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Guidelines for experiments using miScript Target Protectors

For miRNA function research

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Introduction

microRNAs (miRNAs) are a class of endogenous small RNA molecules with similar characteristics to siRNAs. In recent years, it has been discovered that miRNAs play a role in many diverse biological processes such as development, differentiation, and apoptosis. Misregulation of miRNA expression is reported to be associated with several cancers and other diseases.

The miRNA system is an endogenous mechanism of regulation of gene expression. Mature miRNAs contribute to the regulation of endogenous genes, primarily by translational repression. In addition, miRNAs can mediate mRNA destruction by rapid deadenylation and/or decapping. Naturally occurring miRNA-binding sites are typically found in the 3' untranslated regions (UTRs) of target mRNAs. Their partial complementarity has made positive identification of true binding sites difficult and imprecise.

Principle and procedure

Transfection of functional molecules, such as miRNA mimics, miRNA inhibitors, or miRNA target protectors, can be used to elucidate the targets and roles of particular miRNAs. miScript Target Protectors are single-stranded, modified RNAs that specifically interfere with the interaction of an miRNA with a single target, while leaving the regulation of other targets of the same miRNA unaffected. Increased target-gene expression after transfection of a miScript Target Protector, a change in signaling patterns, or an altered phenotype, can all provide evidence that the miRNA and target under study are involved in the pathway or phenotype of interest. The role of miRNAs in various pathways can be studied by examination of specific phenotypes after transfection of different miScript Target Protectors. HiPerFect Transfection Reagent is ideally suited to both low- and high-throughput transfection of small, functional molecules such as miScript Target Protectors.

Identification of potential miRNA binding sites

Due to the complex and only partially understood mechanisms of miRNA target binding, a prediction algorithm is usually necessary to choose potential miRNA–mRNA binding sites. Several such algorithms are publicly available. Some of the more popular prediction algorithms are listed below.

The simplest method to identify a putative binding site is to look for potential miRNA binding partners for an mRNA of interest using a prediction algorithm. The algorithm will list all the miRNAs that can potentially bind to the 3' UTR of the chosen target. The binding sites of a particular miRNA can be viewed by selecting the miRNA in the algorithm interface (usually by clicking on it). To define a binding site and design a miScript Target Protector for the miRNA of interest, the seed region must be identified. Usually the prediction algorithm

marks the seed region for each miRNA binding site, although the way this is shown may differ from algorithm to algorithm (e.g., TargetScan highlights the seed region sequence in white). A single miRNA may have several potential binding sites in the 3' UTR of a particular target.

The chance of successfully predicting a correct binding site may differ between algorithms and between different targets. It is therefore recommended to test several binding sites to find the correct ones for a given miRNA–mRNA pair.

miRNA prediction algorithms and resources

The miRBase Website provides extensive miRNA information and links to popular target prediction sites (www.mirbase.org). In addition, the following is a small selection of Websites available for miRNA information and binding-site prediction.

- TargetScan www.targetscan.org
- miRanda www.microrna.org
- PicTar pictar.mdc-berlin.de
- MicroCosm www.ebi.ac.uk/enright-srv/microcosm

Ordering a miScript Target Protector

miScript Target Protectors can be easily ordered for miRNA targets in many different species. To order, go to www.qiagen.com/miDesign and choose a name for your miScript Target Protector. Next, provide the RefSeq ID of your gene of interest and the design tool will display the 3' UTR sequence. Select the 6 or 7 base miRNA binding site (e.g., ACTGCCT) by either highlighting it in the sequence or entering it in the search field. The design tool will provide a miScript Target Protector ready to order.

Description of protocols

This handbook contains 3 protocols for transfection of adherent cells with miScript Target Protectors. All protocols are Fast-Forward Protocols, which means that cell seeding and transfection are carried out on the same day. Fast-Forward Protocols are quicker and save labor compared to traditional protocols in which cells are seeded the day before transfection. One protocol is for transfection of a miScript Target Protector; the second is for cotransfection of a miScript Target Protector and a miScript miRNA Mimic; the third is for cotransfection of a miScript Target Protector and plasmid DNA.

