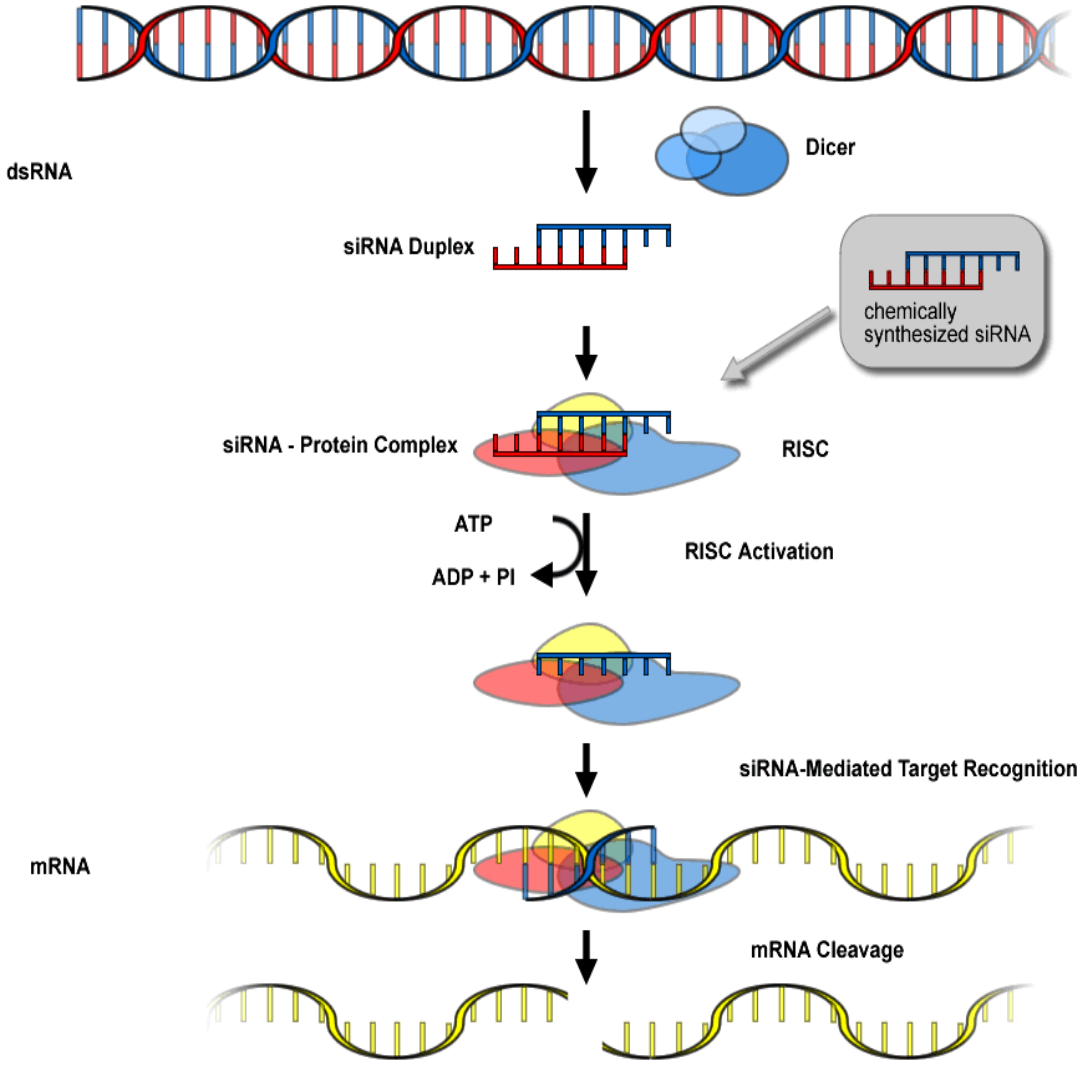
User's Manual and Instructions

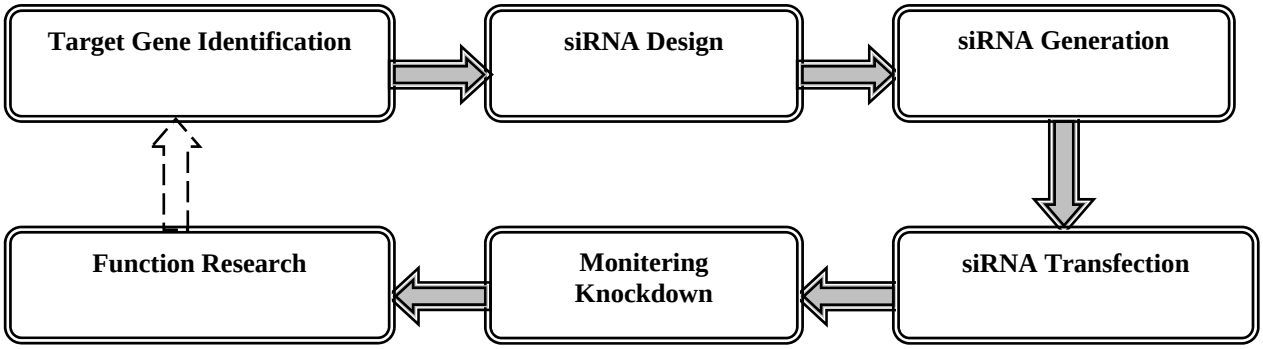
Ⅰ. RNAi Introduction

A. RNAi Experiment Machinery

RNA interfering（RNA interfering，RNAi） phenomenon was first unknowningly observed when RNA was shown to inhibit protein expression in plants and fungi by process, then known respectively as post-transcriptional gene silencing and quelling. In 1998,Fire and Mello first observed that double-stranded RNA was the source of sequence-specific protein inhibition in *C.elegans* known as RNA interference. While the studies in *C.elegans* were encouraging, RNAi was limited in use to lower organisms because delivering long dsRNA for RNAi was non-specifically inhibitory in mammalian cells. Further studies in plants and invertebrate animals demonstrated that actual molecules that lead to RNAi were short double-stranded RNA oligonucleotides,21 to 22 nucleotides in length, processed internally by an enzyme called Dicer. The Dicer cleaverage products are referred to as short (or small)interfering RNA and are today popularly known as siRNA



B. RNAi Workflow Solution



C. RNAi Components

Componets	Reagents Description
siRNA Oligos	Full Complement With mRNA of Your Knock-down Target gene
Transfection Reagents	Such as Cation liposome,Lipofectamin2000，Electricity Perforation
Controls	Negetive Control、Positive control and Mocking transfecion
Monitering Gene Experssion	mRNA Expression In Transcriptional Level Monitoring of Target protein In Translation Level

