PRODUCT GUIDE

Instruction Manual

siIMPORTER™

siRNA and Plasmid DNA Transfection Reagent

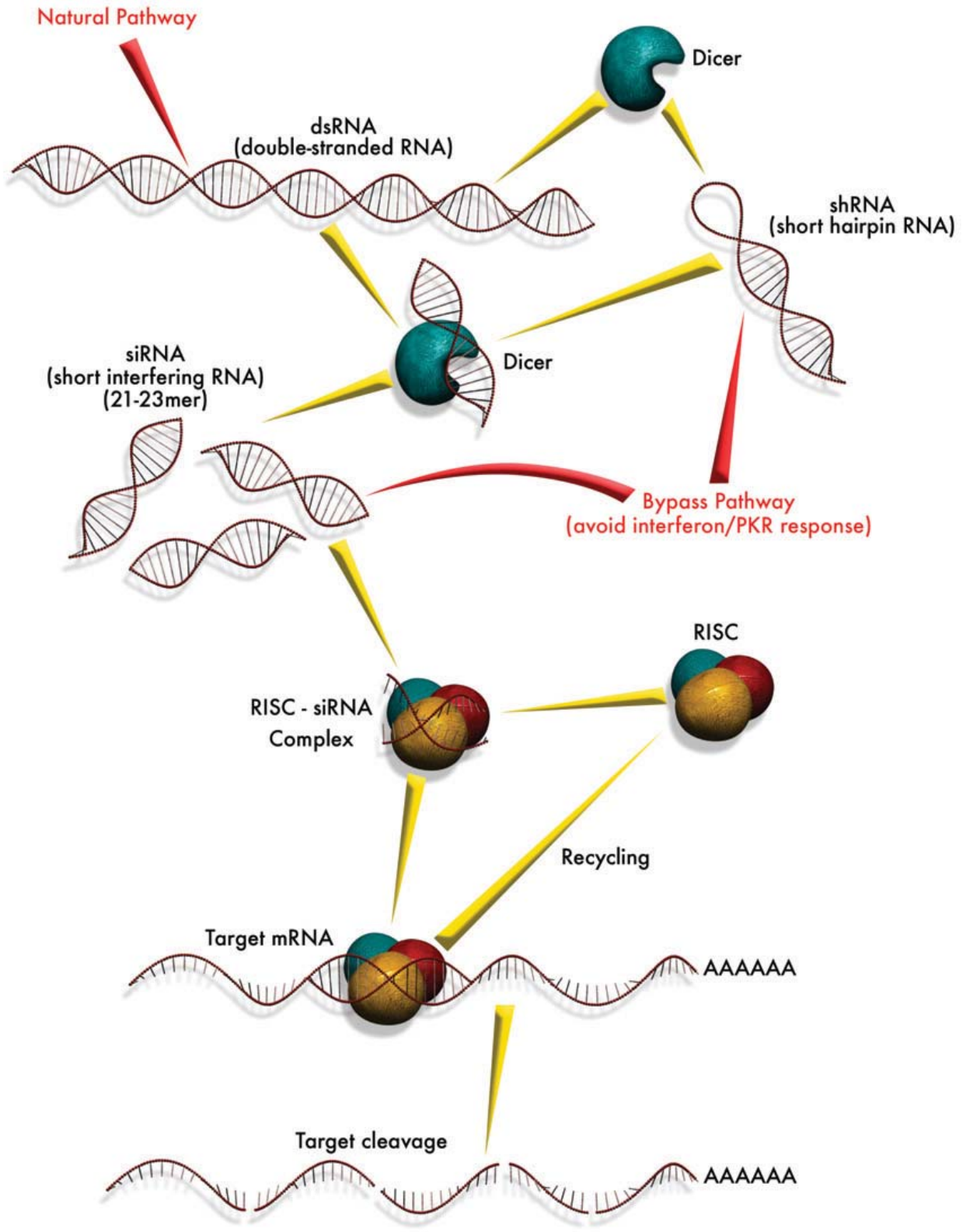
Cat. #64-101

For laboratory research use only.

