Turbo Dicer siRNA Generation Kit

Instruction Manual

Catalog Number

T520001

Genlantis
A Division of Gene Therapy Systems, Inc.
10190 Telesis Court
San Diego, CA 92121
Phone: 888-428-0558 (US. Toll-Free) • 858-457-1919
Fax: 858-623-9494 • 858-558-3617
E-mail: orders@genlantis.com
Web Site: http://www.genlantis.com

PAGE INTENTIONALLY LEFT BLANK

Purchaser Notification

Limited License

The purchase price paid for the Turbo Dicer siRNA Generation Kit by end users grants them a non-transferable, non-exclusive license to use the kit and/or its separate and included components (as listed in the Kit Contents section). This kit is intended **for internal research only** by the purchaser. Such use is limited to protocols described in the product manual. Furthermore, research only use means that this kit and all of its contents are excluded, without limitation, from resale, repackaging, or use for the making or selling of any commercial product or service without the written approval of Genlantis, a division of Gene Therapy Systems, Inc ("GTS").

Purchasers may terminate this License at any time by returning all Turbo Dicer siRNA Generation Kit material and documentation to GTS, or by destroying all Turbo Dicer siRNA Generation Kit components. Purchasers are advised to contact GTS with the notification that a Turbo Dicer siRNA Generation Kit is being returned in order to obtain a refund and/or to expressly terminate a research only license granted through the purchase of the kit.

This document covers in full the terms of the Turbo Dicer siRNA Generation Kit research only license, and does not grant any other express or implied license. The laws of the State of California shall govern the interpretation and enforcement of the terms of this License.

Product Use Limitations

The Turbo Dicer siRNA Generation Kit and all of its components are developed, designed, intended, and sold for research use only. They are not to be used for human diagnostic or included/used in any drug intended for human use. All care and attention should be exercised in the handling of the kit components by following appropriate research lab practices.

For more information, or for any comments on the terms and conditions of this License, please contact:

Director of Licensing
Genlantis, a division of Gene Therapy Systems, Inc.
10190 Telesis Court, San Diego, CA 92121.
Telephone: 858-457-1919
Fax: 858-623-9494.
Email: **licensing@genlantis.com**

Limited Label License for the Recombinant Turbo Dicer Enzyme

This product is covered by several patent applications owned by the Stanford University. The purchase of this product conveys to the buyer the limited, non-exclusive, non-transferable right (without the right to resell, repackage, or further sublicense) under these patent rights to perform the siRNA production methods claimed in those patent applications for research purposes solely in conjunction with this product. No other license is granted to the buyer whether expressly, by implication, by estoppel or otherwise. In particular, the purchase of this product does not include nor carry any right or license to use, develop, or otherwise exploit this product commercially, and no rights are conveyed to the buyer to use the product or components of the product for any other purposes, including without limitation, provision of services to a third party, generation of commercial databases, or clinical diagnostics or therapeutics. In addition, any user that purchases more than $5,000 in any calendar quarter may be outside the above research license and will contact Stanford University for a license. This product is sold pursuant to a license from Stanford University, and Stanford University reserves all other rights under these patent rights. For information on a license to the patent rights for uses other than in conjunction with this product or to use this product for purposes other than research, please contact Stanford University at 650 723-0651. This is Stanford University reference S02-028.

TABLE OF CONTENTS

Page

OVERVIEW

Kit Contents

The Turbo Dicer siRNA Generation Kit contains sufficient reagents for generating small interfering RNAs (siRNAs) from up to 5 different genes and for 50 transfections in 24-well plates.

Contents		Quantity
Recombinant Turbo Dicer Enzyme Kit (50 Units)	Recombinant Turbo Dicer Enzyme	1 tube (100 µl @ 0.5 unit/µl)
	Turbo Dicer Reaction Buffer	1 tube (50 µl)
	50 mM $MgCl_2$ Solution	1 tube (100 µl)
	10 mM ATP	1 tube (50 µl)
	Turbo Dicer Stop Solution	1 tube (100 µl)
	5X BSA	1 tube (50 µl)
	Nuclease-free Water	1 tube (1 ml)
TurboScript™ T7 Transcription Kit (5 Reactions)	T7 Enzyme Mix	1 tube (10 µl)
	T7 Reaction Buffer	1 tube (10 µl)
	NTP Mix	1 tube (40 µl)
	DNase I	1 tube (5 µl)
	2X Gel Loading Buffer	1 tube (175 µl)
	Nuclease-free Water	1 tube (1 ml)
	LiCl Precipitation Solution	1 tube (175 µl)
	GFP Control Plasmid	1 tube (10 µl of gWiz/GFP @ 1 µg/µl)
	5' Control Primer	1 tube (10 µl @ 1 µg/µl)
	3' Control Primer	1 tube (10 µl @ 1 µg/µl)
GeneSilencer® siRNA Transfection Reagent Kit (50 Transfections)	GeneSilencer® siRNA Transfection Reagent	1 tube (180 µl)
	siRNA Diluent	1 bottle (1.5 ml)
RNA Purification Column 1	RNA Purification Column 1	5 columns
	Hydration Buffer	4 ml
RNA Purification Column 2		5 columns

Shipping and Storage

The Turbo Dicer siRNA Generation Kit is shipped frozen. For maximum stability and long-term use, immediately store Recombinant Turbo Dicer Enzyme Kit and TurboScript™ T7 Transcription Kit at –20°C upon receipt. Store the GeneSilencer® siRNA Transfection Kit at 4°C and store RNA Purification Columns 1 and 2 at room temperature. All components are stable for six months when stored properly.