Ⅱ. siRNA Design

A. siRNA Design In

Notice:siRNA design in Mammalian ,as follows:

Efficiency of Gene Expression inhibition depend on selection target sequences。Target sequence may be selected at random, and also by specific sequence in different regions

1. Choose a region of 19 nucleotides.
2. Do not select sequences within the 5' and 3' untranslated regions (UTRs) nor regions near the start codon (within 75 bases) as these may be richer in regulatory protein binding sites (Elbashir et al., 2001).UTR-binding proteins and/or translation initiation complexes may interfere with binding of the RISC.
3. Do not select sequences that contain a consecutive run of 3 or more thymidine residues; a poly(T) tract within the sequence can potentially cause premature termination the shRNA transcript.
4. Calculate the GC content of the selected 19-base oligonucleotide sequence. The GC content should be between 40% and 60%; a GC content of approximately 45% is ideal.
5. Sequences that have at least 3 A or T residues in positions 15–19 of the sense sequence also appear to have increased knockdown activity.
6. Check the 19-base oligonucleotide for secondary structure and long base runs, both of which can interfere with proper annealing. Eliminate candidate sequences that display these characteristics.
7. Compare the remaining candidate sequences to an appropriate genome database to identify sequences that are specific for the gene of interest and show no significant homology to other genes. Candidate sequences that meet these criteria are potential siRNA target sites.
8. To optimize gene silencing, we highly recommend that you test more than one siRNA target sequence per gene.
9. The results show that:UU 3’ end and dTdT 3’ end siRNA have the same knock-donw efficiency

B. BioChain siRNA charachteristics

The company product covers chemical synthesis RNA monomer, common siRNA oligo and chemically modified RNA oligo; fluorescent dye for biology macro-molecule marker;biosynthesis siRN, shRNA; DNA transcribedly coding shRNA, plasmid vector transcribedly coding shRNA; Based on chemical synthesis RNAi complete service; Based on vector regulation RNAi comlete service; siRNA correlated reagents and RNA technology correlated product's sale; The common molecular biology reagent, the experiment material sale, and so on.Our RNAi correlated product not only in domestic occupied the very big market share, moreover continuously is selling to USA, Japan, Taiwan, Singapore, Germany, Sweden, England, France, Korea, the company 's sales at present had surpassed about the half comes from overseas market.

BioChain company have input high-throught MerMade-Ⅳ and ABI394 syntheser and many HPLC purification equipment. The international mainstream purification method - HPLC purification technology, guarantees high-qualified product.

BioChain siRNA Charachteristics	
Quality Control	All our oligos undergo vigorous process monitoring and strict quality control; Produce under ISO9000 quality standard system; Products are strictly quantitied by spectrophotometer.
Purification	HPLC; siRNA content >97%
Modification	3'and 5'end Biotin、FAM and Phosphorate
Length	19～23 base/each dsRNA
Storage and stability	Although oligonucleotides are stable in solution at 4° C for up to 2 weeks, we recommend storage should be at -20° C. Repetitive freeze-thaw cycles should be avoided by storing as aliquots. Storing at concentrations above 20 μM is recommended. We guarantee its oligonucleotides for 6 months, stored under the above conditions.
Technical data sheet	Oligonucleotides are delivered with an Oligonucleotide Technical Data Sheet, which includes oligo name, sequence, concentration, precise quantity in OD and nmols, Tm, MW, size, extinction coefficient and purification data.
Free service	Free design support

C. siRNA Oligo Technical Data

Notice:technical data related with siRNA

1. siRNA MW 13,300.
2. Calculating siRNA oligo OD、nmol and quality；Generally，for a 21 bp siRNA oligo，have a simple relation,as follows:1 OD duplex ＝3.0 nmols＝40 ug
3. 1 OD siRNA is diluted to 20 uM，use 150 uL DEPC H2O to resuspend 1 OD siRNA to make a 20 uM solution.
4. Fluorescent Dye Labeled siRNA Oligo RNA，such as FAM、HEX、TAMRA should be protected from light.

Ⅲ. siRNA Control

A. Common Negative Control

we should choose siRNA sequence which have the same composition as

1. siRNA experiment should have negative control;

negative control siRNA, but not obvious homology with mRNA.(scrambled control) The usual procedure is that we choose mismatch siRNA sequence, which we must guarantees target do not have homology.

2. Ready-to-use and haven't homologous sequence with target gene;
3. Scrambled siRNA sequence which have the same composition as negative control siRNA, but not obvious homology with mRNA;
4. Negative control should haven't homologous sequence with other gene sequence

B. Fluorescent Dye Labeled siRNA Negative Control

6-FAM negative control: Ready-to-use and haven't homologous sequence with target gene

1. RNAi negative control does not have homology with mammal gene
2. Conveniently observing transfection efficiency under fluorescence microscope
3. NC-FAM advantageous to optimization of transfection condition ;
4. The fluorescence is easy to photograph, it has very good pH tolerance, thus is stabler in the living cell

C. siRNA Positive Control

Positive control inspecting experimental system is very important. In other words, when you see siRNA positive control anticipated experimental result, you can guarantee that yours experimental technique, transfection, RNA extraction and detection method are reliable.

siRNA positive control	
1	LaminA/C
2	GFP22
3	Luciferase GL2
4	MAPK1
5	Beta-Actin
6	Vimentin
7	P53
8	GAPDH
9	Cyclophilin B

D. Mock Transfection

For one perfect control system, Mock Transfectionis necessary. Mock Transfectionmay examine Transfectionreagent influence on the toxicity of cell ,survival rate and so on.We recommend using transfection reagent-Lipofectamin2000. (See transfection reagent sheet)

A good way to enhance confidence in RNAi data is to demonstrate a similar effect with two or more siRNA targeted to different sites in the message under study.

E. Multiplicity Control

Ⅳ. siRNA Transfection

A. siRNA Transfection Methods

At present, common method in mammalian transfection includes: Calcium phosphate co-precipitation, Electricity perforation law, DEAE-glycon and polybrene, machinery method (for example, microinjection and gene-gun), cation liposome reagent. Cation liposome reagent is most commonly used transfection method.

Notice: Cation liposome transfection reagent should be:

1. Transfection reagents volumes
2. siRNA Amounts
3. Cell density during transfection
4. Operation order during transfecion
5. The incubation time of cell and tranfection reagents/siRNA complexes

B. Lipofectamin2000 Transfection Reagents

Transfection of primary culture and transformation of cell strain gene;
High-throughput transfection of siRNA /DNA tansfection;
Co-transfection of DNA and siRNA;
In vivo delivery of siRNA oligos(siRNA,DNA and RNA);
Transfection of adherent cell and suspension cells.