Not intended for human or animal diagnostic, therapeutic or other clinical uses.

upstate™
cell signaling solutions

RNA interference pathway



Legend *see page 5*

contents

I. Product Description

The siIMPORTER™ Transfection Reagent is a cationic lipid formulation that has been designed for efficient transfection of siRNA and plasmid DNA into a wide variety of mammalian cell lines. The benefits of siIMPORTER™ are:

- Lowest cytotoxicity
- High efficiency delivery of dsRNA or plasmid DNA into many different mammalian cell lines
- Compatible with and without serum in culture growth media
- Functional gene silencing post transfection

II. System Components

A. Provided Kit Components

siIMPORTER™ (cat. #64-101)
One vial containing **0.75ml** of hydrated siIMPORTER™ cationic lipid reagent for siRNA/plasmid DNA transfection

siRNA Diluent (cat. #20-272)
One vial containing **4ml** of dilution buffer for synthetic siRNA transfection

B. Required Materials Not Provided

- Gene-specific siRNA or plasmid DNA
- Complete cell culture media
- Serum-free cell culture media
- Tissue culture dishes
- Microcentrifuge tubes
- Cell culture incubator
- Variable volume (5- 200µl) pipet + tips

III. Storage and Stability

Storage: The siIMPORTER™ Transfection Reagent is shipped at room temperature. Upon receipt, the kit should be stored at 4ºC.

Stability: If stored properly, all components are stable for 12 months after receipt.

IV. Gene Silencing and siRNA Overview

RNA interference pathway *(See inside front cover for diagram)*
Legend: *RNA interference was originally characterized in lower eukaryotes as a cellular response to the introduction of long pieces of double stranded RNA (dsRNA). In the illustration, this is noted as the "natural pathway". Upon entry to the cell, the*

enzyme "dicer" recognizes the dsRNA and processes it into 21-23 nucleotide duplexes that are called small interfering RNA (siRNA). In mammalian cells, introduction of long dsRNA has been shown to activate the interferon/PKR response which results in the cell shutting down its protein translation machinery. This interferon response can be bypassed in mammalian cells if siRNA or short hairpin RNA (shRNA) are introduced. Synthetic siRNA requires no further processing, but shRNA, it is proposed, is processed by dicer. This processing serves to remove the loop from the shRNA and create a siRNA duplex. siRNA then associates with numerous proteins to form the RNA-induced silencing complex (RISC). In an ATP dependent manner, RISC unwinds the siRNA duplex whereby the antisense strand guides the activated RISC-siRNA complex to its targeted mRNA for hybridization and ultimately its degradation.

The use of siRNA in functional genomics and target validation research has in many ways revolutionized biological science. However, without proper understanding of all of the factors involved in the siRNA-mediated gene silencing process, its efficacy as a tool in the research lab will have noticeable limitations.

There are several critical factors that determine the success of siRNA usage in gene silencing. Transfection efficiency and cytotoxicity of the reagents are two of the most important. Even when using a properly selected and designed siRNA, without proper optimization of transfection, siRNA-mediated knock-down will be very low. Extensive

optimization of transfection conditions is required for the cell line and cell culture conditions being used. siIMPORTER™ was compared to the other siRNA transfection reagents on the market and found to offer the highest transfection efficiency as well as the least amount of cytotoxicity to the cells.

The ability to assess gene function via siRNA-mediated methods represents an exciting and valuable tool that accelerates genome-wide investigations across a broad range of biomedical and biological research. The keys to siRNA-dependent gene silencing in cell culture depends on a number of critical factors:

1. Target sequence selection and siRNA design
2. Cell line and cell culture system
3. Transfection conditions
4. Abundance and turnover rate of the mRNA of interest
5. Protein half-life
6. Accuracy and ease of assaying for mRNA levels, protein levels or phenotype.