Accessory Products

Product Name	Cat. No.	Quantity
Recombinant Human Turbo Dicer Enzyme Kit	T520002	50 Units
TurboScript™ T7 Transcription Kit	T510003	20 Reactions
RNA Purification Column 1	T510004	20 Columns
RNA Purification Column 2	T510005	20 Columns

For efficient and functional siRNA transfection:

Product Name	Cat. No.	Quantity
GeneSilencer® siRNA Transfection Reagent (0.75 ml)	T500750	200 reactions
GeneSilencer® siRNA Transfection Reagent (5 x 0.75 ml)	T505750	5 x 200 reactions

For efficient transfection of DNA into diverse cell lines

Product Name	Cat. No.	Quantity
GenePORTER® 2 Transfection Reagent	T202007	75 reactions (0.75 ml)
GenePORTER® 2 Transfection Reagent	T202015	150 reactions (1.5 ml)
GenePORTER® 2 Transfection Reagent	T202075	750 reactions (5 x 1.5 ml)

For 3-minute transformation into E. coli

Product Name	Cat. No.	Quantity
TurboCells® Chemically Competent *E. coli*	C300020	20 x 50 µl
TurboCells® F′ Chemically Competent *E. coli*	C301020	20 x 50 µl

Product Support

Telephone: 858-457-1919 OR 888-428-0558 (US toll free)	**Fax:** 858-623-9494
E-mail: tech1@genlantis.com	**Web:** http://www.genlantis.com

For a complete list of international distributors, visit our web site at www.genlantis.com.

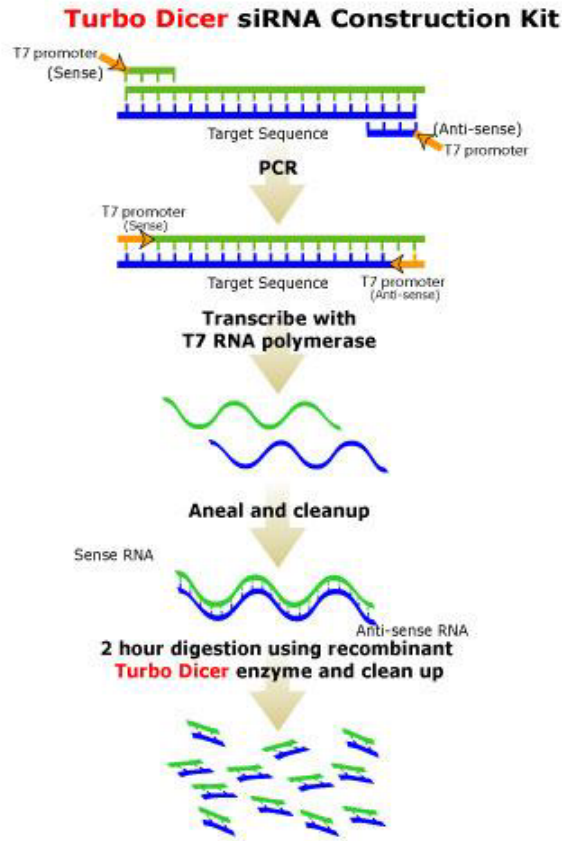
Introduction to RNA Interference

RNA interference (RNAi) has become an important tool for studying gene functions because it allows sequence specific gene suppression in a variety of organisms (*e.g.* plants, insects, and nematodes) and cultured mammalian cell lines. RNAi is characterized by targeted mRNA degradation after introduction of sequence-specific double stranded RNAs (dsRNAs) into cells (1,2,3). Several studies indicate that RNAi is an evolutionarily conserved defense mechanism directed against invading viral genomes or aberrant transcription products (4,5). *In vitro* studies using *Drosophila* lysates revealed that 21-25 nucleotide small interfering RNA duplexes (siRNAs) are the mediators of gene silencing. These siRNAs are derived from processing of the dsRNA by an RNase III-like enzyme (6,7,8). The mechanism involves the recruitment of siRNAs into a multi-protein complex known as RNA Induced Silencing Complex (RISC), which interacts with the target RNA to mediate cleavage in a catalytic fashion (6,9,10). Although cellular uptake of long trigger dsRNAs by organisms such as *C. elegans* and *Drosophila* has proven to be an effective method to induce RNAi, long dsRNAs tend to result in non-specific gene suppression in vertebrate cells due in part to Type I interferon response (11). Subsequent studies using synthetic siRNAs (less than 30 nucleotides) demonstrated that siRNAs can bypass the mammalian interferon response and cause effective gene-specific silencing in mammalian cells (1,12). In addition, the gene silencing effect caused by siRNA can be detected even after many cell divisions. These properties make siRNA a useful tool for a broad range of research areas in mammalian cells.

