelial Cell Medium-2 BulletKit (Cambrex, CC-3162)
- ReagentPack Subculture Reagent Kit (Cambrex, CC-5034)
- Tissue culture treated 75 cm^2 flasks
- Tissue culture treated multi-well plates
- Sterile 50 mL conical tubes
- Bright-Line™ Hemacytometer (Sigma, Z359629)
- Single and/or multichannel pipettes
- Bench-top centrifuge

siRNA transfection

- Target siRNA(s)
- Negative (non-target) control siRNA
- N-TER™ Nanoparticle siRNA Transfection System (Sigma, N2913)
- Water, Molecular Biology Reagent (Sigma, W4502)
- Hanks' Balanced Salt Solution (Sigma, H6648) or Dulbecco's Phosphate Buffered Saline (Sigma, D5837)
- Sterile microcentrifuge tubes
- Single and/or multichannel pipettes
- Ultrasonic cleaner (sonicating water bath), with an operating frequency of 30-40 kHz
- Microcentrifuge

Gene expression assays

- RNA isolation and purification reagents (such as GenElute™ Mammalian Total RNA Miniprep Kit, Sigma, RTN10, RTN70, or RTN350).
- qPCR reaction components (such as SYBR® Green JumpStart™ Taq ReadyMix™, Sigma, S4438)

Steady-state protein levels

- Western Blot
  - Antibody against protein of interest (***www.sigmaaldrich.com/antibodyexplorer***)
  - Chemicals for antibody detection

Phenotypic assays

- Cell-based, enzymatic, or array-based assays can be used

HUVEC cell culture

Thawing the cells

1. Make complete HUVEC medium according to the manufacturer's instructions.
2. Add 15 mL of complete HUVEC medium to each of two tissue culture treated 75 cm^2 flasks.
3. Place the flasks into a 37 °C incubator with 5% CO_2 for at least 30 minutes to allow the medium to equilibrate.
4. Quickly thaw the cells in a 37 °C water bath.
5. Divide the cells evenly between the two flasks.
6. The following day, remove the medium from each flask and replace it with fresh pre-warmed complete HUVEC medium.
7. Repeat step 6 every 48 hours thereafter that until the cells are ~80% confluent.

NOTE: Each 75 cm2 flask of HUVEC cells should yield on average 2.0 x 106 cells when the culture is healthy and ~80% confluent. Depending on the assay design, more than one flask of cells may be needed.

Culturing and plating the cells

1. Allow trypsin, HEPES-BSS, TNS, and complete HUVEC medium to warm to room temperature.
2. Aspirate the medium from each flask.
3. Rinse each flask with 15 mL of HEPES-BSS, then aspirate the HEPES-BSS from each flask.
4. Add 6 mL of trypsin to each flask, and gently rock each flask to coat the cells with the trypsin.
5. Incubate the flask at room temperature until approximately 90% of the cells are rounded and detached. This takes approximately 1 to 2 minutes. Do not trypsinize HUVEC cells at 37 °C, as it will kill the cells.
6. Gently tap the side of the flask to cause the cells to detach.
7. Visually check with a microscope to make sure most of the cells are detached.
8. Add 12 mL of TNS to each flask, and gently rock the flasks to suspend the cells.
9. Transfer the cell suspension to a sterile 50 mL conical tube and centrifuge for 5 minutes at 220 x g.
10. Aspirate the majority of the medium without disturbing the pellet.
11. Gently resuspend the cells in 4 mL of complete HUVEC medium.
12. Count an aliquot of the cells with a hemacytometer.
13. Dilute the cells accordingly in complete HUVEC medium.
14. Seed the cells as indicated in Table 1.

NOTE: We recommend that you seed cells at a density of 1.6 x 104 to 3.1 x 104 cells/cm2 16 to 20 hours before siRNA transfections.

