siRNA-MEDIATED GENE SILENCING

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RNA-interference enjoys a wide range of applications in medical and biological research. In particular, it is used to study functions of genes. One of the most popular approaches to this task is gene knockdown by small interfering RNA (siRNA). Currently there is no unified protocol for this method, which results in low reproducibility of experimental data. In the following article we outline the theoretical bases for this method and provide practical recommendations for its use in siRNA-mediated gene silencing experiments.

Keywords: RNA-interference, knockdown, gene expression

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НОКДАУН ГЕНОВ С ИСПОЛЬЗОВАНИЕМ МАЛЫХ ИНТЕРФЕРИРУЮЩИХ РНК

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РНК-интерференция активно используется для решения различных исследовательских задач в биологии и медицине, в частности, для исследования функции генов. Один из распространенных подходов — нокдаун генов с помощью малых интерферирующих РНК (siРНК). Однако единого протокола для этого метода не существует, и данные, получаемые при использовании различных его вариаций, часто обладают низкой воспроизводимостью. В работе обсуждаются теоретические основы указанного метода и приводятся практические рекомендации для постановки экспериментов по нокдауну генов с siРНК.

Ключевые слова: РНК-интерференция, нокдаун, экспрессия генов

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The human genome contains 19,817 protein-coding genes and 15,787 long noncoding RNA genes [1]. About 40 % of protein-coding genes have not yet been assigned a function. Long noncoding RNAs are terribly understudied: only about a hundred of them have been investigated experimentally so far [2–5]. The simplest and most efficient way to study gene functions is to perform overexpression and knockdown experiments exploring their effects at the molecular and cellular levels.

Methods for gene overexpression were elaborated by the advances in gene engineering and molecular cloning. There are a lot of different techniques aimed to enhance gene expression, as simple (based on the use of plasmid expression vectors) and sophisticated (employing inducible systems, viral vectors, etc.) [6]. Gene silencing methods were developed later. The first silencing tool was based on the use of antisense oligonucleotides [7, 8], which were not effective at first. But discovery of RNA interference revolutionized gene silencing methods. RNA interference is gene-specific mechanism of posttranscriptional gene silencing mediated by small RNA molecules, the so-called endogenous microRNAs (miRNAs) and exogenous small interfering RNAs (siRNAs) [9]. SiRNAs have proved to be efficient and easy instrument to use. So that within a short period of time siRNA-based gene knockdown has put in practice in fundamental research, where it is used to study genes function, and applied research including development of novel gene-specific drugs [10–14].

There are two strategies for RNA interference-based gene knockdown nowadays: the use of siRNAs and hairpin vectors (short hairpin RNAs, shRNAs) [15].

Small interfering RNA is a 20 to 25 nt long double-stranded molecule with two unpaired nucleotides at the 3'-end of each strand. Inside the cell, it is incorporated into the RNA-induced silencing complex (RISC); after that one of the siRNA strands called a passenger strand is decayed and removed from the complex. The remaining guide strand binds complementary to its RNA target. If it is fully complementary to the target, the latter is cleaved causing mRNA degradation and reducing gene expression [16–18]. Small hairpin RNAs are short RNA molecules forming a hairpin-like structure. The length of their stem varies from 19 to 22 bp while the loop contains 4 to 11 nucleotides. ShRNA is a siRNA precursor. ShRNA sequences are delivered into the cell encoded into a bacterial or viral vector.

ShRNA have a few advantages over siRNA: its effect on the target gene expression is more continuous; shRNA can be integrated into the genome for stable heritable shRNA expression; it can be used to create inducible knockdown systems; it can be expressed simultaneously with the reporter gene to control transfection efficiency and detect successfully transfected cells. However, shRNA-based techniques are very labor-consuming. Therefore, for short-term gene silencing (5–7 days) [19] siRNA are recommended.

Knockdown by siRNA is a complex process that takes about a week. There is no standard protocol for gene knockdown: the way how it is performed varies considerably across laboratories. Some researchers do not even care to describe the technique they have used [20–22], which renders results of their experiments hardly reproducible. Knockdown efficiency can be considerably affected by inaccurate work at each step of the process. For example, authors rarely mention efficiency for siRNA transfection, although this factor is crucial for successful knockdown and its subsequent effects. Success of the experiment is largely determined by the number of transfected cells, initial expression levels of the target gene, and techniques used for the very basic steps, such as RNA isolation, reverse transcription, real-time polymerase chain reaction (PCR) and others.

In this article we propose a detailed plan for a siRNA-based gene knockdown experiment (Fig. 1). We discuss problems that may arise at each step and detailed solutions for them. We also describe the knockdown method used in our Laboratory of Functional Genomics of the Research Center of Medical Genetics (Moscow). Controls are provided for each step of the experiment to ensure that all techniques are performed correctly. We hope that both students and research scientists will rely on this plan while preparing to perform knockdown of previously unstudied genes and that their experiments will yield reliable results ready to be published in high-impact academic journals.

I. Design of the experiment

1. Identifying the problem

Before attempting knockdown of the gene of interest in a cell line, it is advisable to analyze the literature and publicly available online data to pick an appropriate biological model for the experiment and identify the nucleotide sequence of the studied gene.

The appropriate biological model. It is important to do a little research on the expression of the target gene in the chosen cell line. A wealth of information is available in specialized databases, such as FANTOM5, GTExPortal, BioGPS, and Human Protein Atlas; RNA-seq data can be accessed using the genome browser UCSC. It is advisable to use cell lines with sufficiently high expression of the studied gene, because knockdown of inherently poorly expressed genes may not produce any tangible effect. Besides, prior to the actual experiment, expression of the studied gene should be measured experimentally in the chosen cell line using a real-time PCR assay.

Analysis of gene sequences. siRNA is supposed to interact with a unique gene region. Therefore, before designing an siRNA molecule, it is important to estimate how suitable is the mRNA sequence of the analyzed gene for targeted knockdown. For this purpose BLAST, genome browsers (such as USCS) and other tools for nucleotide sequence analysis are used. They help to detect pseudogenes, highly homologous paralogs, repeats in the studied sequence and isoforms of the gene of interest.