How Turbo Dicer siRNA Generation Kit Works

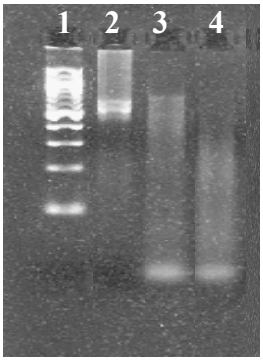
The Turbo Dicer siRNA Generation Kit mimics the natural RNA interference process by using recombinant human Turbo Dicer enzyme, to cleave *in vitro* transcribed dsRNA into a pool of 22 bp siRNAs (Figure 1).

Figure 1. How Turbo Dicer siRNA Generation Kit Works



The Turbo Dicer siRNA Generation Kit relies on two novel technologies for efficient siRNA production. First, the TurboScript™ T7 Transcription Kit utilizes a novel technology to allow rapid synthesis of 10 to 50 times the amount of RNA produced by conventional in vitro transcription reactions. The secret behind high yield is that each DNA template is copied hundreds of times. This ensures that you will have sufficient dsRNA after annealing the transcribed sense and antisense RNA strands. The second key technology is an ultra-active form of human recombinant Turbo Dicer enzyme that can cleave more than 95% of dsRNA template into 22 bp siRNAs within 2 hours under optimized reaction conditions (Figure 2). With abundant supply of dsRNA templates and subsequent efficient dsRNA cleavage you are sure to have sufficient siRNAs in every single species to achieve gene silencing.

Fig. 2 Efficient Digestion of Double-stranded RNA with Recombinant Human Turbo Dicer Enzyme



Lane# Sample

1. 100 bp marker;
2. 700 bp dsRNA, 1μg undigested.
3. 700 bp dsRNA, 1μg digested 15 hours with 1 unit of Dicer Enzyme
4. 700 bp dsRNA, 1μg digested 2 hours with 1 unit of Turbo Dicer Enz me.

Advantages of Turbo Dicer siRNA Generation Kit

Compared to conventional siRNA construction methods such as chemical synthesis and hairpin siRNA expression vectors, the Turbo Dicer siRNA Generation Kit offers the following advantages:

- No guesswork – A mixture of siRNAs has a better chance of success than a single siRNA design.
- Cost effective – No wasted time and money due to failed siRNA designs.
- Fast and effective – effective digestion of dsRNA in two hours.
- High efficiency – More regions of the genes can be screened for silencing.
- Ideal for use with GeneSilencer® siRNA Transfection Reagent.

METHODS AND PROCEDURES

1. Generating Double-Stranded RNA (dsRNA) Template

1.1. Preparation of Template DNA for Transcription

1.1.1 Determining a target region

The region selected for gene silencing does not really seem to matter. You may follow a method suggested by Tuschl *et al.* (13) and pick a region of about 100-150 nucleotides downstream from the start codon, which would prevent the diced-siRNAs (d-siRNAs) from potentially competing with proteins involved in translation initiation.

1.1.2 The size of the target template

The Recombinant Turbo Dicer Enzyme performs best with double-stranded RNA (dsRNA) between 500-1000 bp in length. Turbo Dicer also works with longer dsRNAs, even full-length cDNAs. However, for silencing a single gene, using a 500 bp dsRNA is sufficient. We recommend that you use templates that are 500 – 1000 bp in length.

IMPORTANT *1) The yield of dsRNA might be low if the DNA template is smaller than 300 bp or the gene is GC rich and longer than 2 kb.*
2) The Turbo Dicer enzyme might not digest well if the dsRNA is smaller than 300 bp.

1.1.3 Adding T7 Promoters to the DNA template using PCR

The DNA template must contain the T7 RNA polymerase promoter site at both ends so that it can be used as a template for *in vitro* transcription with the TurboScript™ T7 Transcription Kit.

T7 promoter sequence:

+ 1

TAATACGACTCACTATA**G**GGAGA

NOTE *The underlined sequence shown above is the minimum promoter sequence needed for efficient transcription. The + 1 base (in bold) is the first base incorporated into RNA during transcription. The 20-base T7 promoter region includes 2 bases, which will form the first 2 bases of the transcribed RNA. Yields of transcription product are greatly reduced if the +1 or +2 G residues are changed.*