15. Using the remaining cells, seed new flasks at a density of 2.5 x 103 cells/cm^2.
16. Replace the medium in the flasks 24 hours after seeding and every 48 hours thereafter that until the cells are ~80% confluent.
17. When cells reach the correct confluence, start over with step 1.

For more information on cell maintenance or cell culture technique please consult chapter 12 of the ECACC Handbook.

Table 1: Suggested cell densities and volumes for seeding cells to culture plates

Plate size	Cells per well	Surface area per well (cm^2)†	Volume per well (mL)
96-well	5.0 x 103 - 1.0 x 104	0.32	0.1
48-well	1.5 x 104 – 3.0 x 104	0.95	0.3
24-well	3.0 x 104 – 5.9 x 104	1.90	0.6
12-well	5.9 x 104 – 1.2 x 105	3.80	1.2
6-well	1.5 x 105 – 3.0 x 105	9.50	3.0

† Surface areas listed above are for Corning® Costar® tissue culture treated multi-well plates with flat-bottom wells. The dimensions of other manufacturers' plates may vary.

HUVEC siRNA transfection

Scaling of the N-TER nanoparticle transfection assays

Before beginning the N-TER nanoparticle transfection assay, you will need to take a number of factors into consideration, including: the size of the culture plates that you will use, the range of siRNA concentrations that you wish to screen, and the number of replicates that you wish to perform. These factors will affect the amount of N-TER Peptide/nanoparticle complex (from this point on, referred to as Nanoparticle Formation Solution or NFS) that you need to prepare. Table 2 indicates the amount of NFS required for one well in a range of final siRNA concentrations and culture plate sizes.

First, use Table 2 to determine the amount of NFS required to transfect one well at the desired siRNA concentration(s). Then refer to Equation 1 below to calculate the amount of NFS (NFStotal) that you will need for your assay. Using this equation, you'll multiply the volume of NFS (from Table 2) by the number of wells that you wish to transfect. You'll then multiply that number by 1.2 to ensure that you have enough material for all of your replicates.

Equation 1: NFStotal = (volume of NFS/well) x (number of wells) x 1.2

Table 2: Volume of Nanoparticle Formation Solution (NFS) to add per well for a range of siRNA concentrations‡

	Volume of NFS per well (µL)				
[siRNA]final (nM)	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate
100	24.62	70.77	98.46	184.62	461.54
80	19.69	56.62	78.77	147.69	369.23
60	14.77	42.46	59.08	110.77	276.92
50	12.31	35.38	49.23	92.31	230.77
40	9.85	28.31	39.38	73.85	184.62
30	7.38	21.23	29.54	55.38	138.46
20	4.92	14.15	19.69	36.92	92.31
10	2.46	7.08	9.85	18.46	46.15
5	1.23	3.54	4.92	9.23	23.08

‡ The concentration of the siRNA in the Nanoparticle Formation Solution prior to dilution in culture medium is 650 nM.

Please note that the volumes of NFS in Table 2 are listed to the hundredth of a microliter. This is to aid in the accuracy of scaling calculations. Final calculations should be rounded to the nearest microliter.

Preparation of the Nanoparticle Formation Solutions

Unlike most lipid-based transfection reagents, the ratio of N-TER Peptide to target siRNA remains constant in the N-TER Nanoparticle siRNA Transfection System. When increasing the number of transfections or the volume of transfections, the volumes of reagents used in preparing the Nanoparticle Formation Solution should be scaled accordingly. To scale volumes of reagents to be used in nanoparticle synthesis, multiply the volume of NFS calculated in Equation 1 by each of the numbers in Table 3. These values can then be substituted for the values listed in Tables 4 and 5.