Lipofectamin2000
Applied field:

- ☐ Transfection of primary culture and transformation of cell strain gene
- ☐ High-throughput transfection of siRNA
- ☐ DNA tansfection;Co-transfection of DNA and siRNA
- ☐ In vivo delivery of siRNA oligos(siRNA,DNA and RNA)
- ☐ Transfection of adherent cell and suspension cells

Lipofectamin2000
Features:

- ☐ No necessary to change culture media. Easy to operate. Good repeatability.
- ☐ Transfect siRNA oligos in high efficiency
- ☐ High trnasfection efficiency can be obtained even in culture media containing serum
- ☐ Ready to use, transfection in culture medium with antibiotics
- ☐ Cation liposome reagen ensure that No RNAase
- ☐ High transfection efficiency in vivo

C. Lipofectamin2000 Applied Cell Lines

Lipofectamin2000 can be used for the transfection of DNA and siRNA into many different kind cells. Our new siRNA transf ection reagents have been extensively tested in many different kinds of cell lines originated from different sources, ranging from standard lines, e.g. HeLa,MC-7,Hep3B,COS-7,Neuro-2a,NIKS,B16,DLD-1,NIH/3T3,HT-29,A549, CHO-K1 and 293, and SVRb ag4

D. Cell Culture Before Transfection

It is advised that before starting your transfection experiment ,put your cells

on your cell plate, then add proper culture medium , lastly incubate cells for 24 hrs to be 70%-90% by confluence.

Culture vessel	Surface Area (mm^2/well)	Cell Density	Volume of plating medium (μL /well)
96-well	50	1.5×10^4-5.0×10^4	100 μL
48-well	100	3.0×10^4-1.0×10^5	200 μL
24-well	200	8.0×10^4-2.0×10^5	500 μL
12-well	401	1.6×10^5-4.0×10^5	1.0 mL
6-well	962	3.0×10^5-8.0×10^5	2.0 mL
35 mm	962	3.0×10^5-8.0×10^5	2.0 mL
60 mm	2827	1.0×10^6-2.5×10^6	6.0 mL

E. Lipofectamin2000：Ratio of siRNA/DNA

The appropriate ratio of Lipofectamin2000：siRNA/DNA is very crucial to achieve high efficiency for transfection. It is recommended that the appropriate ratio of DNA：lipofetamin2000 is 1：0.5—1：5（ug:ul），siRNA：lipofectamin2000 is 1：0.01-1：0.1（pmol：ul），generally，you can get good results in the range.

Culture vessel	**siRNA/DNA**	Volume of plating medium (μL /well)	**Lipofectamin2000(siRNA/DNA)**
96-well	5pmol/0.2 μg	100 μL	0.25 μl/0.5 μl
24-well	20pmol/0.8 μg	500 μL	1 μl /2 μl
12-well	40 pmol /1.6 μg	1 mL	2 μl /4 μl
6-well	100 pmol /4.0 μg	2 mL	5 μl /10 μl
35 mm	100 pmol /4.0 μg	2 mL	5 μl /10 μl
60 mm	600 pmol /8.0 μg	5 mL	10 μl /20 μl

F. Adherent Cell Transfection Procedure

IMPORTANT

This procedure is suited to adherent cell transfection using 24-well plate. Choosing healthy cell is very important for enhancing transfection efficiency. The quantity of siRNA(DNA) and DNA and the ratio between the siRNA(DNA) and Lipofectamin2000 can be adjusted slightly within the recommended range.

1. One day before transfection，incubate 4-5 x 104 cell into 24-well plate，add 0.5mL culture medium containing FBS and antibiotics
2. Choose the appropriate cell quantity of primary incubation to make sure that cell fusion can reach 70-90%.
3. Dilute 20pmol siRNA(or 0.8 μg DNA) in 100 μl serum-free medium， add 1 μl Lipofectamin2000 reagent（when transfect DNA, 2. μl Lipofectamin2000 reagent is added）mix thoroughly，incubate at room temperature for 20min in order to form siRNA/Lipofectamin2000 （ or DNA/Lipofectamin2000）complex。
4. Add siRNA/Lipofectamin2000（or DNA/Lipofectamin2000）complex into culture medium and mix up gently。
5. After incubating cells at 37℃ for 24h-48h，continue other steps for transfection.

G. Suspension Cells Transfection Procedure

1. On the day you start transfection,collect cells and centrificate, ，then resuspend in the FBS culture medium.
2. Dilute 20pmol siRNA(or 0.8μg DNA) in 100μl serum-free medium， add 1μl Lipofectamin2000 reagent（when transfect DNA, 2μl Lipofectamin2000 reagent is added）, then add the above mixture into the wells of the 24-well plate.
3. Incubate at room temperature for 30min in order to form siRNA/Lipofectamin2000（or DNA/Lipofectamin2000）complex。
4. Add 400μL cell suspension solution（cell quantity is determined by cell type and the time needed for analysis after transfection）
5. After incubating cells at 37℃ for 24h-48h，continue other steps for transfection.

H. DNA and siRNA Cotransfecion

1. One day before transfection，incubate 4-5 x 104 cell into 24-well plate，add 0.5mL culture medium containing FBS and antibiotics
2. Choose the appropriate cell quantity of primary incubation to make sure that cell fusion can reach 70-90%.
3. Dilute 20pmol siRNA and 0.2μg DNA in 100μl serum-free medium，add 2μl Lipofectamin2000 reagent，mix thoroughly，incubate at room temperature for 20min in order to form siRNA/DNA/Lipofectamin2000 complex。
4. Add siRNA/DNA/Lipofectamin2000 complex into culture medium and mix up gently。
5. After incubate cells at 37℃ for 24h-48h，continue other steps for transfection.

