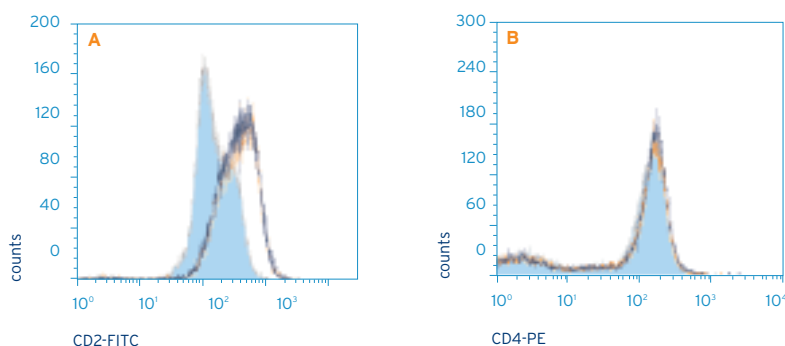


## Establishing siRNA assays in primary human peripheral blood lymphocytes

This protocol is designed to establish siRNA assays in primary human peripheral blood lymphocytes using the Nucleofector® technology. It is suitable for the following cell types:

- Cell types**
- › human T cells
  - › human B cells
  - › human CD34<sup>+</sup> haematopoietic progenitor cells
  - › human DCs
  - › human NK cells



### High specificity of CD2 siRNA in primary human T cells

Primary human T cells were transfected with 1.4 µg siRNA targeted to CD2 (QIAGEN®) using the Human T Cell Nucleofector Kit. 44 hours after delivery cells were stained with anti CD2-FITC antibody (A) or anti CD4-PE antibody (B) and analyzed by flow cytometry. Controls included nucleofection of 1.4 µg control siRNA and nucleofection® without siRNA.

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## Protocol Outline

### Establishing siRNA in human peripheral blood lymphocytes by:

#### Down-regulation of CD2

Suitable for cell types:

- › Human T Cells.
- › Human NK Cells.

#### Down-regulation of CD4

Suitable for cell types:

- › Human CD4<sup>+</sup> T Cells.

#### Down-regulation of Vimentin

Suitable for cell types:

- › Human T Cells.
- › Human B Cells.
- › Human CD34<sup>+</sup> hematopoietic progenitor cells.
- › Human DCs.
- › Human NK Cells.



Choose appropriate Nucleofector Kit for your cell type of interest.



Combine:

- › 100 µl Nucleofector Solution
  - › 2 - 5 x 10<sup>6</sup> cells
  - › 1.5 µg siRNA duplex
- ...and apply optimal Nucleofector program.



Analyze CD2 down-regulation by FACS 24h and 48h after nucleofection.

Analyze CD4 down-regulation by FACS 48h after nucleofection.

Analyze vimentin down-regulation by quantitative RT-PCR after 24h

**2****› Required reagents****2.1****› Positive control siRNA duplexes**

For siRNA applications using the Nucleofector technology we highly recommend the use of QIAGEN siRNA duplexes. QIAGEN® has a leading position in siRNA synthesis technologies and provides custom designed siRNA duplexes as well as a large library of pre-developed siRNA molecules directed against common target genes. For detailed information about QIAGEN's siRNA services, please refer to their website [www.qiagen.com/siRNA](http://www.qiagen.com/siRNA)

<b>CD2 siRNA</b>	Cat.no.	customized
	sense sequence:	GGUGCAGUCUCCAAAGAGAdTdT
	antisense sequence:	UCUCUUUGGAGACUGCACcTdT
	target sequence:	AAGGTGCAGTCTCCAAAGAGA
<b>CD4 siRNA</b>	Cat.no.:	1024675
	sense sequence:	GAUCAAGAGACUCCUCAGUdGdA
	antisense sequence:	ACUGAGGAGUCUCUUGAUcTdG
	target sequence:	CAGATCAAGAGACTCCTCAGT
<b>vimentin siRNA</b>	Cat.no.:	1022055
	sense sequence:	GAAUGGUACAAAUCCAAGUtt
	antisense sequence:	ACUUGGAUUUGUACCAUUCTt
	target sequence:	AAGAATGGTACAAATCCAAGT

**2.2****› Antibodies for FACS analysis of CD2 or CD4 down-regulation**

**anti CD2-** Becton Dickinson Pharmingen, Cat.No. 555347, use as recommended by the FITC supplier or titrate optimal dilution.