All protocols are designed for transfection in a 24-well plate format. We recommend when using 24-well plates that transfection is performed in the order described in this protocol, with cells seeded in wells first followed by addition of miScript miRNA Mimic/miScript Target Protector/plasmid–reagent

complexes. This ensures optimal mixing of cells and complexes. However, transfection can be performed using reverse transfection, with complexes added to wells and cells added on top of complexes, if desired. To perform a reverse transfection, simply change the order in which cells and complexes are added to the plate.

Transfection of miScript Target Protector

If the miRNA under study is endogenously expressed in the cell type, an experiment can be performed in which a miScript Target Protector for the miRNA binding site of interest is transfected, followed by phenotype or gene expression analysis. When establishing this experimental design for the first time, we recommend transfecting a positive transfection control (e.g., AllStars Cell Death Control siRNA) under different conditions to establish the optimal conditions for efficient transfection. These conditions should then be confirmed by transfection of a positive control (e.g., Positive Control miScript Target Protector).

We recommend the following routine controls:

- Negative control (e.g., transfection of Negative Control miScript Target Protector)
- Untransfected cells

Cotransfection of miScript miRNA Mimic and miScript Target Protector

If the miRNA under study is not endogenously expressed or only expressed at low levels in the cell type, an experiment can be performed in which a miScript Target Protector for the miRNA binding site of interest and a miScript miRNA Mimic for the miRNA under study are cotransfected. Cotransfection can be followed by phenotype or gene expression analysis. When establishing this experimental design for the first time, we recommend transfecting a positive transfection control (e.g., AllStars Cell Death Control siRNA) under different conditions to establish the optimal conditions for efficient transfection. These conditions should then be confirmed by transfection of a positive control (e.g., Positive Control miScript Target Protector cotransfected with a miScript miRNA Mimic for miR-15a or miR-16).

We recommend the following routine controls:

- Three negative controls:
    - Cotransfection of Negative Control miScript Target Protector and AllStars Negative Control siRNA
    - Cotransfection of Negative Control miScript Target Protector and the miScript miRNA Mimic of interest

- Cotransfection of the miScript Target Protector of interest and AllStars Negative Control siRNA
- Untransfected cells

Cotransfection of a miScript Target Protector and plasmid reporter construct

If the miRNA target under study is exogenous to the cell type, an experiment can be performed in which a miScript Target Protector for the miRNA binding site of interest and a plasmid reporter construct carrying a reporter (e.g., luciferase) fused to the miRNA binding sequence are cotransfected. Reporter gene expression can be measured with or without target protector cotransfection to determine whether the target protector prevents miRNA downregulation via the miRNA binding site on the plasmid. When establishing this experimental design for the first time, we recommend transfecting a positive transfection control (e.g., AllStars Cell Death Control siRNA) under different conditions to establish the optimal conditions for efficient transfection. These conditions should then be confirmed by transfection of a positive control (e.g., Positive Control miScript Target Protector).

We recommend the following routine controls:

- Negative control (e.g., cotransfection of Negative Control miScript Target Protector and the plasmid reporter construct)
- Untransfected cells
- Transfection of an unregulated plasmid reporter

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Culture medium
- Tools for monitoring miRNA effect at the phenotypic level or by gene expression analysis

Protocol: Fast-Forward Transfection of Adherent Cells with miScript Target Protector in 24-Well Plates

This protocol is provided as a starting point for optimization of miScript Target Protector transfection in a 24-well format. The amounts given are for one well of a 24-well plate. In this protocol, cell seeding and transfection are performed on the same day.