RNA-mediated interference (RNAi) is a well-recognized pathway employed by most eukaryotes as a cellular line of defense directed against invading viral genomes or as a method to clear a cell of aberrant transcription products[1-2].

While the mechanism of successful RNAi-mediated gene silencing remains to be fully elucidated, this method is proving to be an invaluable tool for analysis of gene function and target validation.

In 2001, Dr. Tuschl and his colleagues showed that when short RNA duplexes (19-23 bases in length) were introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA was effected without inducing an interferon response[10]. These short dsRNA, referred to as small interfering RNA (siRNA), act catalytically at sub-molar concentrations and can cleave up to 95% of the target mRNA in the cell. The siRNA-mediated effect has been shown to be relatively stable over time and silencing may be observed through several cell generations (Tuschl *et al*. 2002, siRNA User's Guide, www.rockefeller.edu/labheads/tuschl/sirna.html). Relative to previous antisense technology, these properties make siRNA extremely effective at inhibiting target gene expression.

Cellular uptake of long double stranded RNA (dsRNA) has been shown to induce RNA interference in a diverse group of lower eukaryotic organisms[3-8]. RNAi leads to the inhibition of protein expression by utilizing sequence-specific, dsRNA-mediated degradation of the target messenger RNA (mRNA)[9]. Attempts to induce RNAi using long dsRNA in

mammalian cell lines were first met with limited success, due in part to the induction of the interferon response, which results in a general inhibition of protein synthesis[10].

V. Transfection Procedure

Safety Warnings and Precautions: *The siIMPORTER™ Transfection Reagent is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.*

A. General Notes

1. The source of siRNA may be synthetic siRNA (e.g. a *SMART*pool® siRNA reagent) or an *in vitro* transcribed dsRNA. Additionally, siIMPORTER™ has been shown to work as a plasmid DNA transfection reagent (see section C on page 18).
2. We recommend performing the following experimental controls:
   - Always use a negative control siRNA in every set of transfections (cat. #D-001206) at the same concentration as the experimental siRNA.
   - Include untransfected or mock transfected cells as an additional negative control. Results obtained using a negative control siRNA should mirror those obtained

in untransfected or mock transfected cells.

- In order to determine transfection conditions that result in the highest efficiency, it is recommended to optimize every specific cell culture system with positive control transfection kits such as the Lamin A/C and Cyclophilin A siRNA/siAb™ Starter Kits (cat. #61-001, 61-002, 61-003 and 61-004) as well as a fluorophore-labeled siRNA such as the Cy3-luciferase control *SMART*pool® (cat. #D-001110).

3. Conditions to optimize for the highest transfection efficiency include cell plating density as well as the ratio of siRNA to siIMPORTER™ to siRNA diluent.

4. An additional optimization step is to identify the proper time point after transfection where the greatest siRNA-mediated gene silencing exists. We recommend setting up a time-course to look at the gene target mRNA and/or protein levels at 24, 48, 72 and 96 hours post-transfection. It is important to remember that mRNA knock-down will be seen prior to protein knock down. The half-life of the protein will vary from target to target and will influence the efficacy of knock-down significantly. We have witnessed optimal mRNA knock-down between 24 and 48 hours post-transfection. However, for protein

knock-down, the best results have most often been seen between 72 and 96 hours post-transfection.

5. The siIMPORTER™ siRNA Transfection Reagent should be stored at 4°C and never frozen.

6. To calculate the amount of siRNA, use the following conversion. A 20µM solution of a 21 nucleotide dsRNA is .26µg/µl. ***Note:*** *The molecular weight of a 21 nucleotide dsRNA is 13.4µg/nmol.*

B. siRNA Protocol

Transfection of Adherent Cells:

1. Plate cells the day before transfection so that when transfected, the cells will be approximately 40-60% confluent. Plate cells in media according to the guidelines in **Table 1** (see column 2) below.