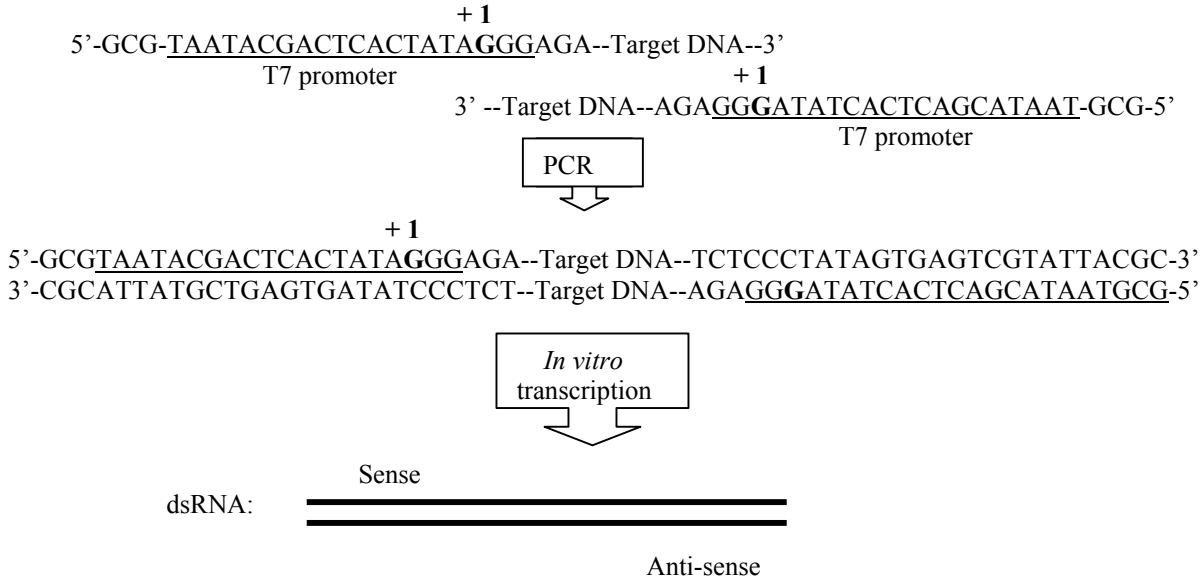
1.1.3.1 Design gene-specific PCR primers by using the diagram below. We recommend using 20 bases that are specific to your gene in each primer.

+ 1

5'- primer: 5'-GCG-TAATACGACTCACTATA**G**GGAGA-NNNNNNNNNN-3'
leader T7 promoter sequence Target DNA (~20 bases)

+ 1

3'- primer: 5'-GCG-TAATACGACTCACTATA**G**GGAGA-NNNNNNNNNN-3'
leader T7 promoter sequence Target DNA (~20 bases)



1.1.3.2 PCR protocol

We recommend that you use the PCR Control Plasmid, which contains a 700 bp GFP gene as positive control template.

Prepare a 100 µl reaction mix as follows:

10 µl	10 x PCR buffer
1 µl	10 mM each dNTP
1 µl	DNA template (50 ng)
1 µl	5' primer (1µg/µl)
1 µl	3' primer (1µg/µl)
x µl	DNA polymerase (Amount varies depending on the supplier)
86-x µl	ddH_2O

PCR program:

94°C for 3 minutes

94°C for 30 seconds
58°C for 30 seconds
68°C for 1 minute/1kb
35 cycles

68°C for 5 minutes
4°C storage

1.1.4 Purification of PCR Product. It is important to proceed with a clean PCR product to ensure high yield of ds-RNA. PCR products can be purified the following method:

1.1.4.1 Add 1/10 volume of 3 M sodium acetate pH 5.3
1.1.4.2 Add 2 volumes of ethanol
1.1.4.3 Mix well and chill at –20°C for 30 min.
1.1.4.4 Pellet the DNA for 15-30 min in a microcentrifuge at top speed.
1.1.4.5 Remove the supernatant carefully. Resuspend the DNA pellet with 100 µl cold 70% ethanol.
1.1.4.6 Spin at top speed for 15 min.
1.1.4.7 Remove the supernatant carefully, and air-dry the DNA pellet.
1.1.4.8 Resuspend the DNA in 20 µl of Nuclease-free Water and quantitate by spectrophotometery. Store at -20°C until later use.

IMPORTANT *If you use other commercially available kits to purify PCR products please make sure that agarose is removed completely before proceeding to the next step.*

1.2 Generation of dsRNA

1.2.1 Transcription Reaction Assembly

1.2.1.1 Thaw the frozen reagents. Place the T7 Enzyme Mix on ice; it is formulated in glycerol and does not freeze at –20°C. Vortex the T7 Reaction Buffer and the NTP Mix until they are completely in solution. Once thawed, store the NTP Mix on ice. **Keep T7 Reaction Buffer at room temperature while assembling the reaction.**

IMPORTANT *All reagents should be microfuged briefly before opening to prevent loss and contamination of material that may be present around the rim of the tube.*

1.2.1.2 Assemble the transcription reaction **at room temperature**. The following amounts are for a single 20 µl reaction. Reactions may be scaled up or down if desired.

Add to 20 µl	Nuclease-free Water
8 µl	NTP mix
2 µl	T7 Reaction Buffer
1 µg	PCR template DNA (from step 1.1.4.8.)
2 µl	T7 Enzyme Mix

IMPORTANT *The spermidine in the T7 Reaction Buffer can co-precipitate the template DNA if the reaction is assembled on ice. Add the T7 Reaction Buffer after the water and the NTP Mix are already in the tube.*

1.2.2 Gently flick the tube or pipette the mixture up and down gently, then microfuge the tube briefly so that the reaction mixture is at the bottom of the tube.