Procedure

1. **Shortly before transfection, seed 0.4–1.6 x 10^5 cells per well of a 24-well plate in 500 µl of an appropriate culture medium containing serum and antibiotics.**
2. **For the short time until transfection, incubate the cells under normal growth conditions (typically 37°C and 5% CO_2).**

   Cells may alternatively be seeded after step 3 of this protocol.
3. **Dilute 3 µl miScript Target Protector (100 µM stock) with 97 µl culture medium without serum (this will give a final concentration of 500 nM miScript Target Protector after adding complexes to cells in step 5). Add 3 µl HiPerFect Transfection Reagent to the diluted miScript Target Protector and mix by vortexing.**

   IMPORTANT: The amount of transfection reagent and miScript Target Protector required for optimal performance may vary, depending on the cell line and gene target.
4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.**
5. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**
6. **Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene expression after an appropriate time (e.g., 24–72 h after transfection, depending on experimental setup). Change the medium as required.**

   Note: The optimal incubation time for analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Fast-Forward Cotransfection of Adherent Cells with miScript Target Protector and miScript miRNA Mimic in 24-Well Plates

This protocol is provided as a starting point for optimization of miScript miRNA Mimic and miScript Target Protector cotransfection in a 24-well format. The amounts given are for one well of a 24-well plate. In this protocol, cell seeding and transfection are performed on the same day.

Procedure

1. **Shortly before transfection, seed 0.4–1.6 x 10^5 cells per well of a 24-well plate in 500 µl of an appropriate culture medium containing serum and antibiotics.**
2. **For the short time until transfection, incubate the cells under normal growth conditions (typically 37°C and 5% CO_2).**

   Cells may alternatively be seeded after step 3 of this protocol.
3. **Dilute 3 µl miScript Target Protector (100 µM stock) and 0.3 µl miScript miRNA Mimic (20 µM stock) with 96.7 µl culture medium without serum (this will give a final concentration of 10 nM miScript miRNA Mimic and 500 nM miScript Target Protector after adding complexes to cells in step 5). Add 3 µl HiPerFect Transfection Reagent to the diluted mimic/target protector and mix by vortexing.**

   IMPORTANT: The amount of transfection reagent and miScript miRNA Mimic and/or miScript Target Protector required for optimal performance may vary, depending on the cell line and gene target.
4. **Incubate the samples for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.**
5. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**
6. **Incubate the cells with the transfection complexes under their normal growth conditions and monitor phenotype after an appropriate time (e.g., 24–72 h after transfection, depending on experimental setup). Change the medium as required.**

   Note: The optimal incubation time for analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Protocol: Fast-Forward Cotransfection of Adherent Cells with miScript Target Protector and Plasmid DNA in 24-Well Plates

This protocol is provided as a starting point for optimization of miScript Target Protector and plasmid DNA cotransfection in a 24-well format. The amounts given are for one well of a 24-well plate. In this protocol, cell seeding and transfection are performed on the same day.

Procedure

1. **Shortly before transfection, seed 0.4–1.6 x 10^5 cells per well of a 24-well plate in 500 µl of an appropriate culture medium containing serum and antibiotics.**
2. **For the short time until transfection, incubate the cells under normal growth conditions (typically 37°C and 5% CO_2).**

   Cells may alternatively be seeded after step 3 of this protocol.
3. **Dilute 3 µl plasmid DNA (100 ng/µl stock) and 3 µl miScript Target Protector (100 µM stock) with 54 µl culture medium without serum (this will give 300 ng plasmid and a final concentration of 500 nM miScript Target Protector after adding complexes to cells in step 5). Add 1.5 µl Attractene Transfection Reagent to the diluted miScript Target Protector and mix by pipetting up and down.**

   IMPORTANT: The amount of transfection reagent, plasmid DNA, and miScript Target Protector required for optimal performance may vary, depending on the cell line and gene target.
4. **Incubate the samples for 10–15 min at room temperature (15–25°C) to allow the formation of transfection complexes.**
5. **Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.**
6. **Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene expression after an appropriate time (e.g., 24–72 h after transfection, depending on experimental setup). Change the medium as required.**

   Note: The optimal incubation time for analysis depends on the cell type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise.