**anti CD4-PE** Becton Dickinson Pharmingen, Cat.No. 555347, use as recommended by the supplier or titrate optimal dilution.

**2.3**› **Reagents for quantitative vimentin RT-PCR****Primer 1** 5' - ACC AGG TCC GTG TCC TCG T - 3'**Primer 2** 5' - CTG CCC AGG CTG TAG GTG - 3'**PCR product** 119 bp**Isolation of total RNA** For isolation of total RNA, we recommend the usage of "High Pure Isolation Kit" (Roche Cat.No.1828665).**First Strand cDNA synthesis** For the synthesis of cDNA from RNA by reverse transcription, we recommend the usage of "First Strand cDNA synthesis kit for RT-PCR (AMV)" (Roche Cat.No.1483188).**3**› **Cell preparation and culture**

For detailed recommendations about cell isolation, purification and culture prior to nucleofection, please refer to the Optimized Protocol for the appropriate cell type.

**It is strongly recommended that purified cell populations are used, especially if your analysis method is RT-PCR**, where the choice to discriminate between cell populations is not possible.**4**› **Important controls**

Besides the sample with your siRNA duplex of choice we recommend that you perform two control samples as follows:

**negative control** Nucleofection of the recommended amount of cells in Nucleofector Solution with a scrambled or unspecific siRNA duplex.**Mock control (reference)** Nucleofection of the recommended amount of cells in Nucleofector Solution without an siRNA duplex. This serves as your reference sample to quantify down-regulation by the specific siRNA duplex.

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› **Nucleofection protocol**

**Preparation of Nucleofector™ Solution**

Add 0.5 ml Supplement to 2.25 ml Nucleofector Solution and mix gently. The Nucleofector Solution is now ready to use and is stable for 3 months at 4°C.

**Note the date of addition on the vial.**

**One nucleofection sample contains**

› **Always work in an RNase-free environment**

› **2 - 5 x 10<sup>6</sup> cells**

› **1.5 µg siRNA**

› **100 µl Nucleofector Solution**

For more details about the nucleofection of siRNA:

[www.amaxa.com/RNAi](http://www.amaxa.com/RNAi)

**Preparation of samples**

1. Cultivate the required number of cells.
2. Prepare **1.5 µg** siRNA for each sample.
3. Pre-warm the supplemented Nucleofector Solution recommended by amaxa to room temperature. Pre-warm an aliquot of culture medium containing serum and supplements at 37°C in a 50 ml tube (100 µl per sample).
4. Prepare 12-well plates by filling the appropriate number of wells with 1.5 ml of culture medium containing serum and supplements and pre-incubate plates in a humidified 37°C/5% CO<sub>2</sub> incubator.
5. Take an aliquot of cell culture and count the cells to determine the cell density.
6. Centrifuge the required number of cells (**2 - 5x10<sup>6</sup> cells** per nucleofection sample) at **200xg for 10 min**. Discard supernatant completely so that no residual medium covers the cell pellet.
7. Resuspend the pellet in room temperature Nucleofector Solution to a final concentration of **2 - 5x10<sup>6</sup> cells/100 µl**. Avoid storing the cell suspension longer than 15-20 min in Nucleofector Solution, as this reduces cell viability and gene transfer efficiency.