1.2.3 Incubate at 37°C for 2-4 hours.

TIP *The first time a new template is transcribed, the recommended incubation time is 2–4 hours. To determine the optimum incubation time for maximum yield with a given template, a time-course experiment can be done. To do this, set up a TurboScript™ T7 Transcription reaction, and remove aliquots of the reaction at various intervals (for example after 1 hour, 2 hours, 4 hours, 6 hours, and overnight incubations).*

1.2.4 Add 1 µl DNase I to each 20 µl T7 Reaction. Mix well and incubate for 15 min at 37°C.

NOTE *The DNase I treatment removes the template DNA.*

1.2.5 Check the dsRNA on a 1% agarose gel (TAE) by using the 2X Gel Loading Buffer.

NOTE *dsRNA will migrate like DNA i.e. a 500 bp dsRNA will migrate at the same rate as a 500 bp band in a DNA ladder. ssRNA will migrate much faster than a dsDNA of the equivalent size. You may see faint slower-migrating bands above the full-length transcript on non-denaturing gels. These may be the result of secondary structures within the transcript and should be ignored.*

1.3 Recovery of dsRNA

dsRNA can be directly use for the Recombinant Turbo Dicer Enzyme Kit without purification. However, dsRNA purified using the following procedure can give slightly better result.

1.3.1 Precipitate the RNA by adding 30 µl Nuclease-free Water and 30 µl LiCl Precipitation Solution to the mixture from Step 1.2.4.

1.3.2 Mix thoroughly. Chill for $\geq$ 30 min at –20°C.

1.3.3 Centrifuge at 4°C for 15 minutes at maximum speed to pellet the RNA.

1.3.4 Carefully remove the supernatant. Wash the pellet once with ~1 ml 70% ethanol and centrifuge again to maximize removal of unincorporated nucleotides.

1.3.5 Carefully remove the 70% ethanol, and resuspend the RNA in Nuclease-free Water or TE Buffer. Determine the RNA concentration and store at –20°C or –70°C.

IMPORTANT *Lithium chloride precipitation may not efficiently precipitate RNAs smaller than 300 nucleotides. Also, the concentration of RNA should be at least 0.1 µg/µl to assure efficient precipitation. To precipitate from TurboScript™ reactions that are thought to have very low yields of RNA, do not dilute the transcription reaction with water prior to adding the LiCl Precipitation Solution.*

1.4 Quantitation of dsRNAs

1.4.1. Quantitation by UV light absorbance

Reading the A_{260} of a diluted aliquot of the reaction is clearly the simplest way to determine yield, but any unincorporated nucleotides and/or template DNA in the mixture will contribute to the reading. Typically, a 1:500 dilution of an aliquot of a TurboScript™ reaction will give an absorbance reading in the linear range of a spectrophotometer.

For RNA molecules, 1 A_{260} unit corresponds to 40 µg/ml, so the RNA yield can be calculated as follows:

A_{260} X dilution factor X 40 = µg/ml RNA

1.4.2. Assessing dsRNA yield with RiboGreen®

If you have a fluorometer, or a fluorescence microplate reader, Molecular Probes' RiboGreen® fluorescence-based assay for RNA quantitation is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for using RiboGreen®.

1.4.3. Quantitation by ethidium bromide fluorescence

The intensity of ethidium bromide staining of dsRNA in an agarose gel can be used to get a rough estimation of the RNA yield.

1.4.3.1. Ethidium bromide spot assay

If unincorporated nucleotides have been removed, an ethidium bromide spot assay can be used to quantitate RNA concentration. Make a standard curve with several 2 fold dilutions of an RNA solution of known concentration. Start at about 80 ng/µl, and go down to about 1.25 ng/µl. Make a few dilutions of the unknown RNA, and add ethidium bromide to 1 ng/µl to each dilution of both RNAs. Spot 2 µl of the control RNA samples and the unknown RNA dilutions onto plastic wrap placed on a UV transilluminator. Compare the fluorescence of the RNAs to estimate the concentration of the sample RNA. Make sure that the sample dilutions are in the linear range of ethidium bromide fluorescence. This assay will detect as little as 5 ng of RNA with an error of about 2 fold.

1.4.3.2. Denaturing gel electrophoresis

If unincorporated nucleotides have not been removed from the reaction, an aliquot of the TurboScript™ reaction should be run on a denaturing agarose or acrylamide gel alongside an aliquot of RNA of known concentration. Stain the samples with ethidium bromide, and simply compare the intensity of the unknown sample to that of the known RNA sample to estimate its concentration.

2. Generating siRNAs Using Recombinant Turbo Dicer Enzyme

2.1 Turbo Dicer Reaction

2.1.1 ***Keep the Turbo Dicer Reaction Buffer at room temperature while assembling the reaction***. The following amounts are for a single 10 µl reaction.

3 µl – x	Nuclease-free water
x µl	dsRNA (1 µg)
1 µl	10 mM ATP
1 µl	5X BSA
2 µl	50 mM $MgCl_2$
1 µl	10X Dicer Reaction Buffer
2 µl	Recombinant Turbo Dicer Enzyme (0.5 unit/µl)

IMPORTANT *1. Avoid using excess recombinant Turbo Dicer enzyme, as it may decrease the amount of diced-siRNA (d-siRNA) that can be generated from the digestion reaction.*
2. 1 µg of dsRNA control template will yield about 0.5 µg of d-siRNA, which is sufficient for one or two transfections in 24-well plates using the GeneSilencer siRNA Transfection Reagent. Adjust the reaction volume accordingly if you need more d-siRNAs.

2.1.2 Incubate for two hours at 37°C; ***avoid overdigesting dsRNA for longer than 2 hours.***

2.1.3 Stop the reaction by adding 2 µl Turbo Dicer Stop Solution

2.1.4 Check d-siRNA (~22 base pairs) by using one of the following methods:

a. 3 % agarose Gel (TAE)
b. 15% native polyacrylamide gel (29:1, cast in 1X and electrophoresed in 0.5X TBE). Run at 10 Watts and 4°C. Visualize RNA by staining with ethidium bromide.