I. Transduction In Vivo

1. Appropriate amount siRNA and DNA is dissolved in RNAase-free sterile water， mix up gently， because volume of the injection is limited， we suggest high concentration siRNA or DNA，generally DNA is 2μg / μL、siRNA is 10μg / μL.
2. Mix up appropriate amount DNA、siRNA or siRNA\DNA complex with Lipofectamin2000。For example，in No.1 tube add 0.5μL of DNA（1μg）and 0.5μL of siRNA（5μg），in No.2 tube add 0.55μL of Lipofectamin2000

Tel: 1-888-762-2568 Fax: 1-510-783-5386 Email: info@biochain.com

（24μg） and 0.45μL of RNAase-free sterile water，the collect No.1 tube solution and add them into No.2 tube，incubate at room temperature for 30min，in order to form siRNA/Lipofectamin2000 （ or DNA/Lipofectamin2000）complex。

3. Preparation siRNA/DNA-Lipofectamin2000 complex can be used to transduct siRNA、DNA siRNA\DNA in vivo.

J．siRNA FAQs and Suggestion

Low transfection efficiency	Suggestion
Not optimized Lipofectamin2000：siRNA(DNA) ratio	Optimize DNA：lipofetamin2000 is 1：0.5—1：5（ug:ul），siRNA：lipofectamin2000 is 1：0.01-1：0.1（pmol：ul）
Concentration of siRNA/Lipofectamin2000（or DNA/Lipofectamin2000）complex is too low	Slightly increase concentration of siRNA/Lipofectamin2000（ or DNA/Lipofectamin2000）complex
Condition of cell growth is bad	Cells with non-optimal conditions decrease Transfectionefficiency. Suggest that cell fusion can reach 40-70% in 24hrs after incubation，finishing transfection operation in 24hrs。
Purify of DNA or siRNA is too low	Use high-purify DNA or siRNA，ideally use column purified DNA and HPP grade siRNA
The culture medium used to dilute the DNA or siRNA containing serum	Generally，serum can not depress the formation of siRNA/Lipofectamin2000（or DNA/Lipofectamin2000）complex dramatically，suggest using serum-free culture medium to dilute DNA or siRNA
The repeatability is bad	**Suggestion**
Cell confluent is not uniform	Using the same amount of master cell, cultural time and cultural conditions after incubating must be uniform
Times of cell subculture is too many	Using low subculture times cells
Cells died apparently	**Suggestion**
Key gene related to cell survive is shut down	Re-design the experiment
Cell conditions is not very good	Using low subculture times cells and cell fusion can reach 40-70% in 24hrs after incubation，finishing transfection operation within 24hrs。
Concentration of siRNA/Lipofectamin2000（or DNA/Lipofectamin2000）comlex is too high	Generally, siRNA/Lipofectamin2000（or DNA/Lipofectamin2000）complex would not affect cell growth ,but when concentration is too high ,sometimes it may produce some cell toxicity
Gene expression or gene silencing efficiency is lower	**Suggestion**
Expression vector design is not correct or siRNA design is not correct	Re-design the experiment
Cultural time is too short after transfection	Gene expression need time, so prolong the culture time appropriately in necessary

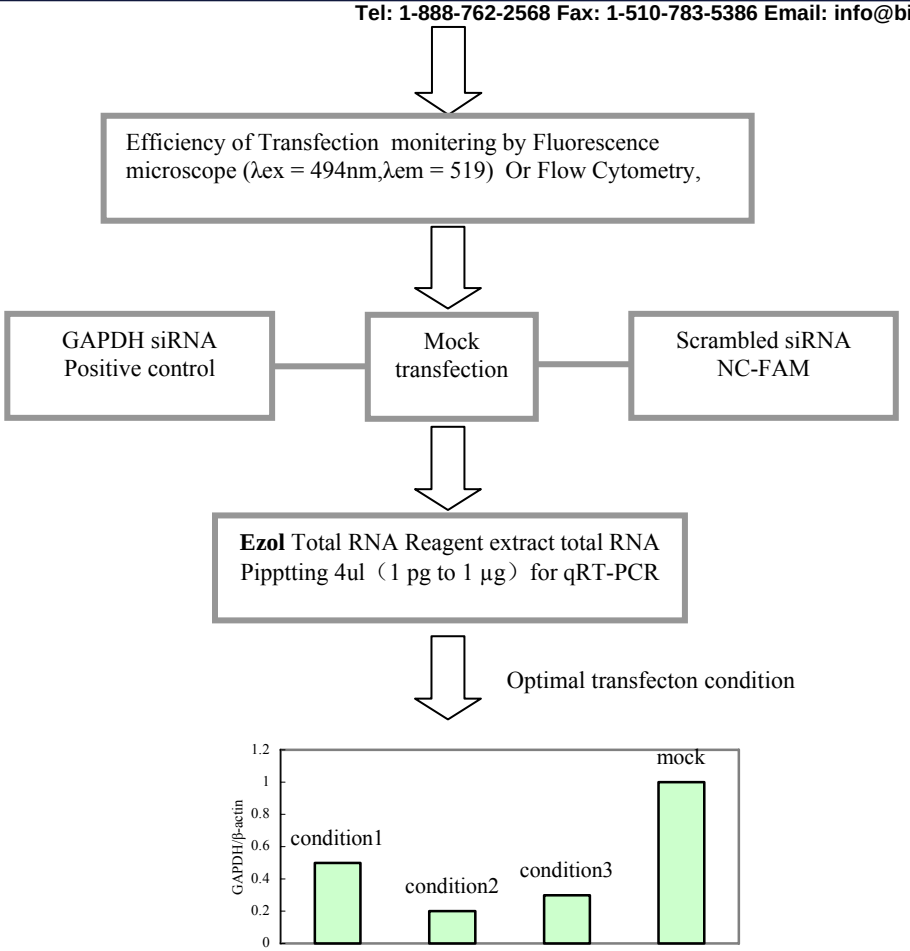
Ⅴ. mRNA Expression In Transcriptional Level

A. siRNA siRNA Cell TransfectionOptimization

The best method of validated siRNA and transfection:specific-gene treated cells and negative control treated cells,monitor mRNA expression in transcriptional level by qRT-PCR.

1. Optimal Procedure of Cell Transfection （RNAi-Startup GAPDH Control Kit）

Culture vessel	siRNA	siRNA range	Vol. of dilution medium	Lipofectamin2000	Lipofectamin2000 range
96-well	5pmo	2.5-10pmol	100μL	0.25μl	0.1-0.5μL
24-well	20pmol	10-40pmol	500μL	1μl	0. 5-2μL
12-well	40 pmol	20-80pmol	1 mL	2μl	1-4μL
6-well	100 pmol	50-200pmol	2 mL	5μl	2.5-10μL
35 mm	100 pmol	50-200pmol	2 mL	5μl	2.5-10μL
60 mm	600 pmol	0.3-1.2nmol	5 mL	10μl /	5-20μL



2. RNAi-Startup GAPDH Control Kit Components

The kit makes enough reagents for 20 reactions in transfection optimization,also for Endogenous control of RNAi experiment。By Fluorescent dye labeled dsRNA,directly observe cell transfection efficiency ,and furturemore,by Real-Time PCR evaluate inhibition efficieny of GAPDH before and after transfecion.