Table 1: Transfection Format

Tissue Culture Plate Format	Volume of Media (µl) Per Well	siRNA/siIMPORTER™ Mixture (µl)	Total Volume Per Well (µl)
96 wells	93	7	100
24 wells	232.5	17.5	250
12 wells	465	35	500
6 wells	930	70	1,000

2. Using a 1.6ml microcentrifuge tube, dilute the siIMPORTER™ reagent into serum-free medium that has been warmed to 37°C according to **Table 2** below.

Table 2: siIMPORTER™ Dilutions for Adherent Cells

Tissue Culture Plate Format	siIMPORTER™ siRNA Transfection Reagent (µl) + Serum-Free Medium (µl) per well
96 wells	0.5 + 2.5
24 wells	1.25 + 6.25
12 wells	2.5 + 12.5
6 wells	5.0 + 25

3. Mix gently by pipetting.

4. In a second 1.6ml microcentrifuge tube, prepare the siRNA solution by first mixing the siRNA diluent (cat. #20-272) with serum-free medium according to **Table 3** on the following page. The siRNA diluent aides complex formation between siRNA and siIMPORTER™. Add the siRNA amount recommended in **Table 3** to the diluent/serum-free medium solution. The amount of siRNA utilized will give a final concentration in the reaction of 100nM.

Table 3: siRNA Dilutions for Adherent Cells

Tissue Culture Plate Format	siRNA Diluent (µl) + Serum-Free Medium (µl) per Well	Amount of 20µM siRNA to use (µl) per Well
96 wells	2.5 + 1	0.50
24 wells	6.25 + 2.5	1.25
12 wells	12.5 + 5	2.50
6 wells	25.0 + 10	5.00

5. Mix gently by pipetting. Do not vortex.
6. Add the siRNA solution prepared in step 4 to the diluted siIMPORTER™ solution prepared in step 2. Incubate this mixture at room temperature for 5 minutes. This allows siRNA/lipid complexes to form. **You may incubate longer than 5 minutes, but do not incubate longer than 30 minutes, as this will be detrimental to the transfection efficiency.**
7. Add the siRNA/siIMPORTER™ mixture to cells growing in serum-containing medium. Incubate at 37°C for 24 hours or over a time course to identify the most efficacious harvest time point. Some cell types such as HeLa, MDCK and CHO-K1 may result in higher transfection efficiencies if serum is not present in the

transfection for the first 4 hours. If this is performed, after 4 hours add one volume of media containing 20% serum and proceed to Step 8.

8. Add fresh tissue culture media to growing cells as needed. Most RNA interference can be detected within 24 to 96 hours post transfection. Look for decreases in targeted message levels between 24 and 48 hours post transfection and decreases in targeted protein levels between 72 and 96 hours post transfection as a general rule. Adjust times as necessary for the specific gene target studied.

Transfection of Suspension Cells:

1. Passage cells the day before transfection so that on the day of transfection the cells are in a log-phase of growth.
2. Using a 1.6ml microcentrifuge tube, dilute the siIMPORTER™ reagent into serum free medium that has been warmed to 37°C according to **Table 4** on the following page.

Table 4: siIMPORTER™ Dilutions for Suspension Cells

Tissue Culture Plate Format	siIMPORTER™ siRNA Transfection Reagent (µl) + Serum-Free Medium (µl) per well
96 wells	0.5 + 2.5
24 wells	1.25 + 6.25
12 wells	2.5 + 12.5
6 wells	5.0 + 25

3. Mix gently by pipetting.
4. In a second 1.6ml microcentrifuge tube, prepare the siRNA solution by first mixing the siRNA diluent (cat. #20-272) with serum-free medium according to **Table 5** below. Add the recommended amount of siRNA to the diluent/serum-free medium mixture (see Table 5). The amount of siRNA utilized will give a final reaction of 100nM.