2. siRNA design

Currently, siRNA design comes down to selecting an optimal binding site on a target transcript corresponding to the sense strand of siRNA. The antisense strand is complementary to the

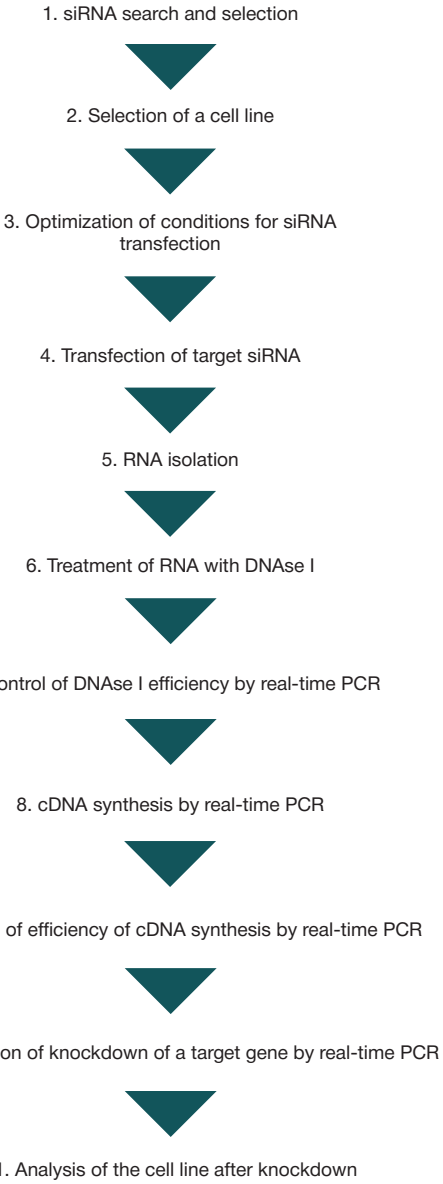

Fig. 1. Knockdown experiment: the plan

sense strand and forms a stable bond with RISC. There is no unified algorithm for selecting a target-effective siRNA sequence [23, 24]. Online search tools rely on different algorithms (empirical rules, BLAST data, neuronal networks) and therefore may return different results for one and the same input [16, 23, 25]. After the sequence of the studied gene has been analyzed by the software, a list of siRNAs is generated from which the user is free to select 3 or 4 most suitable sequences. Basic rules of siRNA design are provided below.

1. Sequence length should be limited to 20–25 nucleotides (normally the sequence is 21 to 23 nt long).
2. G/C content should be 35–55 %.
3. The 5′-end of the antisense siRNA strand should contain more A/U-nucleotides, because a strand with a less thermodynamically stable 5′-end binds to RISC more effectively.
4. An unpaired 3′ dTdT overhang on a siRNA strand enhances stability of the duplex and facilitates siRNA loading into RISC.
5. siRNA must be gene-specific. It means that no transcripts of other genes are expected to be detected by BLAST, fully complementary to the selected siRNA.
6. siRNA complementarity to other transcripts must be limited to a 16-nt long sequence.
7. siRNA must downregulate expression of all isoforms of the target transcript, unless intended otherwise.
8. siRNA should not contain repeated nucleotide motifs and sequences of >3 identical nucleotides.
9. There are a few empirical rules aimed at improving siRNA efficiency, one of them related to the positions of nucleotides, exemplified by Table 1. Further details are available in the article by Lagana at al. [26]. Software tools for siRNA design are listed in Table 2.

3. Controls

Efficiency of siRNA delivery into the cell is monitored using fluorescently labeled siRNA molecules. We prefer 5'-end -FAM labeled siRNA oligos (siFlu).

Negative control of knockdown specificity and off-target effects is normally a non-target siRNA that has no effect on gene expression. Many manufacturers offer ready-to-use non-target siRNAs (such as Negative control siRNA by Qiagen, Germany, or Silencer Negative Control by Invitrogen, USA). Another option is scrambled siRNA. It is composed of the same nucleotides as the target RNA arranged into a different sequence. Inconveniently, the use of scrambled siRNA requires preparation and validation of a new control for each knockdown experiment, proved to have no off-target effects. This may be quite labor-consuming if more than one experiment is planned. In our laboratory we use nonspecific siRNA controls, namely siMax siRNAs, designed from the sequence obtained from a publicly available source [38].

Positive control is what makes you certain of the accuracy of your experiment. Positive controls are siRNA molecules that effectively silence easily detectable genes, such as p53, GAPDH, or lamin-coding genes (the achieved knockdown levels are >70 %). Their sequences can be found in the literature or purchased from commercial sources.

Quality control should be performed at all stages of the experiment, including RNA isolation, treatment of RNA with DNAse 1, synthesis of cDNA, real-time PCR assays. These controls will be discussed further below.

II. siRNA delivery in to the cell

The most common methods of siRNA delivery into the cell are chemical transfection and electroporation. Chemical transfection can be performed using a variety of different techniques, lipofection being the most popular. Lipofection is transfection by cationic liposomes [11, 39–41]. Among its advantages are high reproducibility, simplicity and high efficiency. Lipofection is, however, almost ineffective for non-dividing cells, therefore slowly dividing or primary cells that are considered hard to transfect might call for a different delivery method, such as electroporation. The latter may be less beneficial, though, because of substantial cell death and a

Table 1. Rules for nucleotides in siRNA sequence. AS is the antisense strand, S is the sense strand. Grey cells represent positions of siRNA nucleotides (Lagana et al., [26])

AS	3'	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	5'
	G/T			G/C no A	C no A no G	no A			U no G	U no G		U	A			U no C				no C	U	A/U	
		G/C no U		A no C		no C	A/U	no C	U no G		A/U no C	no G		G/C no G	no G		A	no G		A/U no G no C		no C	G/T
S	5'	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	3'

Table 2. The most popular software tools used for siRNA design

Software name	URL	Reference
OptiRNAi 2.0	http://rnai.nci.nih.gov	[27]
siDirect 2	http://sidirect2.rnai.jp	[28]
siRNA Scales	http://gesteland.genetics.utah.edu/siPHK_scales	[29]
siExplorer	http://rna.chem.t.u-tokyo.ac.jp/cgi/siexplorer.htm	[30]
RFRCDB-siRNA	http://www.bioinf.seu.edu.cn/siPHK/index.htm	[31]
OligoWalk	http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi	[32]
Sfold	http://sfold.wadsworth.org	[33]
DSIR	http://biodev.cea.fr/DSIR/	[34]
siRNA Scan	http://bioinfo2.noble.org/RNAiScan.htm	[35]
RNAxs	http://rna.tbi.univie.ac.at/cgi-bin/RNAxs/RNAxs.cgi	[36]
i-Score	http://www.med.nagoya-u.ac.jp/neurogenetics/i_Score/i_score.html	[37]

large number of parameters that may want optimization. In the light of the above, lipofection is preferable in the experiments on readily transfectable cells. There are a lot of commercial reagent kits for transfection available on the market, such as Lipofectamine (Invitrogen) and Metafectene, which we used in this work (Biontex, Germany).