2.2 Purification of siRNAs

2.2.1 Use RNA Purification Column 1 to remove salts and unincorporated nucleotides

IMPORTANT *A fixed-angle-rotor microcentrifuge is required in this step. On a variable speed microcentrifuge, DO NOT use the pulse button, which overrides the speed setting and takes the rotor to maximum g-force.*

2.2.1.1 Tap the RNA Purification Column 1 to settle the dry gel in the bottom of the spin column.

2.2.1.2 Hydrate the column with 650 µl of Hydration Buffer. Cap, vortex, tap out air bubbles, and hydrate at room temperature 5–15 min.

2.2.1.3 (Optional) Once hydrated, these columns can be stored in the refrigerator up to 3 days.

2.2.1.4 Spin the column at 750 x g for 4 min to remove excess interstitial fluid, keeping track of the orientation of the column in the rotor by marking the column.

2.2.1.5 Discard the wash tube and immediately apply the sample (20–100 µl) DIRECTLY TO THE CENTER OF THE GEL BED at the top of the column without disturbing the gel surface or contacting the sides of the column with the pipette tip or reaction mixture.

2.2.1.6 Place the column in the sample collection tube and place in the rotor, maintaining the same orientation as in Step 2.2.1.4.

2.2.1.7 Spin the column in the tube at 750 x g for 3 min. Your sample will be in the collection tube.

2.2.2 Use RNA Purification Column 2 to remove the undigested dsRNA.

2.2.2.1 Insert the sample reservoir into one of the vials provided.

2.2.2.2 Add 10 µl of nuclease free water into the sample reservoir without touching the membrane with the pipette tip. Seal the attached cap.

2.2.2.3 Place the assembly in a microcentrifuge and counterbalance with a similar device. Spin at 500 x g for 2 minutes. Empty any nuclease free water in the collection tube.

2.2.2.4 Add the samples from step 2.2.1.7 into the sample reservoir without touching the membrane with the pipette tip. Seal with the attached cap.

2.2.2.5 Place assembly in a microcentrifuge and counterbalance with a similar device. Spin at 500 x g for 15 minutes

IMPORTANT *Do not centrifuge more than 15 minutes.*

2.2.2.6 Remove assembly from centrifuge. Separate collection vial from sample reservoir. **Purified d-siRNA is in the collection vial**, undigested large dsRNAs remain in the sample reservoir.

2.2.2.7 Store the d-siRNA at –20°C.

2.2.3 Qualification of Purification Products

For RNA molecules, 1 A_{260} unit corresponds to 40 µg/ml, so the siRNA yield can be calculated as follows:

A_{260} X dilution factor X 40 = µg/ml RNA

NOTE *Typically, each microgram of dsRNA will yield about 0.5 µg of d-siRNA*

3. Transfect d-siRNA with the GeneSilencer® siRNA Transfection Reagent

IMPORTANT *Transfection Optimization Guidelines*

a. Adherent Cells
Although GeneSilencer® 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 GeneSilencer™/siRNA ratio and the siRNA quantity. To optimize these two variables:

a1. Determine the best GeneSilencer®/siRNA ratio by using 0.5 - 7 µl of reagent for each 100 ng of siRNA. Use a low siRNA quantity to optimize this parameter.

a2. 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 GeneSilencer®/siRNA ratio is higher.

NOTE *Gene suppression was observed with GFP d-siRNA generated from the GFP Control Plasmid. Co-transfection of 1 µg of GFP Control Plasmid and 500 ng of d-siRNA resulted in 60% suppression of GFP expression in transiently transfected NIH 3T3 cells.*

3.1 Transfection of Adherent Cells

3.1.1 The day before transfection, plate cells so that they will be 50-70% confluent on the day of transfection.

3.1.2 Prepare the GeneSilencer® Reagent by diluting in serum free medium according to the recommended amount in Table 1 below.

Table 1: GeneSilencer® Dilutions For Adherent Cells

Tissue Culture Plate or Dish Type	GeneSilencer® Reagent (µl) + Serum Free Medium (µl) per well
96 wells	1.0 + 25
48 wells	1.75 + 25
24 wells	3.5 + 25

3.1.3 Prepare the siRNA solution by first mixing the siRNA Diluent and serum free medium (SFM) according to Table 2 below. Use the Diluent/SFM mix to dilute the recommended amount of d-siRNA in Table 2. Mix well by pipetting up and down several times. Incubate at room temperature for 5 minutes.

IMPORTANT *Avoid vortexing the siRNA/Diluent mix.*

Table 2: siRNA Dilutions For Adherent Cells

Tissue Culture plate type	Recommended Amount of d-siRNA to use (ng) per well	siRNA Diluent (µl)+ Serum Free Medium (µl) per well	Final transfection volume (µl)
96 wells	125	2.5 + 15	100
48 wells	250	5.0 + 15	200
24 wells	500	10.0 + 15	500

3.1.4 Add the RNA solution from Step 3.1.3 to the diluted GeneSilencer solution from Step 3.1.2. Incubate at room temperature for 5 minutes to allow the siRNA/lipid complexes to form.