Table 5: siRNA Dilutions for Suspension Cells

Tissue Culture Plate Format	siRNA Diluent (µl) + Serum-Free Medium (µl) Per Well	Amount of 20µM siRNA to Use Per Well (µl)
96 wells	2.5 + 1	0.50
24 wells	6.25 + 2.5	1.25
12 wells	12.5 + 5	2.50
6 wells	25 + 10	5.00

5. Mix gently by pipetting. Do not vortex. Incubate at room temperature for 5 minutes.
6. Add the siRNA solution prepared in step 4 to the diluted siIMPORTER™ solution prepared in step 2. Incubate this mixture at room temperature for 5 minutes. This allows siRNA/lipid complexes to form. **You may incubate longer than 5 minutes, but do not incubate longer than 30 minutes, as this will be detrimental to the transfection efficiency.**
7. While the siRNA/siIMPORTER™ mix is incubating, spin down the cells from Step 1, remove the growth medium and then resuspend the cells in the appropriate growth medium to achieve a final density of 10^6 cells/ml.
8. Transfer resuspended cells to culture plates according to **Table 6** on the following page.

Table 6: Volume and Number of Cells to Transfer Into Culture Dishes

Tissue Culture Plate Format	Volume of Resuspended Cells to Transfer to Each Well (µl)	Number of Cells Transferred to Each Well (approximate)
96 wells	93.0	1×10^5
24 wells	232.5	2.5×10^5
12 wells	465.0	5×10^5
6 wells	930.0	1×10^6

9. Add the siRNA/siIMPORTER™ mixture to the resuspended cells from **Table 6** above. Gently mix by pipetting. This step is important to avoid cell clumping. Incubate plates at 37°C until cells are ready for harvest.

10. Add fresh tissue culture media to growing cells as needed. Most RNA interference can be detected within 24 to 96 hours post transfection. Look for decreases in targeted message levels between 24 and 48 hours post transfection and decreases in targeted protein levels between 72 and 96 hours post transfection as a general rule. Adjust times as necessary for the specific gene target studied.

C. Plasmid DNA Protocol

Transfection of Adherent Cells:

1. Plate cells the day before transfection so that when transfected, the cells will be approximately 60-80% confluent.
2. Using a 1.6ml microcentrifuge tube, mix the recommended amount of plasmid DNA with serum-free medium according to **Table 7** below. ***Note:*** *Optimization of the DNA concentration utilized may be necessary to obtain the highest transfection efficiency with each cell type.*

Table 7: Plasmid DNA Dilution

Tissue Culture Plate Format	Recommended Amt. of Plasmid DNA to Use Per Well (ng)	Total Volume (µl): Plasmid DNA + Serum-Free Medium
96 wells	50	4
24 wells	125	10
12 wells	250	20
6 wells	500	40

3. Mix by pipetting and spin briefly. Set tube aside until Step 7.

4. Replace the media in each well of the tissue culture plate with pre-warmed serum-free medium according to **Table 8** below. ***Note:*** *Optimization of this step may be necessary since some cell types can be transfected with serum-containing media.*

Table 8: Media Requirements

Tissue Culture Plate Format	Serum-Free Medium to Add Per Well (µl)
96 wells	193
24 wells	482.5
12 wells	965
6 wells	1,930

5. In a second 1.6ml microcentrifuge tube, dilute the siIMPORTER™ reagent into pre-warmed serum-free medium according to **Table 9** on page 20.

Table 9: siIMPORTER™ Dilution

Tissue Culture Plate Format	siIMPORTER™ reagent Per Well (µl)	Serum-Free Medium Per Well (µl)
96 wells	0.50	2.50
24 wells	1.25	6.25
12 wells	2.50	12.5
6 wells	5.00	25.0

6. Mix gently by pipetting. **Do not vortex.**
7. Add the plasmid DNA mixture prepared in step 2 to the diluted siIMPORTER™ solution prepared in step 5. Incubate this mixture at room temperature for 5 minutes. This allows DNA/lipid complexes to form. **You may incubate longer than 5 minutes, but do not incubate longer than 30 minutes, as this will be detrimental to the transfection efficiency.**
8. Add the plasmid DNA/siIMPORTER™ mixture to the cells from step 4. Rock plate gently for 20 seconds. Incubate at 37°C.
9. Four hours later, remove the media and replace it with media-containing serum. Add fresh culture media to the growing cells as needed.
10. Incubate cells at 37°C until they are ready for harvesting.