Table 3: Preparation of target siRNA and control dilutions

Reagent	siRNA	N-TER Peptide
5 µM siRNA working stock (µL)	0.13 x (NFStotal)	-
N-TER Peptide (µL)	-	0.08 x (NFStotal)
N-TER Buffer (µL)	0.37 x (NFStotal)	-
Water (µL)	-	0.42 x (NFStotal)
FINAL VOLUME (µL)	0.50 x (NFStotal)	0.50 x (NFStotal)

The protocol detailed below is for a standard 100 µL nanoparticle formation reaction. This provides sufficient NFS for the transfection of 20 wells of a 96-well plate, 7 wells of a 48-well plate, 5 wells of a 24-well plate, 2 wells of a 12-well plate, or 1 well of a 6-well plate at a final siRNA concentration of 20 nM.

1. Thaw the N-TER Peptide (Sigma, N2788) and 5 µM siRNA working stocks at room temperature for approximately 10 minutes. Briefly vortex each tube and pulse-spin in a microcentrifuge. Store the siRNA working stocks on ice until they are needed.
2. Incubate the thawed N-TER Peptide in a sonicating water bath at maximum output and continuous power for 3-5 minutes.

NOTE: Incubation of the N-TER Peptide in a sonicating water bath is optional. However, this step decreases possible aggregation of the peptide and can reduce the variability of transfection efficiency.

3. While the N-TER Peptide is in the sonicating water bath, prepare the target siRNA, negative control siRNA, and cells only control in sterile tubes (see Table 4). Add the 5 µM target and negative control siRNA working stocks to Tubes 1A and 2A, respectively. Then, add the appropriate amount of N-TER Buffer (Sigma, N0413) to Tubes 1A through 3A. Briefly vortex each tube and pulse-spin in a microcentrifuge. Store the diluted target and negative control siRNAs and cells only control on ice until they are needed.

Table 4: Preparation of target siRNA and control dilutions

Reagent	Tube 1A TargetsiRNA	Tube 2A Neg. control siRNA	Tube 3A Cells only control
5 mM target siRNA working stock (µL)	13	0	0
5 mM negative control siRNA working stock (µL)	0	13	0
N-TER Buffer (µL)	37	37	50
FINAL VOLUME (µL)	50	50	50

4. Prepare the N-TER Peptide dilutions in sterile tubes (see Table 5). Add the N-TER Peptide to Tubes 1B and 2B. Then, add the appropriate amount of water to Tubes 1B through 3B. Briefly vortex each tube and pulse-spin in a microcentrifuge. Incubate the diluted N-TER Peptide in a sonicating water bath at maximum output and continuous power for 3-5 minutes.

Table 5: Preparation of N-TER Peptide

Reagent	Tube 1B TargetsiRNA	Tube 2B Neg. control siRNA	Tube 3B Cells only control
N-TER Peptide (µL)	8	8	0
Water (µL)	42	42	50
FINAL VOLUME (µL)	50	50	50

5. Repeat step 5 with Tubes 2 and 3 for the negative control siRNA NFS and the cells only control, respectively.

Table 6: Preparation of target and control Nanoparticle Formation Solutions

Reagent	Tube 1 TargetsiRNA	Tube 2 Neg. control siRNA	Tube 3 Cells only control
Tube A (µL)	50	50	50
Tube B (µL)	50	50	50
FINAL VOLUME (µL)	100	100	100

6. Incubate Tubes 1, 2, and 3 at 37 °C for 30-45 minutes to allow the N-TER Peptide/siRNA nanoparticles to form.

NOTE: The concentration of the siRNA in the Nanoparticle Formation Solution is 650 nM at this point.

Dilution of the Nanoparticle Formation Solutions

The NFS must be diluted to the appropriate volume in 0.5´ N-TER Buffer before it is added to the culture plate to ensure that you have enough liquid to cover the bottom of each well. This volume will vary depending on the number of wells in the culture plate.

NOTE: 0.5´ N-TER Buffer can be prepared by mixing water and N-TER Buffer in a 1:1 ratio.

Use the appropriate equation in Table 7 to calculate the volume of 0.5´ N-TER Buffer needed to dilute the NFS for one well of the culture plate that you are using. Refer to Table 2 to find the volume of NFS that is required for the desired plate size and [siRNA]final.