	Comments and suggestions
Low transfection efficiency	
a) Suboptimal HiPerFect Transfection Reagent:miScript miRNA Mimic/miScript Target Protector ratio	Although fixed volumes of HiPerFect Transfection Reagent usually work very well with a range of miScript miRNA Mimic/miScript Target Protector concentrations, it could occur that the overall charge of the complexes is negative, neutral, or strongly positive, which can lead to inefficient adsorption to the cell surface. For optimal adsorption, complexes should be weakly positive. To optimize the HiPerFect Transfection Reagent to miScript miRNA Mimic/miScript Target Protector ratio, perform systematic titrations of HiPerFect Transfection Reagent (for more information on optimization, consult the *HiPerFect Transfection Reagent Handbook* at www.qiagen.com/HB/HiPerFectTransfectionReagent_EN).
b) Suboptimal cell density	If cell density at the time of addition of HiPerFect Transfection Reagent–miScript miRNA Mimic/miScript Target Protector complexes is not at an optimal level, cells may not be in the optimal growth phase for transfection. This can lead to insufficient uptake of complexes into the cells or inefficient processing of the miScript miRNA Mimic/miScript Target Protector.
Excessive cell death (unless desired phenotypic effect)	
a) Concentration of HiPerFect Transfection Reagent– miScript miRNA Mimic/miScript Target Protector complexes is too high	Decrease the amount of HiPerFect Transfection Reagent– miScript miRNA Mimic/miScript Target Protector complexes added to the cells.

	Comments and suggestions
b) Cells are stressed	Avoid stressing cells with temperature shifts and long periods without medium during washing steps. It is particularly important for transfection of miScript miRNA Mimic/miScript Target Protector that the cells are in good condition. Therefore, ensure that cell density is not too low at transfection.
c) Key gene is silenced or key miRNA is inhibited	If the gene/miRNA targeted is important for the survival of the cell, silencing/inhibiting this gene/miRNA may lead to cell death.

Variable transfection efficiencies in replicate experiments

a) Inconsistent cell confluencies in replicate experiments	Count cells before seeding to ensure that the same number of cells is seeded for each experiment. Keep the incubation time between seeding and complex addition consistent between experiments.
b) Possible mycoplasma contamination	Mycoplasma contamination influences transfection efficiency. Variations in the growth behavior of mycoplasma-infected cells will lead to different transfection efficiencies between replicate experiments.
c) Cells have been passaged too many times	Cells that have been passaged a large number of times tend to change their growth behavior and morphology, and are less susceptible to transfection. When cells with high passage numbers are used for replicate experiments, decreased transfection efficiencies may be observed in later experiments. We recommend using cells with a low passage number (<50 splitting cycles).
d) Concentration of miScript miRNA Mimic or miScript Target Protector is too low	Increase the concentration of miScript miRNA Mimic or miScript Target Protector used for transfection.

No or very small effect after miScript miRNA Mimic transfection

a) Incubation time after transfection too short	The gene silencing effect observed at the protein level is dependent on the expression level of the protein and its rate of turnover within the cell. Perform a time-course experiment to determine the optimal time point for analysis.