Amount	Component	Storage
500μl	Real-time PCR Master Mix（5×）	4℃
1nmol	GAPDH siRNA Positive control	-20℃
125μl	Scrambled siRNA NC-FAM（20μM）	-20℃*
60μl	GAPDH mix（10μM）	-20℃*
60μl	β -actin mix（10μM）	-20℃*
25μl	Taq DNA polymerase （5U/μl）	-20℃
1ml	1X RNA Dilution buffer	4℃

* Keep in dark place and avoid multiple freeze thaw cycles

B. Evaluation of RNAi Efficiency by Real-Time PCR

Real-Time PCR adds fluorescent dye labeled probes based on traditional PCR reaction system,and monitors real-time process of PCR reaction by detecting changes of fluorescent signals in tube.Monitoring RNAi efficiencyc by real-time PCR only have two steps:I.Total RNA extraction;II.qRT-PCR.

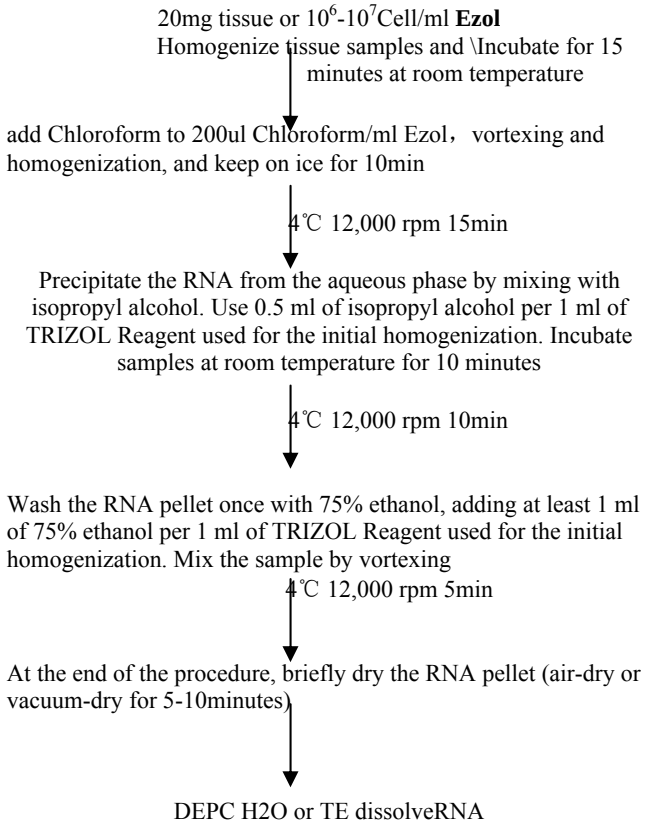
1. Cell Total RNA Extraction（Ezol Total RNA Extraction Reagent）

☐EZOL

performs well total RNA extraction from human, animal, plant, or bacterial origin. The simplicity of the EZOL Reagent method allows simultaneous processing of a large number of samples. The entire procedure can be completed in one hour. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase. Copurification of the DNA may be useful for normalizing RNA yields from sample to sample.

☐Prodution characteristics

Isolating high-purity and high-yields RNA
Performing well RNA extraction with many typies of cell lines and tissues
performing well with small quantities of tissue (50-100 mg) and cells (5 × 106), and large quantities of tissue (≥1 g) and cells (>107)



2. RT Reaction for cDNA Synthesis

Preparing RT Reaction master mix should keep on ice;All reagents homogenization.

Table. **RT reaction system components**

Amount per reaction	Component
5 μl	5×RT-Buffer
0.75 μl	10mM dNTP
3 μl	25mM Mg^{2+}
0.125 μl	Oligo dT_{15} （50 μM）
— μl（10～25ng）	RNA Extracts
0.25 μl	RNAsin（40U/ul）
0.5 μl	M-MLV（200U/ul）
To 25 μl total volume	RNase Free H_2O

If you doubt that your templates have secondary strutures,you can increase RT temperature to

42℃。

☐ Incubate in 37℃ for 30 min ,RT reaction；
☐ Inactivate reverse transcriptionase in 85℃ 10 min;
☐ If your are not read to use, plase store at –20℃；

3. Real-Time PCR reactions

Per rxn	Component
8 μl	5×PCR Master Mix（including PCR primer，Fluorescent dye labled probes）
4 μl	RT products
0.4 μl	Taq DNA polymerase（5U/ μl）
To 40 μl	dd H_2O

IMPORTANT

When using TaqMan Probes or SYBR Green1 monitoring, collect fluorescent signal during PCR extention phase;; when use Beacon probes,, collect fluorescent during PCR annealing phase

Real-Time PCR reactions conditions：

94_℃ for 2 min， 94_℃ for 20 s， 60℃30 s 40 cycles

C. Real-Time PCR Results Analysis

As a RNAi experiment,we determined if siRNA oligos have knockdown target gene by monitoring gene expression assay duiring siRNA transfecting into certen cell line. By RT-PCR, two methods can be used to achieve above results:

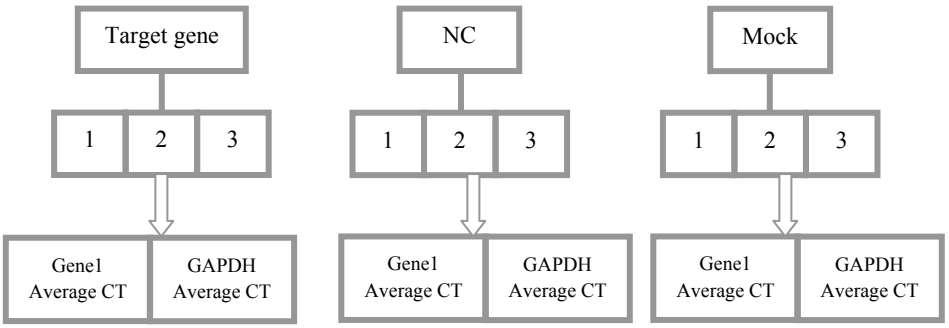
- One is that successful transfection of siRNA into cells causes a reduction in the levels of target gene mRNA ,named absolute quantitation of gene expression.
- Another is that amplification of an endogenous control may be performed to standardize the amount of sample RNA or DNA added to a reaction. For the quantitation of gene expression, researchers have used β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA (rRNA), or other RNAs as this endogenous control.