IMPORTANT *You can incubate the siRNA/GeneSilencer® mix for longer than 5 minutes, but make sure not to exceed 30 minutes in order to maintain maximum siRNA transfection efficiency.*

3.1.5 Add the siRNA/GeneSilencer® mix to cells growing in serum-containing medium. Incubate at 37°C for 24 hours. See Table 2 for final transfection volume.

TIPS *For some cell lines like HeLa, MDCK, and CHO-K1, transfection efficiencies may be higher if serum is omitted from the medium during the first 4 hours of transfection. After this step, add one volume of medium containing 20% serum, then proceed to step 3.1.6.*

3.1.6 Add fresh tissue culture medium to growing cells as needed. Most RNA interference can be detected within 48 hours post transfection.

3.2 Transfection of Suspension Cells

3.2.1 The day before transfection, split the cells as necessary to optimize their health and achieve log-growth by transfection time.

3.2.2 Prepare the GeneSilencer® Reagent by diluting in serum free medium according to the recommended amount in Table 3 below.

Table 3: GeneSilencer® Dilutions For Suspension Cells

Tissue Culture plate type	GeneSilencer™ Reagent (µl) + Serum Free Medium (µl) per well
96 wells	1.0 + 25
48 wells	1.75 + 25
24 wells	3.5 +25

3.2.3 Prepare the d-siRNA solution by first mixing the siRNA Diluent and serum free medium (SFM) according to Table 4 below. Use the Diluent/SFM mix to dilute the recommended amount of d-siRNA in Table 4. Mix well by pipetting up and down several times. Incubate at room temperature for 5 minutes.

IMPORTANT *Avoid vortexing the siRNA Diluent mix.*

Table 4: siRNA Dilutions For Suspension Cells

Tissue Culture plate or dish type	Recommended Amount of d-siRNA to use (ng) per well	siRNA Diluent (µl)+ serum free medium (µl) per well
96 wells	125	2.5 + 15
48 wells	250	5.0 + 15
24 wells	500	10.0 + 15

3.2.4 Add the d-siRNA solution from Step 3.2.3 to the diluted GeneSilencer® solution from Step 3.2.2. Incubate at room temperature for 5 minutes to allow the siRNA/lipid complexes to form.

IMPORTANT *You can incubate the siRNA/GeneSilencer mix for longer than 5 minutes, but make sure not to exceed 30 minutes in order to maintain maximum siRNA transfection efficiency.*

3.2.5 While the siRNA/GeneSilencer mix is incubating, spin down the cells from Step 3.2.1, remove the growth medium and re-suspend the cells in the appropriate growth medium (serum-free or serum-containing) to achieve a final cell density listed in Table 5.

3.2.6 Transfer resuspended cells to culture plates according to Table 5 below.

Table 5: Volume and Number of Cells to Transfer Into Culture Dishes.

Tissue Culture plate or dish type	Volume of resuspended cells to transfer to each well (ml)	Number of cells transferred to each well (approximate).
96 wells	0.1	1×10^5
48 wells	0.2	2×10^5
24 wells	0.5	5×10^5

3.2.7 Add the siRNA/GeneSilencer® mix to resuspended cells in Table 5 above. Gently mix the cells by pipetting up and down several times to avoid cell clumping. Incubate at 37°C for 24 hours.

3.2.8 Add fresh tissue culture medium to growing cells as needed. Most RNA interference can be detected within 48 hours post transfection.

APPENDIX

Quality Control

All components in the TurboScript™ T7 Transcription Kit are functionally tested by using PCR product amplified from the GFP Control Plasmid. Each 20 µl reaction yields at least 50–80 µg of dsRNA after a 4 hour incubation. All components in the Recombinant Turbo Dicer Enzyme Kit are tested using a 10µl reaction containing 1 µg of dsRNA (generated from the GFP Control Plasmid). At least 0.5 µg of d-siRNA must be produced after 2 hours of incubation. The GeneSilencer® siRNA Transfection Kit is functionally qualified by transfecting fluorescent-labeled siRNA in cultured cells.

Troubleshooting Guide

Problem	Possible Causes	Recommended Solutions
I have difficulty obtaining PCR products for the full-length gene of interest	Sub-optimal primer design.	Re-design primers by changing the gene-specific portions of the primers and optimize the PCR conditions.
	The gene is too long.	Amplify portions of the gene in 500 to 700 bp fragments and proceed to transcribe dsRNAs from these fragments.
Neither my template nor the control reaction works in generating dsRNA.	Expired or defective kit component.	Double check that you have followed the procedure accurately, and consider trying the control reaction a second time. If the kit control still doesn't work, it is an indication that something else may be wrong with the kit. Call GTS Technical Support for further troubleshooting.
The control reaction works with the TurboScript™ T7 Transcription Kit, but my template gives low dsRNA yield.	Wrong amount of DNA template or poor DNA quality.	Check the amount and quality of template . Also, check an aliquot of the template DNA on an agarose gel to make sure it is intact and that it is the expected size.
	PCR products were of poor quality.	Use a different DNA polymerase if possible and/or extend reaction time
	DNA template has high G/C content	Optimize transcription reaction condition by doubling the amount of GTP and CTP, performing the reaction at 15°C, and adding 0.5-1% DMSO.
d-siRNA yield is low.	No dsRNA or poor dsRNA quality.	Check the amount of dsRNA added. Make sure that the correct fractions from the RNA Purification Columns are used and/or purify dsRNA before Turbo Dicer reaction.
	Too much Recombinant Turbo Dicer Enzyme was added.	Use 1 unit of the enzyme for every microgram of dsRNA.
	10 mM ATP is old.	Use fresh ATP solution.