Table 7: Determination of 0.5´ N-TER Buffer volume (BV) needed to dilute NFS

Plate size	Equation for calculating 5´ N-TER Buffer volume
96-well plate	30 – NFS
48-well plate	80 – NFS
24-well plate	120 – NFS
12-well plate	200 – NFS
6-well plate	350 – NFS

1. Add the appropriate volume of target siRNA NFS (as indicated in Table 2) to a new tube. To dilute, add the volume of 0.5´ N-TER Buffer calculate using Table 7. Briefly vortex each tube and pulse-spin in a microcentrifuge.
2. Repeat step 1 with the negative control siRNA NFS and cells only control.

Transfection of the HUVEC cells with the Nanoparticle Formation Solutions

1. Carefully remove the complete HUVEC medium from each well. Then wash each well with Hank's Balanced Salt Solution or Dulbecco's Phosphate Buffered Saline. Be careful to avoid disturbing the cell layer on the bottoms of the wells.
2. Transfer the appropriate volume of the diluted target NFS to the culture plate(s), and gently rock the plate(s) to evenly distribute the liquid over the surface of the cells. Incubate at room temperature for 3-5 minutes.
3. Repeat steps 1-2 with the diluted negative control NFS and cells only control.
4. Carefully add the appropriate amount of serum-free HUVEC medium to each well as indicated in Table 8. Incubate the plate(s) under standard cell culture conditions, typically 37 °C and 5% CO_2, for 2-4 hours.
5. Add the appropriate amount of complete medium to each well as indicated in Table 8.
6. Incubate the plates under standard cell culture conditions, typically 37 °C and 5% CO_2, for 24-48 hours.

To calculate the volumes required to transfect multiple wells, multiply each volume in Table 8 by the number of wells that you wish to transfect. Then multiply that number by 1.2 to ensure that you have enough material for all of your replicates.

NOTE: Table A1 in the Appendix shows the volumes of NFS, 0.5´ N-TER Buffer, and complete HUVEC medium required to transfect three wells in a range of culture plate sizes.

Table 8: Suggested volumes of NFS and medium for the transfection of cells in culture plates

	Volume per well (µL)				
Reagent (µL)	**96-well plate**	**48-well plate**	**24-well plate**	**12-well plate**	**6-well plate**
NFSdilute	30	80	120	200	350
NFS	NFS	NFS	NFS	NFS	NFS
0.5´ N-TER Buffer	30 - NFS	80 - NFS	120 - NFS	200 - NFS	350 - NFS
Serum-free HUVEC medium	30	80	120	200	350
Complete HUVEC medium	100	300	400	800	2300
Final Volume	**160**	**460**	**640**	**1200**	**3000**

Appendix

Reagent volumes required to transfect three wells at suggested siRNA concentrations

The volumes of NFS, 0.5´ N-TER Buffer, and complete HUVEC medium listed in Table A1 provide enough material to transfect three wells in a range of culture plate sizes.

Table A1: Volume of Nanoparticle Formation Solution (NFS) to add for three wells at an [siRNA]final of 10 or 20 nM

Reagent (µL)	**96-well plate**		**48-well plate**		**24-well plate**		**12-well plate**		**6-well plate**	
	10 nM	**20 nM**	**10 nM**	**20 nM**	**10 nM**	**20 nM**	**10 nM**	**20 nM**	**10 nM**	**20 nM**
NFSdilute	108	108	288	288	432	432	720	720	1260	1260
NFS	9	18	25	51	35	71	66	133	166	332
0.5´ N-TER Buffer	99	90	263	237	397	361	654	587	1094	928
Serum-free HUVEC medium	108	108	288	288	432	432	720	720	1260	1260
Complete HUVEC medium	360	360	1080	1080	1440	1440	2880	2880	8280	8280
Final Volume	576	576	1656	1656	2304	2304	4320	4320	10800	10800

070650
0128