The mechanism of lipofection can be briefly described as follows. Lipids mixture assembles in liposomes or micelles with an overall positive charge at physiological pH and are able to form complexes (lipoplexes) with negatively charged nucleic acids. When the resulting solution is mixed with the cells, liposomes attach to and fuse with the cell membrane releasing siRNA into the cell, where it eventually interacts with mRNA in the cytoplasm.

1. Optimization of siRNA-based lipofection

Success of lipofection is determined by multiple parameters that may vary depending on the cell line. It is therefore critical to optimize conditions for lipofection before the actual knockdown experiment in order to facilitate siRNA delivery into the cells. Lipofection can be performed in different plates and dishes (6-, 12-, 24-, or 96-well plates; 6- or 10 cm dishes) depending on the goal of the experiment. We tend to use 96-well plates; therefore, we are going to show how to optimize lipofection parameters for this dish format. To find information on other dish formats, please refer to the protocol for Metafectene-based transfection [42]. In general, the following parameters need to be optimized regardless of the dish format:

1. siRNA/liposome ratio (μg/μl) (expected to range from 1 : 1 to 1 : 8).

2. The absolute amount of transfected complexes (siRNA + liposomes). For a 96-well plate siRNA amount may vary from 0.04 to 0.3 μg, the amount of liposomes may vary from 0.2 to 4 μl.

3. The number of cells. Transfection should be performed once the cells have entered a stage of logarithmical growth. Optimal confluency for transfection is 30–60 % [42]. The number of cells used may vary depending on the cell type or size. For a 96-well plate the number of cells may vary from 5×10^3 to 60×10^3.

Other parameters may also influence the efficiency of the procedure, such as: 1) general health of cells at the time of transfection (cells must be healthy and actively dividing); 2) the presence of serum supplements in the medium (for most cultures, transfection is efficient with 10 % serum content); 3) the duration of incubation with the transfection complex (usually 3–6 hours but can be increased up to 72 hours); 4) the use of a transfecting solution within an hour after seeding can make transfection more efficient.

Lipofection efficiency is estimated using FAM-labeled siRNAs (siFlu), varying the parameters described above. Control experiments with liposomes and without siRNA are a must. Some cells should be left untreated to estimate toxicity of transfecting reagents and transfection efficiency. The latter is evaluated 24 hours after transfection by calculating the proportion of fluorescently labeled cells to the total number of cells. Toxicity of transfecting reagents is also evaluated by comparing the number of cells that have survived transfection to the number of untreated controls.

Table 3 explains how to optimize conditions for transfection in a 96-well plate. A detailed transfection protocol is described in Section 3 (*Lipofection protocol*).

After transfection conditions have been optimized, their suitability for knockdown should be assessed. Transfection efficiency gives a rough idea of whether post-knockdown changes in target gene expression can be detected by real time PCR (Fig. 2). For example, if transfection efficiency is 75 % and a 40 % knockdown is expected, then ΔΔCt for the target gene will be <0.5, meaning that real time PCR assays will be very unlikely to detect post-knockdown changes. If a 90 % knockdown is expected, then ΔΔCt may be as high as 1.5, which can be detected by real-time PCR.

2. Transfection with siRNA

After transfection conditions have been optimized, the transfection with siRNA for target gene can be performed. The experiment should be carried out in 5 to 7 biological replicates to ensure accurate statistical processing. The experiment should include the following transfections:

- with fluorescently labeled siRNA (siFlu) to determine transfection efficiency;
- with gene-nonspecific siRNA (sicontrol);
- with siRNA targeting the gene of interest;
- without siRNA (untreated control).

In this work transfected cells were incubated for 120 hours to evaluate the effect of knockdown at the cellular level.

3. Lipofection protocol

Below we provide a protocol for HEK293 transfection using the Metafectene reagent [42].

Reagents:

1. complete growth medium for the chosen cell line (depending on the cell line),
2. phosphate buffered saline (PBS),
3. Metafectene,
4. siRNA solutions,
5. cell culture.

Equipment:

1. Goryaev chamber or fluorescence-based flow cytometer,
2. laminar flow cabinet for eukaryotic cells,
3. CO_2 incubator.

The protocol:

1. Calculate the amounts of reagents required for all planned transfections (Table 4).

2. Prepare the cells: take a small aliquot of cells to count their number, then adjust plating density per well. For example, you will need to prepare 4 ml of 67×10^3 cells/ml solution for 25 wells of a 96-well plate and subsequently add 150 μl of the solution into each well.

3. Prepare the reagents: thaw siRNA, prepare metafectene and the tubes.

4. Prepare solutions A and B as shown in Table 4. Note that solution A can be mixed by vigorous pipetting, while solution B can be pipetted only once.

5. Combine solutions A and B carefully, pipet once to avoid degradation of liposomes. Incubate at room temperature for 15 minutes.

6. Seed the cells into the wells. You will need to reserve a few wells for metafectene and a control well for untreated cells (viability control).

7. Add the A+B mix dropwise into the appropriate wells. Mix the solution by moving the plate gently. Incubate for 6 hours at 37 °C in 5 % CO_2.

8. After 6 hours, visually inspect the cells under the microscope. If adherent cells have attached to the surface, carefully replace the medium (metafectene can be toxic for cells). Incubate for 18 hours at 37 °C in 5 % CO_2.