**Important: Steps 8-12 should be performed for each sample separately.**

8. Mix 100 µl of cell suspension with **1.5 µg siRNA**.
9. Transfer the sample into an amaxa certified cuvette. Make sure that the sample covers the bottom of the cuvette, avoid air bubbles while pipetting. Close the cuvette with the blue cap.
10. Select the appropriate Nucleofector program (see Nucleofector Manual for details). Insert the cuvette into the cuvette holder (Nucleofector I : rotate the carousel clockwise to the final position) and press the "X" button to start the program.



**Nucleofection**

**Cultivation post  
Nucleofection**

11. **To avoid damage to the cells remove the sample from the cuvette immediately after the program has finished** (display showing "OK"). Take the cuvette out of the holder. To transfer the cells from the cuvettes, we strongly recommend using the plastic pipettes provided in the kit to prevent damage and loss of cells. Add 500 µl of the pre-warmed culture medium containing serum and supplements to the cuvette and transfer the sample into the prepared 12-well plates. Alternatively, transfer the sample into a 1.5 ml microcentrifuge tube and place it in a 37°C heat block.
12. Press the "X" button to reset the Nucleofector.
13. Repeat steps 8-12 for the remaining samples.
14. If you have incubated the samples in 1.5 ml microcentrifuge tubes transfer them into the prepared 12-well plates.
15. Incubate cells in a humidified 37°C/5% CO<sub>2</sub> incubator. Perform flow cytometric analysis after 24h and 48h, RT-PCR after 24h.

**6**› **Analysis methods****6.1**› Analysis of CD2 or CD4 down-regulation by flow cytometry

The easiest method for quantitative analysis of CD2 or CD4 down-regulation is flow cytometry. For the first experiment, we recommend performing the flow cytometric analysis **24h** (for CD2) and better at **48h** (for CD4) after nucleofection.

1. In each well, re-suspend cells and transfer 600 µl cell suspension into a 1.5 ml reaction tube.
2. Centrifuge cells at 90xg, 4°C for 10 min and discard the supernatant.
3. Resuspend the cell pellet in 100 µl of PBS/0.5% BSA and add a fluorescent dye-conjugated antibody directed against the surface marker you are using (see chapter 2.2).
4. Incubate for 10 min on ice in the dark.
5. Add 1 ml PBS/0.5% BSA.
6. Centrifuge cells at 90xg, 4°C for 10 mins and discard the supernatant.
7. If you need to stain with a secondary antibody, repeat steps 3 - 7.

8. Re-suspend the pellet in 400 µl PBS/0.5 BSA.
9. Add 1 µl propidium iodide (10 µg/ml) to stain dead cells.
10. Analyze cells by flow cytometry: Gene silencing is often easier to detect by monitoring the mean fluorescent intensity (i.e. average expression level per single cell) instead of exclusively consulting the total number of positive cells.

## 6.2 › Analysis of vimentin down-regulation by Quantitative RT-PCR

A common method to validate efficient gene knock-down is quantitative RT-PCR. Before you perform the first siRNA experiment, establish the optimal PCR conditions according to your PCR equipment supplier. We recommend the usage of the kits and primers given in chapter 2.3. If you are working with the Light Cycler (Roche) please contact our Scientific Support team for further details. For the first experiment we recommend performing the quantitative RT-PCR analysis 24h after nucleofection.

## 6.3 › Analysis of vimentin down regulation by Western Blot

An alternative method of analyzing efficient gene knock-down is by Western Blot. Before you perform the first siRNA experiment, establish the optimal blotting conditions according to your equipment supplier. We recommend the usage of the antibodies given in chapter 2.4. For the first siRNA experiment, we recommend performing the Western Blot analysis, **24h** and **48h** after nucleofection.

\* amaxa's Nucleofector® Process, Nucleofector® Device and Nucleofector® Solutions are covered by PCT applications PCT/EP01/07348, PCT/DE02/01489, PCT/DE02/01483 and other patents in addition to domestic or foreign applications corresponding thereto.

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