VI. Optimization

A. Adherent Cells

Although siIMPORTER™ consistently delivers high transfection efficiencies in a wide range of cell types, to obtain maximum efficiency in particular cell lines, some optimization may be needed. The two critical variables are the siIMPORTER™/siRNA ratio and the siRNA quantity. To optimize these two variables:

1. Determine the best siIMPORTER™/siRNA ratio by varying the amount of siIMPORTER™ while keeping a constant amount of siRNA.
2. Once the optimal ratio has been established, vary the siRNA quantity over the suggested range. At this point, cell number can also be optimized.

B. Suspension Cells

For suspension cells the optimization procedure is the same as for adherent cells except that the siIMPORTER™/siRNA ratio can be higher.

VII. Troubleshooting

Problem	Possible Causes	Recommended Solutions
Low transfection efficiency	Suboptimal siIMPORTER™/siRNA ratio	Optimize the siIMPORTER™/siRNA ratio by varying the amount of siIMPORTER™, while keeping a constant amount of siRNA.
	Suboptimal siRNA concentration	After establishing the optimal siIMPORTER™/siRNA ratio, vary the siRNA quantity over the ranges suggested in the Protocol section.
	Poor siRNA quality	Use RNase-free handling procedures and plastic ware. Check for siRNA degradation on acrylamide gels.
	Denatured siRNA	Use recommended buffer (cat. #20-272) to dilute siRNA. Water is not recommended as it can denature siRNA.
	Cells have been in continuous passage for > 2 months	Thaw and culture fresh vial of cells that are from a low passage number. Avoid using high passage number cells.
	Suboptimal cell density	Use cells that are 40-60% confluent on the day of transfection. Optimal cell density may vary depending on cell type.

Problem	Possible Causes	Recommended Solutions
Low transfection efficiency *continued*	Improper storage	siIMPORTER™ is very stable but long exposure to elevated temperatures and/or freeze/thaw cycles may cause degradation of the reagent. Store siIMPORTER™ at 4°C.
	Wrong medium	Be sure to use serum-free medium when forming the siIMPORTER™/siRNA complex.
	Serum in media on cells	Use serum-free medium during transfection and boost with serum-containing media 4 hours later.
	Adverse siRNA diluent effect	In some cell types, the diluent causes decreased transfection efficiencies.
	Cell line is difficult to transfect	Optimize siIMPORTER™/siRNA ratio and siRNA amount as indicated in the Optimization section VI.
	siIMPORTER™/siRNA complexes were not freshly prepared	Prepare siIMPORTER™/siRNA complexes just before use and store them no longer than 30 minutes.

Problem	Possible Causes	Recommended Solutions
Low transfection efficiency *continued*	Suboptimal siIMPORTER™/siRNA ratio used	Too much siIMPORTER™ or too much siRNA could cause aggregation. Adjust the ratio as outlined above.
Aggregation	Excess siIMPORTER™ used	Decrease amount of siIMPORTER™ used.
Cytotoxicity	Unhealthy cells	1. Check cells for contamination. 2. Thaw a new batch of cells. 3. Cells are too confluent or cell density too low. 4. Check culture medium (pH, type used, last time checked, etc.) 5. Check materials used for proper function (culture plates, incubator temperatures, etc.)
	siIMPORTER™ concentration too high	1. Reduce siIMPORTER™ concentration in 20-30% increments. 2. Replace media 4 hours after transfection.

VII. References

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