9. Twenty-four hours after transfection wash control cells with PBS, trypsinize, neutralize trypsin with serum, centrifuge, resuspend and count the cells using the flow cytometer to evaluate transfection efficiency and toxicity of the reagents.

III. Evaluating knockdown efficiency

1. Isolation of total RNA from a cell culture

The next step includes preparation of lysates from transfected cells and RNA isolation. The importance of this step should not be underestimated. There are a lot of various methods for RNA isolation. Among the oldest ones is isolation in CsCl gradient. Although it ensures highly reliable results, it is difficult, time-consuming and very expensive and therefore is rarely used. In contrast, silica sorbents are very popular, because they are easy to use and ensure faster RNA extraction. Reagents for silica-based absorption are plentiful, but relatively expensive and do not guarantee the best extraction quality. Another option is phenol-chloroform extraction. It is the cheapest, fastest and most reliable method. A commercial reagent for this method is called TRIzol [43]. In our lab we prefer classic phenol chloroform extraction [44, 45].

Extracted RNA can be contaminated by environmental RNAse. Therefore, RNA extraction must be performed in a clean space using RNAse-free reagents.

Cells are lysed using the guanidine thiocyanate buffer. Guanidine thiocyanate enters the cell easilyand inactivates RNAses. It is important to keep transfected cells cold before lysis. Freshly prepared lysates must be stored on ice to prevent RNA degradation. After removing debris from the samples, lysates should be divided into two portions, one of which can be stored at –70 °C for up to 6 months. The other will be used for RNA extraction. If anything goes wrong at some stage of the experiment, the frozen lysates will always come in handy.

During phenol-chloroform extraction phenol and aqueous phases are separated. Nucleic acids remain in the upper (aqueous) phase. Some of the proteins migrate to the phenol phase, while the rest sit at the interface. DNA and RNA are separated by acid phenol (pH 4.4) which retains RNA in the upper phase (RNA is stable in acidic pH) and prompts DNA and proteins to migrate to the interface.

Reagents:

1. acid phenol saturated with citrate buffer (pH 4.4),
2. chloroform, 96 % and 70 % ethanol,
3. guanidine thiocyanate buffer (GTB): 4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5 %-Lauroylsarcosine sodium salt, 0.1 M β-mercaptoethanol (add to the buffer immediately before use),
4. phosphate buffered saline (PBS),
5. RNAse-free water.

Equipment:

1. sonicator,
2. centrifuge with a cooling system,
3. electrophoresis chamber.

Below is the detailed RNA extraction protocol [44, 45].

1. Pre-cool the tubes in an ice bath, sign the tubes.
2. Remove the medium from wells containing adherent cells (aspirate off the medium, carefully add cold PBS, then aspirate off PBS). Resuspend suspension cells, centrifuge at 1.5 krpm for 1 min, remove the medium, wash with PBS, remove PBS.
3. Cover the cells with 1 ml GTB, transfer the lysate immediately to the clean pre-cooled tubes. Vortex vigorously.
4. Homogenize the cells. *Note*. Cells can be homogenized by passing the lysate through a sterile syringe needle, but this method is not very effective. Sonication on ice is more reliable. We sonicate the cells at 130 Watt for 30 s [46].
5. Once again vortex the samples.
6. Centrifuge the lysates at 10,000 g at 4 °C for 5–20 min to precipitate cell debris that may hinder RNA isolation.
7. Transfer the supernatant to clean tubes (be careful not to disturb the pellet).
8. Divide the lysate from each sample into 2 aliquots of equal volume: one will be used for extraction; the other should be reserved for future use and stored at –70 °C.
9. Add 1/10 volume (50 µl) of 2 M sodium acetate pH 4.2 to 500 µl of the lysate. Stir gently.
10. Add an equal volume of acid phenol (500 µl) to the lysate, stir gently, and incubate at room temperature for 5 min until protein-nucleic acid complexes are completely dissolved.
11. Add 1/5 volume (100 µl) of chloroform, vortex vigorously.
12. Centrifuge for 20 min at 10,000 g.
13. Transfer the upper phase to a clean tube; be careful not to disturb the interface.
14. If there is a large interface, repeat extraction until the interface is completely gone. Note that the upper phase will shrink every time the sample is centrifuged, therefore it should be replenished with GTB to maintain a 500 µl volume.
15. Add the equal volume of the acid phenol : chloroform mix (1 : 1, 500 µl, mix in advance, because water is released when these two reagents are mixed and the solution volume changes). Vortex vigorously, centrifuge for 10 min at 10,000 g.
16. Carefully transfer the upper phase to a clean tube.
17. Add one volume of chloroform (500 µl) to the upper phase, vortex vigorously, centrifuge for 10 min at 10,000 g. Carefully transfer the upper phase to a clean tube.
18. Add 2.5 volumes of 96 % ethanol (1,250 µl) to the upper phase. For better visibility of the pellet add precipitating agents, such as glycogen, after centrifugation.
19. Incubate at –20 °C for at least 1 h or leave overnight at –20 °C (for nucleic acid precipitation).
20. Centrifuge for 20 min at 10,000g at +4 °C.
21. Decant the supernatant; wash the pellets with cold

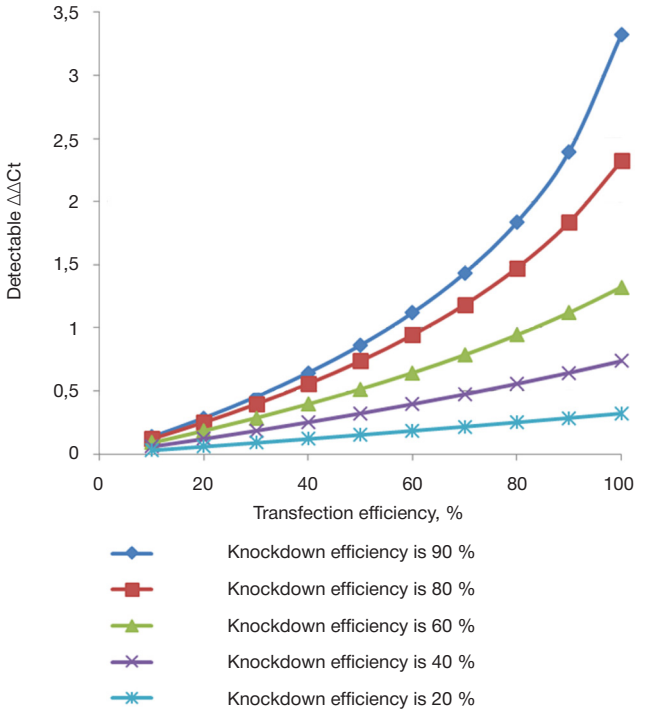

Fig. 2. Predicted ΔΔCt detectable by real-time PCR. Prediction is based on different transfection and knockdown efficiencies