Normally monitoring knockdown of target gene by relative quantitation.

1. Real-Time PCR Experiment Design

Real-Time PCR Experiments design should be including three groups:experimental group(siRNA transfect into cells);Negative Control）and Mock Transfaction.Each group has three repeations at leas. Eevry group should monitoring target gene and house keeping gene Ct value at the same

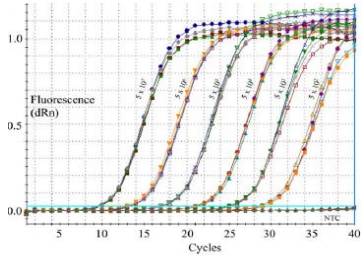
time.Next example set GAPDH as house keeping gene.



2. Real-Time PCR Ct Value for siRNA Transfection of Target Gene and House-keeping Gene

IMPORTANT

Between each repeated group difference of Ct value should be little.Generally, difference of Ct value in range of 1 is acceptable.



Sample		Negative control		Mock Transfection	
Target Gene	GAPDH	Target Gene	GAPDH	Target Gene	GAPDH
30.40	23.63	24.21	22.66	26.21	24.60
30.35	23.40	24.60	22.56	26.15	24.31
30.41	23.52	24.66	22.48	26.35	24.72

3. Real-Time PCR data analysis

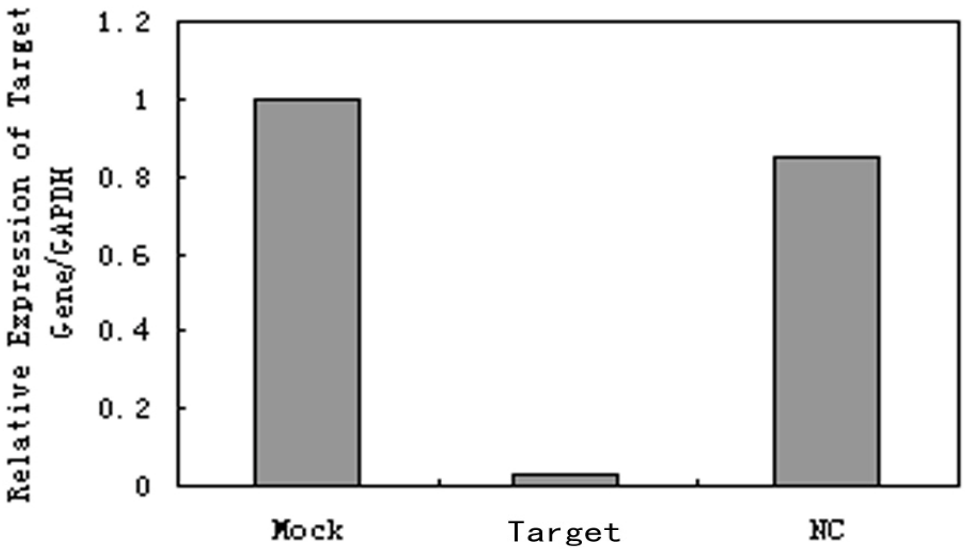
Designated Mock Tansfection as the Calibrator，GAPDH gene for Normalizer，ΔΔCt=($Ct_{Target\ gene}$-Ct_{GAPDH})Target Gene —($Ct_{Target\ Gene}$-Ct_{GAPDH})Calibrator

	Target Gene Average Ct	GAPDH Average Ct	ΔCt Target Gene–GAPDH	ΔΔCt ΔCt–ΔCt, Mock	Target Gene Rel. to Mock
Mock	26.24±0.10	24.54±0.21	1.7±0.21	0.00±0.21	1.0(1.16—0.86)
Target	30.39±0.09*[1]	23.51±0.15*[2]	6.88±0.17*[3]	5.18±0.17	0.027(0.024—0.031)
NC	24.49±0.24	22.56±0.09	1.93±0.25	0.23±0.25	0.85(0.71—1.01)

注：*1 The calculation Ct standard deviation，using Excel ;

*2 The calculation using following formula: $s = \sqrt{s_1^2 + s_2^2}$ ， as a example: $\sqrt{(0.15)^2 + (0.09)^2} = 0.17$

*3 The calculation using following formula:$2^{-\triangle\triangle Ct+S}$ and $2^{-\triangle\triangle Ct-S}$，S is △△Ct standard deviation，as a example: $2^{-(-2.5+0.10)} = 5.3$



Ⅵ. Monitoring of Target protein In Translation Level

A. Western-blot Machinery

Western Blot:
The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin, et al. in 1979 and is now a routine technique for protein analysis. The specificity of the antibody-antigen interaction enables a single protein to be identified in the midst of a complex protein mixture. Western blotting is commonly used to positively identify a specific protein in a complex mixture and to obtain qualitative and semiquantitative data about that protein.

Western immunoblotting（Western Blot）transferred protein onto a nitrocellulose or polyvinylidene Difluoride (PVDF) membrane, then an antibody is used to specifically detect its antigen.

B. Western-blot Procedure

1. Collect cells and wash with 1X PBS

a. Collect the cells from a 24 well tissue culture plate using trypsin-EDTA and transfer to a 1.5 ml microcentrifuge tube.

b. Centrifuge the cells at 600 x g for 5 min, and discard the supernatant.

c. Wash with PBS by adding 500 µl 1X PBS and centrifuging as in the
previous step. Discard the PBS wash.

2. Lyse the cells on ice for 15 min

a. Add 100 µl of lysis buffer to each sample and vortex for 15 sec.

b. Incubate for 15 min on ice.

3. Collect the lysate and determine the total protein concentration

a. Centrifuge at 16,000 x g at 4°C for 10 min to pellet cellular debris
and transfer the supernatant to a fresh tube. Store the clarified lysate
on ice for immediate use; alternatively store at –20°C.

b. Determine the total protein concentration.

4. Perform PAGE, and transfer protein to a blotting membrane	a. Mix clarified lysates with gel loading buffer. The protein amount and sample volume will depend on the size of the gel, for minigels, we recommend running samples containing ~5 µg total protein per well. Analyze equal protein amounts of each sample. b. Heat samples to 95°C for 3 min to denature proteins, then store on ice while you set up the gel apparatus. c. Load and run the gel. d. Transfer the protein to a support membrane.
5. Detect GAPDH on the membrane using Anti-GAPDH MAb	a. Block nonspecific binding by immersing the membrane in blocking reagent for 1 hr. b. Wash the membrane with PBST (0.1% Tween 20, 1X PBS) three times for 5 min each. c. Add 1 µg/ml Anti-GAPDH MAb diluted in fresh blocking solution to the membrane and incubate for 1 hr. d. Wash the membrane with PBST three times for 5 min each. e. Add the secondary antibody diluted according to the supplier's recommendations in fresh blocking solution and incubate for 1 hr. f. Wash the membrane with PBST three times for 5 min each. g. Detect the Anti-GAPDH MAb using an appropriate detection method for the conjugated secondary antibody.