Troubleshooting Guide (Continued)

Problem	Possible Causes	Recommended Solutions
Low Transfection Efficiency	Sub-optimal GeneSilencer /siRNA ratio or Suboptimal siRNA concentration.	Optimize the GeneSilencer/siRNA ratio by using 0.5-7 µl of GeneSilencer for each 100 ng of siRNA. Use a low siRNA quantity to optimize this parameter. After establishing the optimal GeneSilencer /siRNA ratio, vary the siRNA quantity over the ranges suggested in the Methods and Procedures section.
	Over-digestion of dsRNA	Make sure you digest the dsRNA with Turbo Dicer for no longer than 2 hours as recommended.
	Denatured siRNA	Use recommended buffer (100 mM NaCl, 50 mM Tris, pH 7.5 in RNase-free water) to dilute siRNA. Do not use water as it can denature the siRNA.
	Cells have been in continuous passage for > 2 months	Thaw out a fresh aliquot of cells and passage once (or more) before transfecting. Avoid using cells that have been in culture or have been passaged for excessive periods of time.
	Sub-optimal cell density.	Use cells that are 50-70% confluent on the day of transfection. Optimal cell density may vary depending on cell type.
	Improper storage.	GeneSilencer reagent is very stable but long exposure to elevated temperatures and/or excessive freeze/thaw cycles may cause degradation of the reagent. Store GeneSilencer Reagent at 4° C.
	Wrong medium.	Be sure to use serum-free medium when forming the GeneSilencer/siRNA complex.
	Cell line is difficult to transfect.	Optimize GeneSilencer/siRNA ratio and siRNA amount as indicated on page 15.
	GeneSilencer/ siRNA complexes not freshly prepared.	GeneSilencer/siRNA complexes should be freshly prepared. If complexes have been prepared and stored for longer than 45 minutes, aggregation may occur.
	Sub-optimal GeneSilencer /siRNA ratio used.	Too much GeneSilencer or too much siRNA could cause aggregation; Adjust the ratio as outlined above.
Aggregation	Excess GeneSilencer used.	Decrease the amount of GeneSilencer reagent.
Cytotoxicity	Unhealthy cells.	- Check cells for contamination. - Thaw a new batch of cells. - Cells too confluent or cell density too low. - Check culture medium (pH, kind used, last time changed). - Check materials used for proper function (culture plates, incubators, etc.).
	GeneSilencer concentration too high	Reduce GeneSilencer concentration in 20-30% increments.

References:

1. Caplen, N.J., S. Parrish, F. Imani, A. Fire and R.A. Morgan. 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci U S A* **98:**9742-9747

2. Grishok, A., A.E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D.L. Baillie, A. Fire, G. Ruvkun and C.C. Mello. 2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* **106:**23-34

3. Parrish, S., J. Fleenor, S. Xu, C. Mello and A. Fire. 2000. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Mol Cell* **6:**1077-1087.

4. Hamilton, A.J. and D.C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286:**950-952.

5. Sijen, T., J. Fleenor, F. Simmer, K.L. Thijssen, S. Parrish, L. Timmons, R.H. Plasterk and A. Fire. 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107:**465-476.

6. Bernstein, E., A.A. Caudy, S.M. Hammond and G.J. Hannon. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409:**363-366.

7. Hutvagner, G., J. McLachlan, A.E. Pasquinelli, E. Balint, T. Tuschl and P.D. Zamore. 2001. A cellular function for the RNA-interference enzyme Turbo Dicer in the maturation of the let-7 small temporal RNA. *Science* **293:**834-838.

8. Knight, S.W. and B.L. Bass. 2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science* **293:**2269-2271.

9. Boutla, A., C. Delidakis, I. Livadaras, M. Tsagris and M. Tabler. 2001. Short 5'-phosphorylated double-stranded RNAs induce RNA interference in Drosophila. *Curr Biol* **11:**1776-1780.

10. Hammond, S.M., E. Bernstein, D. Beach and G.J. Hannon. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404:**293-296.

11. Ui-Tei, K., S. Zenno, Y. Miyata and K. Saigo. 2000. Sensitive assay of RNA interference in Drosophila and Chinese hamster cultured cells using firefly luciferase gene as target. *FEBS Lett* **479:**79-82.

12. Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:**494-498.

13. Elbashir SM, Harborth J, Weber K, Tuschl T. 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods.* **26(2):**199-213.

For additional troubleshooting assistance, please contact Genlantis Technical Support Dept. at:

Telephone: 858-457-1919 OR 888-428-0558 (US toll free)	**Fax:** 858-623-9494
E-mail: tech1@genlantis.com	**Web:** http://www.genlantis.com

For a complete list of GTS international distributors, visit our web site at
http://www.genetherapysystems.com