Table 3. Optimizing transfection for a 96-well plate

		10×10^3 cells per well				20×10^3 cells per well			
siFlu/Met	siRNA Metafectene siRNA : Metafectene	0.1 μg (~15 pmol) 0.1 μl 1 : 1	0.1 μg (~15 pmol) 0.2 μl 1 : 2	0.1 μg (~15 pmol) 0.4 μl 1 : 4	0.1 μg (~15 pmol) 0.8 μl 1 : 8	0.1 μg (~15 pmol) 0.1 μl 1 : 1	0.1 μg (~15 pmol) 0.2 μl 1 : 2	0.1 μg (~15 pmol) 0.4 μl 1 : 4	0.1 μg (~15 pmol) 0.8 μl 1 : 8
	siRNA Metafectene siRNA : Metafectene	0.2 μg (~30 pmol) 0.2 μl 1 : 1	0.2 μg (~30 pmol) 0.4 μl 1 : 2	0.2 μg (~30 pmol) 0.8 μl 1 : 4	0.2 μg (~30 pmol) 0.6 μl 1 : 8	0.2 μg (~30 pmol) 0.2 μl 1 : 1	0.2 μg (~30 pmol) 0.4 μl 1 : 2	0.2 μg (~30 pmol) 0.8 μl 1 : 4	0.2 μg (~30 pmol) 0.6 μl 1 : 8
	siRNA Metafectene siRNA : Metafectene	0.3 μg (~45 pmol) 0.3 μl 1 : 1	0.3 μg (~45 pmol) 0.6 μl 1 : 2	0.3 μg (~45 pmol) 1.2 μl 1 : 4	0.3 μg (~45 pmol) 2.4 μl 1 : 8	0.3 μg (~45 pmol) 0.3 μl 1 : 1	0.3 μg (~45 pmol) 0.6 μl 1 : 2	0.3 μg (~45 pmol) 1.2 μl 1 : 4	0.3 μg (~45 pmol) 2.4 μl 1 : 8
Without siRNA	siRNA Metafectene siRNA : Metafectene	– 0.3 μl –	– 0.6 μl –	– 1.2 μl –	– 2.4 μl –	– 0.3 μl –	– 0.6 μl –	– 1.2 μl –	– 2.4 μl –
Untreated cells	siRNA Metafectene siRNA : Metafectene	–	–	–	–	–	–	–	–

Table 4. Calculating the amount of siRNA and Metafectene for different plate types per transfection

Plate type	6 wells	24 wells	96 wells
Total volume, ml	2.2	0.56	0.21
Cell suspension volume, ml	2.0	0.50	0.15
Solution A (siPHK (30μM) + PBS, μl), volume per well	5.0 + 100.0	2.5 + 30.0	0.5 + 30.0
Solution B (lipid + PBS, μl), volume per well	5.0 + 100.0	2.5 + 30.0	0.4 + 30.0
A+B mixed, μl, volume per well	200.0	60.0	60
Number of cells per well ($\times10^4$)	25	10	1

70 % ethanol. Make sure that ethanol comes in contact with every part of the tube wall to wash away salts that may inhibit further enzymatic reactions. Centrifuge for 10 min 10,000g at +4 °C.

22. Air-dry the pellet. Leave the tubes open for a few minutes until liquid is gone and visible pellet becomes transparent. Dissolve the pellet in nuclease-free water.

23. Use electrophoresis to check for RNA degradation and genomic DNA contamination. If the pellet is very visible, run one sample on the gel twice, but use different sample volumes (for example, 1 and 5 μl).

24. During agarose gel electrophoresis the intact RNA will produce two distinct bands corresponding to18S and 28S rRNAs; the 28S band should be twice as intense as the 18S band (Fig. 3). A faint smear should be visible all through the gel lane, representing high molecular weight mRNA. Note that a band at the bottom will indicate RNA degradation. If it is there and is quite intense, read through the protocol carefully once again and repeat extraction. The presence of the genomic DNA band running at 10 kpb (DNA ladder size) and upwards indicates contamination. In case of genomic DNA contamination, repeat RNA extraction paying attention to phenol buffering and be careful when collecting the aqueous phase.

Quality and quantity of the obtained RNA can be assessed instrumentally using the spectrophotometer. RNA concentrations are measured by absorbance at 260 nm. Additional measurements at 240 and 280 nm will provide information about protein contamination of the sample. The purity of the sample is determined by the A260/280 ratio ranging from 0.5 to 2.0. The less protein-contaminated is the RNA, the higher is the ratio. The A260/230 ratio is calculated to detect the presence of organic contaminants, such as phenol or its salts or other salts used during RNA extraction. Ideally, this value should be about 2.0. If the RNA sample is not pure enough, it can be additionally purified by ethanol-induced precipitation. Isolated RNA must be stored at –70 °C and thawed in the ice bath before use.

2. Treatment of RNA samples with DNAse I

DNA contamination of RNA samples may render real-time PCR data inaccurate, causing formation of non-specific by-products. Primers selected for target gene amplification should sit on different exons, otherwise genomic DNA will be co-amplified with cDNA, skewing the results. To sum up, RNA should be free of contaminating genomic DNA. Unfortunately, even commercial reagent kits for RNA isolation do not guarantee a perfect result. Therefore, it is recommended to treat RNA with DNAse I to eliminate contamination.

We recommend attempting DNAse treatment on a portion of the sample. Do not treat the whole sample – reserve a portion in case something goes wrong.