C. western-blot Required Reagents and Materials:

- Routine cell culture supplies and reagents such as 1X PBS, trypsin-EDTA, pipettes, plates, tubes, etc.
- Lysis buffer: 50 mM HEPES pH 8.3, 420 mM KCl, 0.1% NP-40, 1 mM EDTA
- Reagent to measure total protein concentration such as Lowry reagent
- Protein gel electrophoresis equipment and supplies: we recommend using 12% acrylamide/bisacrylamide (29:1) SDS gels with a stacking gel for good separation of the 36 kDa GAPDH protein
- Western blotting reagents and equipment such as transfer membrane and apparatus, rocker platform agitator, blocking buffer: e.g., 1% dry milk in 1X PBS, PBST: 0.1% Tween 20 in 1X PBS, anti-mouse secondary antibody conjugated to the enzyme or ligand of choice, and appropriate detection reagents/equipment

Ⅶ. FAQs

What is the best location on the duplex to incorporate a dye label?
Modification at the 5'-end of the antisense strand has been shown to interfere with siRNA silencing activity and therefore this position is not recommended for modification. Modifications at the other three termini have been shown to have minimal to no effect on silencing activity. Modification on the 5'-end of the sense strand is the position that provides the most efficient chemical synthesis and thus is recommended.

If I have the protein sequence for a particular gene product, can an siRNA be designed to target this protein?
siRNAs function at the mRNA level, not at the protein level. In order to design an siRNA, the precise target mRNA nucleotide sequence is required. Due to the degenerate nature of the genetic code and codon bias, it is impossible to accurately predict the correct nucleotide sequence from the peptide sequence. Additionally, since the function of siRNAs is to cleave mRNA sequences, it is important to use the mRNA nucleotide sequence and not the genomic sequence for siRNA design. Pre-mRNA processing removes intron sequences that are contained in the genomic sequence. Designs using genomic information might inadvertently target introns and as a result the siRNA would not be functional for silencing the corresponding mRNA.

Will an siRNA designed to target the human version of a particular gene work in a different species?
siRNAs designed specifically to target human genes are not expected to silence homologs in other species. Thus far, of the siRNAs designed to be species-specific, few cross-target another species, even in cases where a high identity exists between the gene sequences of two species. However, there are instances where siRNAs can be specifically designed to be functional in two or more species, but this involves careful siRNA design and bioinformatics analysis.

What is the best method of delivering siRNAs into the cell?
There are several methods that are used for delivering siRNAs into cells including lipid-based transfection, electroporation, calcium phosphate co-precipitation, microinjection and vector delivery techniques. The choice between these methods is often a result of several factors including the ability of the cells to tolerate the delivery method, susceptibility to viral infection, and the growth properties of the cells. Although lipid-based transfection is one of the more commonly used methods for adherent cells, suspension cells are often more difficult to transfect and generally have higher rates of delivery with electroporation techniques.

Which transfection reagent is recommended for delivery of siRNA?
The choice of a transfection reagent often depends more upon the particular cell line than the substance being delivered into the cells. Further optimization for siRNA delivery may be necessary.

Is there a quick method for monitoring transfection efficiency?
The uptake of siRNAs can be visualized with the appropriate filters on a confocal microscope or by flow cytometry. Alternatively, fluorescent-labeled siRNA is cytotoxic when successfully delivered into cells. Cells that have efficiently taken up this transfection control typically undergo apoptosis within 24 to 48 hours. This phenotypic outcome can easily be monitored using standard cell viability methods (e.g., alamarBlue, MTT cytotoxicity, Trypan Blue dye exclusion, JC1 dye, or other appropriate assays).

I see a fair amount of cell death when I transfect. What can I do about this?
Extensive cell death following transfection is an indication that delivery conditions need to be further optimized. Basic parameters to consider when optimizing transfections include transfection reagent and cell specific conditions such as the ratio of siRNA:lipid reagent, the lot/batch of transfection reagent, duration of transfection, cell passage number and cell density at transfection. Often

decreasing the amount of lipid present during transfection and/or the total duration of transfection will help minimize the toxic effect to the cells. Additionally, it is not uncommon to observe significant variability from one tube of transfection reagent to another, and this may also represent a source of experimental variability. If the problem persists, we recommend that other transfection reagents be considered

Why is optimizing transfection efficiency important?
Transfection efficiency is a measure of the proportion of cells that successfully internalized the siRNA. Low transfection efficiency will result in lower observed silencing, not because the siRNA is a poor silencer, but because mRNA expression from untransfected cells will contribute to the total observed mRNA level.

I transfected my siRNA and I'm seeing less than 75% silencing at the mRNA level. What is wrong?
The two most frequent causes of poor silencing are low transfection efficiency and poor siRNA sequence design. Often the lack of silencing is related to inefficient delivery of siRNA into the cells, suggesting that transfection conditions need to be optimized. If these procedures to optimize transfection conditions have already been performed and poor or moderate silencing persists, an alternative transfection reagent or technique (e.g., electroporation) may provide better delivery for that particular cell line.

If transfection efficiencies have been optimized and poor silencing results are still observed, the siRNA sequence design may be the cause. Conventional siRNA design rules and publicly available design tools vary significantly in their ability to select functional sequences for difficult-to-silence genes. In addition, poor to moderate siRNA duplexes have been shown to exhibit variability in silencing efficiency.

What is the best method for monitoring siRNA functionality?
siRNA-mediated silencing occurs as a result of target mRNA recognition and cleavage. The status of the target mRNA level is critical for understanding the experimental system and for isolating potential issues while troubleshooting. Because of the variability in protein stability and turnover rates in biological systems, the time course and degree of protein reduction may differ significantly from that of the target mRNA. Thus, while protein measurement provides important experimental information, measurement of the silencing effect at the mRNA level is the most important and reliable indication of siRNA-mediated silencing efficiency. Once successful silencing has been confirmed at the mRNA level, subsequent measurement of target protein levels can assist in correlating mRNA reduction with phenotypic effects. Time-course studies to assess maximal protein reduction may need to be performed to further optimize transfection protocols for protein-level studies.