	Comments and suggestions
b) Concentration of miScript miRNA Mimic is too low	Increase the concentration of miScript miRNA Mimic used for transfection.
c) Inappropriate experimental approach	For many targets, miRNA effects cannot be detected at the transcript level. If possible, repeat experiments using a different experimental approach (e.g., if currently detecting transcript levels using PCR, try to detect protein levels instead). If possible, include both positive and negative controls in your experiments.
d) miRNA has only modulatory function	miRNA regulatory effects may be less obvious than gene silencing by siRNA. miRNAs can also have a modulatory function in which they regulate gene expression without completely silencing the gene. Several miRNAs may have different regulatory effects on the same target.
e) Target under study is not regulated by chosen miRNA	miRNAs are naturally occurring noncoding RNAs with binding patterns that differ from those of siRNAs. This makes target prediction difficult, and a high score on available target prediction software does not guarantee that the investigated target is regulated by the miRNA of interest. If possible, include both positive and negative controls in your experiments.
f) Multiple target transcripts exist with different 3' UTRs	miRNA regulates translational repression by binding to the 3' UTR of the target mRNA. Many cellular proteins are translated from 2 or more different transcripts. Often the 3' UTRs of these transcripts differ significantly, with one transcript having a specific miRNA binding site in the 3' UTR, while the other does not.
No or very small effect after miScript Target Protector transfection	
a) Incubation time after transfection too short	The inhibitory effect observed at the protein level is dependent on the rate of target protein synthesis. Perform a time-course experiment to determine the optimal time point for analysis.

	Comments and suggestions
b) Concentration of miScript Target Protector is too low	Increase the concentration of miScript Target Protector used for transfection. Although 500 nM or less miScript Target Protector is usually efficient, in some cases, especially if performing experiments with a plasmid reporter construct, 1 µM or higher miScript Target Protector may be necessary.
c) Inappropriate experimental approach	For many targets, miRNA effects can not be detected at the transcript level. If possible, repeat experiments using a different experimental approach (e.g., if currently detecting transcript levels using PCR, try to detect protein levels instead). If possible, include both positive and negative controls in your experiments.
d) Target miRNA has only modulatory function	miRNA regulatory effects may be less obvious than gene silencing by siRNA. miRNAs can also have a modulatory function in which they regulate gene expression without completely silencing the gene. For this reason, inhibiting a specific miRNA may not lead to significant changes at the transcript or protein level.
e) More than one miRNA regulates the target	Many targets are regulated by more than one miRNA, therefore inhibiting one miRNA may not abolish translational repression by the other miRNAs targeting the same gene. If possible, use a cell system with only few of the endogenous miRNAs that target your gene of interest. Alternatively, select a target that is regulated by only few endogenous miRNAs. It is also possible to cotransfect different miScript Target Protectors to prevent other miRNAs from regulating the target gene.
f) Target under study is not regulated by the chosen miRNA	miRNAs are naturally occurring noncoding RNAs with binding patterns that differ from those of siRNAs. This makes target prediction difficult, and a high score on available target prediction software does not guarantee that the investigated target is regulated by the miRNA of interest. If possible, include both positive and negative controls in your experiments.

	Comments and suggestions
g) The prediction of the miRNA binding site is not correct, or there is more than one binding site	The mechanisms of miRNA binding are still not fully understood. miScript Target Protectors will only work correctly if they target the correct binding site for the miRNA. Sometimes an miRNA has more than one binding site for a single target, or the commonly used algorithms to predict binding sites do not agree in their prediction. Blocking of more than one miRNA binding site using different miScript Target Protectors is often necessary to see a full effect. If possible, include both positive and negative controls in your experiments.

Ordering Information

Product	Contents	Cat. no.
miScript Target Protector	5 nmol cell-culture–grade target protector	Varies*
Negative Control miScript Target Protector	5 nmol cell-culture–grade target protector with no homology to any known mammalian gene	Varies*
Positive Control miScript Target Protector	5 nmol cell-culture–grade target protector for the miR-15a and miR-16 binding sites of BCL2	Varies*
miScript miRNA Mimic	1 nmol or 5 nmol cell-culture–grade mimic or 20 nmol animal-grade mimic	Varies*
miScript miRNA Inhibitor	1 nmol or 5 nmol cell-culture–grade inhibitor or 20 nmol animal-grade inhibitor (option of phosphorothioate modification)	Varies*
HiPerFect Transfection Reagent (0.5 ml)†	Reagent for miRNA/siRNA transfection	301704
Attractene Transfection Reagent (0.5 ml)†	Reagent for DNA transfection and DNA-miRNA cotransfection	301004

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