Treating samples with DNAse I is easy [47]: add a reaction buffer and an enzyme provided by the same manufacturer to an RNA aliquot. It is not recommended to vortex DNAse I as vortexing may result in the loss of DNase activity. Incubation with the enzyme lasts for at least one hour, which is normally enough to remove contaminating DNA. DNAse I can be inactivated by EDTA that chelates Mg^{2+} ions and heating to 60 °C.

Reagents:

1. Mg^{2+}-containing buffer,

2. 50 mM EDTA,
3. RNAse-free water.
Equipment: thermostat.
We suggest the following protocol:
1. Thaw RNA in an ice bath.
2. Take an RNA aliquot (2–3 μg) and add RNAse-free water to bring its volume to 8 μl.
3. Add 1 μl of the 10x Mg^{2+}-containing buffer, vortex.
4. Add 1 μl of the enzyme, pipet down (do not vortex).
5. Incubate for 1 hour at 37 °C.
6. Add 1 μl of 50 mM EDTA and incubate for 10 min at 60 °C.

3. DNAse I treatment control

To make sure genomic DNA has been degraded by DNAse, the sample should be tested for the presence of DNA molecules. It is done by running a real-time PCR assay using a pair of primers annealing to genomic DNA. Untreated RNA and genomic DNA should be used for control. The Ct value of the DNAse-treated sample must be > than the Ct values of the untreated RNA and genomic DNA samples. If Ct is >37, the treatment is considered successful, meaning that genomic DNA will not significantly affect the accuracy of the post-knockdown expression analysis.

Reagents:
1. RNAse-free water,
2. PCR mix (5x): buffer for polymerase (5x), 12.5 mM $MgCl_2$, a mix of 4 deoxynucleotides (1mM each),
3. Taq-polymerase,
4. EvaGreen dye,
5. PCR primers for the *HPRT1* gene:
HPRT f3 — ACCACCGTGTGTTAGAAAAGTA,
HPRT r3 — AGGGAACTGCTGACAAAGATT.
Equipment:
1. real-time PCR amplifier,
2. electrophoresis chamber.

To amplify genomic DNA targets by control PCR, we use *HPRT1* primers. The protocol is provided below.

1. Prepare the following PCR mix for each sample:
- 4 μl of the PCR buffer (5x),
- 2 μl of each primer (2.5 μM),
- 1 μl of EvaGreen (20x),
- 0.25 μl of Taq-polymerase (5 units per μl),
- 9.75 μl of water,
- 1 μl of the template.

Note that the PCR buffer contains the Taq-polymerase buffer, 12.5 mM of Mg^{2+}, and 2 mM deoxyribonucleotides.

2. Thermocycling conditions: 95 °C for 1 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s. Fluorescence is recorded during the elongation step; the melting curve is recorded between 60 °C and 95 °C, with measuring points at 0.3 °C intervals
3. Analyze the curves, calculate Ct.

After RNA samples have been treated with DNAse, RNA should be run on the agarose gel again, as described above, to check for contamination.

4. cDNA synthesis by reverse transcription

Although this step is technically easy, it is very important for further analysis, because poorly performed reverse transcription may affect data accuracy during the expression analysis.

To date, there are two major techniques for cDNA synthesis based on the use of random hexanucleotides (hexamers) and the so-called oligo-dT-primers. Random hexanucleotides anneal to random complementary sites of the RNA molecule producing a library of cDNA fragments corresponding to all RNA sequences. Oligo-dT-primes are annealed to the poly(A) tail of mRNA, producing a library of polyadenylated transcripts. Primers for reverse transcription (RT) are selected considering the nature of the target gene and the available primers for its amplification. Random hexanucleotides facilitate synthesis of cDNAs that represent all RNA sequences, making primer selection easier. The 5'-ends of the resulting RNA libraries are often overrepresented, though. In contrast, oligo-dT-primers allow to effectively obtain cDNA fragments corresponding to the 3'-ends of the RNA molecule [48]. Besides, in this case the resulting library will be rich in polyadenylated mRNAs. To sum up, the choice of the technique depends on the mRNA length and the site the primers will be annealing to.

The protocol for reverse transcription includes 3 steps: two- or three-stage primer annealing to RNA, reverse transcription, and its inactivation.

Reagents:
1. reverse transcriptase,
2. reverse transcriptase buffer,
3. deoxyribonucleotides (dNTPs),
4. 25 mM $MgCl_2$,
5. nuclease-free water.
Equipment: thermostat and ice bath.
The protocol for reverse transcription [49] is provided below.
1. Set the thermostat to 70 °C, prepare the ice bath.
2. Calculate the amounts of the reagents for the reaction mix based on RNA concentration (Table 5). The final reaction volume should be 10–30 μl. If RNA concentration is low and the volume of RNA is too large, volume of reaction mixture should be minimized.
3. Add 10 pmol of the oligodT (5'–$(T)_{25}$VN–3') primer or 100 pmol of random hexanucleotides to the volume containing 1 μg RNA.
4. Incubate for 3 min at 70 °C.
5. Transfer to the ice immediately, leave in ice for 3 min.
6. Prepare the reaction mix, add it to RNA.
7. Incubate for 2 min at 42 °C (this step can be skipped).
8. Add 1 μl of ImProm-II Reverse Transcriptase (200 units).
9. Incubate for 15 min at 20 °C (for random hexanucleotides only).
10. Incubate for 1.5 h at 42 °C (reverse transcription).
11. Inactivate revertase: 70 °C, 20 min.
12. Dilute cDNA down 10- to 20-fold with water.