I plan on using RT-PCR to detect knockdown of my target gene expression. Is there anything I need to take into consideration when designing my primers?
We recommend that primers be designed to bracket one of the siRNA cleavage sites as this will help eliminate possible bias in the data (i.e., one of the primers should be upstream of the cleavage site, the other should be downstream of the cleavage site). Bias may be introduced into the experiment if the PCR amplifies either 5' or 3' of a cleavage site, in part because it is difficult to anticipate how long the cleaved mRNA product may persist prior to being degraded. If the amplified region contains the cleavage site, then no amplification can occur if the siRNA has performed its function.

What is the best time-frame for monitoring siRNA-dependent decreases in target mRNA expression levels? In target protein levels?
Generally, target mRNA levels are decreased after 24 hours post-transfection following transfection of a gene-specific siRNA duplex. However, maximal silencing may be reached at a later time point, so it is advisable to assay target mRNA in a time-course study. In most cases, silencing will be maximal at 24 to 48 hours following transfection.

Cellular target protein levels should be examined starting at 24 hours and assayed until a minimum level is noted, often 48 to 96 hours or greater. As always, it is important to verify transfection efficiency using an appropriate positive control. If there is no decrease in protein levels within this

time frame, it may be necessary to perform a second siRNA transfection, use a stabilized siRNA, or develop a vector-based silencing cassette that can continuously produce the siRNA for extended periods of time.

I transfected my siRNA at 100 nM and only saw 50% silencing. Should I increase the siRNA concentration to 200 nM or even 400 nM?
Increasing the concentration of a moderately functional siRNA generally does not improve silencing ability. In addition, higher concentrations of siRNA can lead to significant off-target effects and can ultimately be toxic to the cells. In contrast, highly functional siRNAs that result from rational design algorithms typically generate 75% or greater silencing at 100 nM or lower concentrations. We recommend that the concentration and integrity of the siRNA be assessed as a first step in troubleshooting. The absorbance at 260 nm should be used together with the provided extinction coefficient to confirm concentration. The integrity of the duplex may be verified on a non-denaturing PAGE where a single band representing the duplex should be observed. Finally, the lack of significant silencing can be a result of poor transfection efficiency, suggesting that siRNA delivery should be further optimized.

How are fluorescently labeled siRNAs detected?
Fluorophore labeled duplexes are popular items for optimizing transfection conditions. Uptake of the labeled pre-synthesized siRNAs is readily detected either by flow cytometry or by fluorescence confocal microscopy. We recommend that you use protocols and procedures that are specific for your optical system and software package to detect the fluorescently labeled cells. It is also important to consider that fluorescence may not correlate with siRNA function unless the siRNA has been modified specifically for nuclease resistance.

How do I calculate the amount of ug siRNA in each well if I use 6 uL of a 10 uM stock siRNA solution?
There are several steps to completing this calculation. First, calculate how many nmol siRNA are in each well:

a. equation: ? nmol = (6 uL)(10 umol/L)
b. unit conversions: ? nmol = (6 uL)(10 umol/L)(1 L/10exp6 uL)(1000 nmol/umol)
c. answer: ? nmol = 0.06 nmol
Therefore, there is 0.06 nmol of siRNA in each well.

Next, use the molecular weight of siRNA to convert between nmol and ug. If the specific molecular weight is not known, you may use the average molecular weight of siRNA, which is 13,300 g/mol.

a. equation: ? ug = (0.06 nmol)(13,300 g/mol)
b. unit conversions: ? ug = (0.06 nmol)(13,300 g/mol)(mol/10exp9 nmol)(10exp6 ug/g)
c. answer: ? ug = 0.798 ug, or 0.8 ug
Therefore, there is 0.8 ug of siRNA in each well, when 6 uL of a 10 uM siRNA stock solution is used.

How do I convert between nmol and ug of siRNA?
Use the molecular weight of siRNA to convert between nmol and ug. If the specific molecular weight is not known, you may use the average molecular weight of siRNA, which is 13,300 g/mol.
If, for example, you have 5 nmol of siRNA, the conversion would be performed using the following steps:
a. equation: ? ug = (5 nmol)(13,300 g/mol)
b. unit conversions: ? ug = (5 nmol)(13,300 g/mol)(mol/10exp9 nmol)(10exp6 ug/g)
c. answer: ? ug = 66.5 ug
Therefore, a 5 nmol quantity of siRNA is 66.5 ug.

Why are positive controls important for an RNAi experiment?
A positive control is useful as an experimental systems check. That is, when you see the expected results with a positive control siRNA, you have reasonable assurance that your transfections, as well as your RNA extraction and assay, are reliable and robust in your experimental model.

Tel: 1-888-762-2568 Fax: 1-510-783-5386 Email: info@biochain.com

Are negative controls really necessary?

A non-targeting siRNA control is important to help establish that any decrease in gene expression levels observed with a gene-specific siRNA is related to a sequence-specific RNAi event. Global down-regulation events may be due to cellular stress responses to a certain transfection reagent or technique. Without negative controls, a researcher might mistakenly interpret this broad, non-specific silencing as true gene-specific silencing. To control for non-RNAi related or non-specific effects,BioChain offers a variety of products for RNAi experiments.

I heard I should use a scrambled control that has the same GC content as my siRNA. Isn't that better than a random sequence?

A true scrambled control containing the same base composition as your target specific siRNA might be considered a good non-specific control, provided that the scrambled siRNA has been verified to not inadvertently target another gene. However, experimental data indicate that even when no known target is identified, certain non-targeting siRNAs result in some cellular toxicity. With respect to preserving the overall base content (or GC content), BioChain has found that this is less critical as the relative position of the bases will also have an impact on interactions with the silencing machinery. Until a better method is available for predicting the potential for toxicity or non-specific effects, it is recommended that a non-targeting siRNA that has been validated and determined to be non-functional or "inert" be used as a negative control.

How do I store my siRNA samples?

siRNAs should be stored at -20°C or -70°C in a non-frost free freezer, either as a dried pellet or resuspended in an RNAse-free solution buffered to pH 7.4 - 7.6 to help with stability during freeze-thaw cycles. We recommend that the siRNAs be resuspended to a convenient stock concentration (20-100 uM) and stored in small aliquots to avoid multiple freeze thaw cycles. siRNAs should not go through more than 5 freeze thaw cycles. When stored under these conditions and using good RNase-free technique, they typically remain stable for more than 6 months. If degradation is a concern, the integrity of the siRNA duplexes can be evaluated on an analytical PAGE gel.