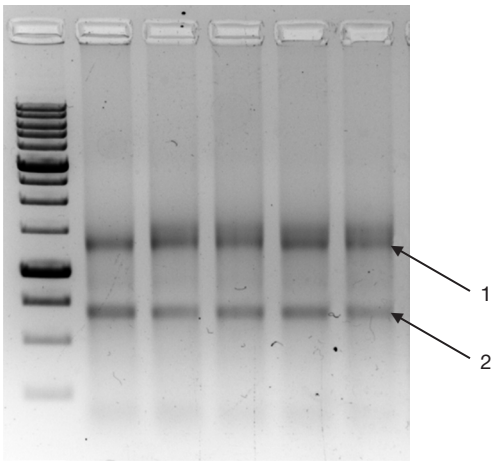

Fig. 3. Electrophoresis of high quality RNA extracts obtained from the lysate. 1 — the 28S rRNA band, 2 — the 18SrRNA band

5. cDNA quality control

Although reverse transcription is easy to perform, cDNA yield can still be low in spite of the seemingly good RNA quality. There are a few reasons for that: RNA was not purified properly after phenol extraction; RNA pellet was not properly washed after precipitation, which resulted in salt contamination; RNA pellet was underdried after ethanol wash. It is recommended to monitor the quality of the obtained cDNA by real-time PCR using primers for housekeeping genes. There is no need for technical replicates, but negative (water) and positive (cDNA) controls are a must. Calculated meaning Ct help to estimate reverse transcription efficiency. Ideally, the difference between the meanings of Ct of the tested sample and the control should not be greater than 1 cycle. If the difference is greater than 4 cycles, reverse transcription should be performed again. Sometimes it might be necessary to purify RNA by ethanol precipitation once again.

Reagents:

1. RNAse-free water,
2. PCR mix (5x): buffer for polymerase (5x), 12.5 mM $MgCl_2$, a mix of 4 deoxynucleotides (1 mM each),
3. Taq-polymerase,
4. EvaGreen or SybrGreen dye,
5. PCR primers for gene HPRT1:

HPRT f4 — TCAGGCAGTATAATCCAAAGATGGT,

HPRT r4 — AGTCTGGCTTATATCCAACACTTCG.

Equipment: real-time PCR amplifier.

We normally perform cDNA quality control with primers for housekeeping genes, such as B2M or HPRT1. The protocol is provided below.

1. The following PCR mix should be prepared for each cDNA sample:
   - 4 µl of the PCR buffer (5x),
   - 2 µl of each primer (2.5 µM),
   - 1 µl of EvaGreen (20x),
   - 0.25 µl of Taq-polymerase (5 units per µl),
   - 2.75 µl of water,
   - 10 µl of the template.
2. Thermocycling conditions: 95 °C for 1 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s; 72 °C for 10 s. Fluorescence is recorded during the elongation step; the melting curve is recorded from 60 to 95 °C with measuring points at 0.3 °C intervals.
3. Analyze the curves, calculate Ct.
4. The Ct value of *HPRT1* must be equal to that of cDNA (~25 cycles). If Ct meaning of the studied cDNA and the control sample are almost equal, reverse transcription is considered successful and the obtained cDNA can be used in further experiments.

6. Evaluation of knockdown efficiency using real-time PCR assay

PCR efficiency is known to be dependent on a number of factors, including template concentrations. Before measuring post-knockdown expression, cDNA concentrations should be equalized in all samples by diluting cDNA down with water, considering the Ct values from the previous step. For all cDNA samples the difference between meanings of Ct should not be greater than 1 cycle to minimize data dispersion during final analysis.

Once cDNA quantities have been equalized, gene expression can be analyzed using real-time PCR. Expression of the target genes must be compared to that of the housekeeping genes. According to the MIQE Guidelines [50], three or more reference genes should be used to achieve accurate normalization. The more reference genes are used in the experiment, the more accurate and reliable is the analysis of post-knockdown expression of the target genes. While selecting reference genes, it should be kept in mind that their expression levels may vary, which means that lowly expressed or overexpressed genes should be opted out. To normalize gene expression data, we often use genes *HPRT1*, *TFRC*, *B2M*, and *TBP*.

Real time PCR is usually performed with three technical replicates per sample. PCR product can be detected by either intercalating dyes -or specific probes. TaqMan probes are very target-specific and easy to use. But intercalating dyes are much cheaper and could be a good alternative to the probes if you are going to conduct only a few experiments [51].

Whether intercalators or TaqMan probes are used, the reaction must be optimized using control cDNA samples because of possible primer-associated problems during the PCR assay.

Reagents:

1. RNAse-free water,
2. PCR mix (5x): buffer for polymerase (5x), 12.5 mM MgCl2, a mix of 4 deoxynucleotides (1 mM each),
3. Taq- polymerase,
4. EvaGreen dye.

Equipment: real-time PCR amplifier.

We described preparation of the PCR reaction mix above. For the majority of the amplified loci we normally use the following thermocycling conditions: 95 °C for 1 min; 40 cycles of 95 °C for 10 s, 60 °C for 10 s 72 °C for 10 s. Fluorescence is recorded during the elongation step; the melting curve is recorded between 60 and 95 °C with measuring points at 0.3 °C intervals.

PCR is usually followed by data analysis, including construction of the melting curves, to make sure that

Table 5. The reaction mix for reverse transcription

Component	Stock solution concentration	Volume per reaction (20 µl)
RNA	–	0,5–1,0 µg
Primer	10µM (oligodT) 100 µM (random hexanucleotides)	1 µl 1 µl
ImProm-II™ Reaction Buffer	5x	4 µl
dNTPs mix	2mM (of each dNTP)	2 µl
$MgCl_2$	25mM	2 µl
ImProm-II Reverse Transcriptase	200 un/ µl	1 µl
Nuclease-free water (MQ)	–	Up to 20 µl

amplification was gene-specific and no primer-dimers were formed. Usually, technical replicates of the same sample are compared. If there are >0.3 differences between the Cts of the replicates, PCR should be performed again strictly according to the protocol. Data are analyzed using mean Ct values from technical replicates.

Now, relative expression levels of the target genes can be calculated. Geometric mean is calculated for the expression of reference genes [52]. Further calculations are based on ΔΔCt:

1. calculate Ct(ref) geometric mean for housekeeping genes;
2. calculate ΔCt = Ct(target gene) – Ct(ref),
3. calculate mean ΔCt(med) and standard deviation SD,
4. calculate ΔΔCt using the following formula: ΔΔCt = ΔCt(med)control – ΔCt(med)target,
5. relative expression is calculated as $2^{-\Delta\Delta Ct}$,
6. for sicontrol relative expression equals 1.

The obtained value shows how expression of the target gene has changed after knockdown in comparison with the control sample. To see if the obtained value is statistically significant, the margin of error has to be calculated. The confidence interval is calculated as $2^{-(\Delta\Delta Ct \pm Sd)}$, where ΔΔCt is calculated for the target gene in the samples treated with target siRNA, relative to the control; SD is standard deviation of ΔCt of biological replicates for target siRNA. The margin of error is the difference between the minimum or maximum values of the confidence interval and the ΔΔCt. Based on these calculations, bar charts with error bars are constructed (Fig. 4).

Statistical significance of data is usually evaluated by the nonparametric Kolmogorov–Smirnov test and Mann–Whitney U test.

IV. Analyzing the effect of knockdown

Depending on the goal of the experiment, different measurements can be taken after gene knockdown to study its effect at the molecular (e. g., expression of other genes) and cellular levels. The effect of gene knockdown on the life of cells can be analyzed using various tests aimed to evaluate cell proliferation, apoptosis, migration, growth, or analyze the cell cycle.

Cell viability assays are commonly used tests employed by similar research studies to determine the number of viable cells in the sample and thus to estimate cell death or proliferation rates after exposure to various factors. Cell viability can be assessed using tetrazolium dyes (MTT, MTS, XTT, WST-1), resazurin, and protease activity markers (GF-AFC), or by measuring ATP levels, etc. All these methods have their own advantages and drawbacks. At the moment, the most popular tool for measuring cell viability is the MTT assay based on the reduction of the MTT tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). It is cheap and simple, but not that sensitive as other methods, therefore it is often used for primary screening [53].

Living cells are capable of reducing MTT to formazan (it is water-insoluble, max absorbance is reached at 570 nm) whose concentrations are measured by the assay. MTT is a commercially available reagent. We normally use the MTT powder supplied by PanEco (Russia).

If the experiment aims to investigate the effect of knockdown on cell viability, the latter is usually measured in the samples treated with control siRNA and target siRNA. Measurements for each sample are taken in five biological replicates. Besides, cell viability must be measured over a period of time at different time points (for example, once every 5 days; time points may vary depending on the goal of the experiment). Thus, every experiment, starting with transfection, has to be repeated as many times as there are time points, because cells die in the course of the MTT assay.

The protocol for the MTT assay is provided below (based on [53, 54]).

Reagents:

1. MTT working solution: 5 mg/ml MTT in PBS pH 7.4. The solution must be filter-sterilized through a 0.2 μM filter and stored in the dark frozen (up to 6 months) or at +4 °C up to two weeks,
2. DMSO solvent (100 % dimethyl sulfoxide).

Equipment:

1. plate reader for measuring absorbance at wavelengths of 570 and 670 nm,
2. plate shaker (optional).

The protocol:

1. Perform transfection in a 96-well flat-bottom plate.
2. The volume of the fresh complete medium replacing the old medium should be 150 μl per well. Note that wells containing the medium without cells should be reserved for control (further referred to as empty wells). Their number should be equal to the number of biological replicates. The assay should be performed on the empty wells in exactly the same manner as on the experimental samples.
3. Add 20 μl of the working solution (5 mg/ml MTT) to each plate. Pipet gently.

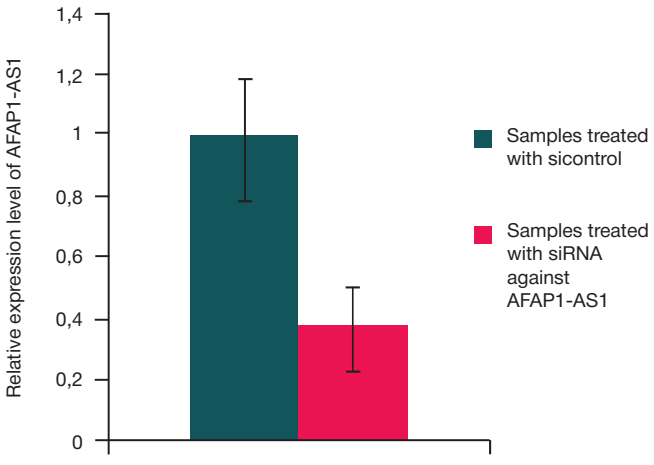

Fig. 4. A bar chart representing real-time PCR data

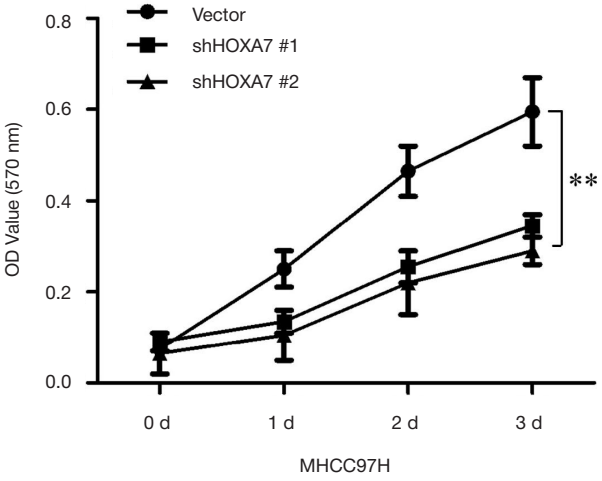

Fig. 5. The results of the MTT-assay following *HOXA7* knockdown (Tang et al. [55])

4. Incubate for 3–4 h at 37 °C and 5 % CO_2.

5. Remove the medium and leave the wells to dry a little.

6. Dissolve the formazan in 200 µl of DMSO. Agitate it on the plate shaker (if available) for 10 min at room temperature to make sure the formazan is dissolved evenly and quickly.

7. Using the plate reader, measure optical densities of the solutions in every plate at 570 nm and 670 nm (for background signals).

Data analysis:

1. Calculate the corrected optical density for each well (D_{cor}) using the formula: D_{cor} =D (570 nm) – D (670 nm), where D is the optical density.

2. For each cell-containing well, subtract mean Dcor of the empty wells from Dcor.

3. For each sample, calculate means for the obtained values of optical density and standard deviations.

4. For each sample, construct the graphs to illustrate dependence of optical density on time elapsed after transfection (Fig. 5).

CONCLUSIONS

Knockdown by siRNA is a difficult multi-step process. There are pitfalls at every step that the researcher should be aware of. Failure to understand or adhere to the proposed guidelines may result in serious mistakes at each step of the experiment, unreliable results or inaccurate interpretations.

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