Silencer™ siRNA Labeling Kit (Cat #1632, 1634)

Instruction Manual

Manual Version 0208a

Literature Citation When describing a procedure for publication using this product, we would appreciate that you refer to it as the *Silencer*™ siRNA Labeling Kit.

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I. Introduction

A. Background

RNAi and siRNAs

Post transcriptional gene silencing or RNA interference (RNAi), originally shown to exist in only a few organisms, has now been observed in evolutionarily diverse organisms. It is one of the most approachable methods for studying gene function in mammals. Be sure to visit Ambion's RNA Interference Resource on the web at http://www.ambion.com/RNAi/index.html; it includes the most relevant new scientific articles regarding RNA interference (including siRNA), the latest information on Ambion's own RNAi and siRNA studies, links to the best RNAi web sites, and details on the hottest new products for your RNAi research.

The observation that dsRNA can cause a reduction in the expression of the corresponding gene that is significantly greater than when sense or antisense RNA is used, was made in a seminal paper by Fire et al. Subsequent studies using in vitro systems have elucidated some of the mechanistic aspects of RNAi. Long double-strand RNA (dsRNA) is processed by a specific ribonuclease (Ketting et al. 2001) into short dsRNA that contains a 2 nucleotide 3' overhang and a 5' phosphate. This short dsRNA, also called short interfering RNA (siRNA), next associates with RNA-induced silencing complex (RISC) that guides the siRNA to its target mRNA through base-pairing interactions (Hammond et al. 2001). Once the siRNA is associated with its mRNA target, nucleases cleave the mRNA (Tuschl et al. 1999, Zamore et al. 2000). Understanding the RNAi mechanism has lead to the advancements which enable RNAi to be performed in mammalian cells (Elbashir et al. 2001).

Product description

The *Silencer* Cy™3 and fluorescein (FAM) RNA Labeling Kits (patent pending) enable researchers to label siRNA produced by chemical or enzymatic synthesis. Using these kits, either single-stranded RNA (ssRNA) oligonucleotides can be labeled individually, or duplex siRNAs can be labeled. A successful labeling reaction can label up to 45–50% of the siRNA using the recommended protocol. Whether siRNAs are labeled on one or both strands, they retain complete functionality and can be used to reduce the expression of the corresponding target gene (Byrom et al. 2002). Labeled siRNA can be used in analysis of siRNA trans-

fection efficiencies and distribution for pharmacokinetic and metabolism studies. The nucleic acid labeling reagents are designed to covalently attach either Cy3 or fluorescein to RNA in a one step reaction that takes approximately 2 hours. Reagents are supplied to label up to 65 µg of siRNA. This is enough siRNA for over 150 transfections in a 24 well dish at a 100 nM final concentration.

B. Materials Provided with the Kit

The *Silencer* siRNA Labeling provides reagents to label up to 65 µg of siRNA.

Cat #1632	Cat #1634	Component	Storage
500 µl	500 µl	10X Labeling Buffer	–20°C
100 µl	100 µl	Reconstitution Solution	–20°C
1.75 ml	1.75 ml	Nuclease-free Water	–20°C
500 µl	500 µl	5M NaCl	–20°C
500 µl	500 µl	5X siRNA Annealing Buffer	–20°C
40 µl	40 µl	GAPDH siRNA 20 µM solution: ~10 µg total	–20°C*
40 µg	– –	Cy3 Labeling Reagent†	–20°C
– –	60 µg	Fluorescein Labeling Reagent†	–20°C

* ≤–70°C for long term storage (over 6 months)

† Manufactured for Ambion by Mirus Corporation.

Properly stored kits are guaranteed for 6 months from the date received.

C. Materials Not Provided with the Kit

siRNA to be labeled

siRNA must be in a low salt buffer or water for efficient labeling with this procedure (see section II.A on page 5 for more information).

Either ssRNA oligonucleotides, or duplex siRNA can be labeled using the *Silencer* siRNA Labeling Kit. These can be made enzymatically or by chemical synthesis. For information on sources of RNA oligonucleotides and siRNA design, see section *I.D. Related products Available from Ambion:* on page 3 and Ambion's RNA Interference Resource on the web at http://www.ambion.com/RNAi/index.html.

100% ethanol

Use ACS grade or better 100% ethanol. The protocol calls for cold 100% ethanol, so store some ethanol at –20°C.

The protocol also requires 70% ethanol. Make 70% ethanol by diluting 95–100% ethanol with nuclease-free water; store it at room temp.

(optional) Reagents and equipment to verify labeling

Labeling can be checked by either running part of the reaction on a 20% acrylamide gel (instructions in section IV.D on page 16), or by measuring the absorbance of the labeled RNA solution and calculating the labeling efficiency (follow the instructions in section IV.E on page 17, and use the calculator on Ambion's web site at http://www.ambion.com/techlib/append/base_dye.html).

D. Related products Available from Ambion:

RNase*Zap*® Cat #9780–84	RNase Decontamination Solution. RNase*Zap* is simply sprayed or poured onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*.
***Silencer*™ siRNA Construction Kit** Cat #1620	The *Silencer* siRNA Construction Kit (patent pending) synthesizes siRNA by in vitro transcription, producing transfection-ready siRNA at a fraction of the cost of chemical synthesis. The *Silencer* siRNA Construction Kit includes all reagents for transcription, hybridization, nuclease digestion, and clean up of siRNA (except gene specific oligonucleotides for template construction)
Custom siRNA Chemical Synthesis Cat #16000	Ambion is a licensed manufacturer of synthetic RNA oligonucleotides for siRNA research and will synthesize custom siRNA at the 0.2 µM scale, which yields enough siRNA for hundreds to thousands of transfections. To ensure that you receive a quality product, optimized deprotection and reverse phase desalting are provided at no charge. For the highest possible purity, a PAGE purification option is available that yields siRNA with greater than 95% purity.
***Silencer*™ Transfection Kit** Cat #1630	The *Silencer* siRNA Transfection Kit contains both siPORT™ *Amine* and siPORT *Lipid* Transfection Agents and a well-characterized GAPDH siRNA, ideal for developing an optimal transfection protocol for your cells. Also included are the corresponding GAPDH negative control siRNA, and a detailed instruction manual.
RNAqueous™ RNA Isolation Kits Cat #1911, 1912, 1914, 1920	RNAqueous Kits employ a simple and rapid procedure to purify total RNA from source material without using organic solvents (such as phenol). There are specialized RNAqueous Kits for several different applications. RNAqueous and RNAqueous-Midi are designed for isolating RNA on a small or large scale respectively. RNAqueous-4PCR is the kit of choice to isolate RNA that will be used in RT-PCR; it incorporates a DNase digestion and a novel reagent for rapid and safe removal of the DNase and divalent cations. RNAqueous-96 brings the RNAqueous system to 96 well plate configuration.

NorthernMax™ Kits Cat #1940, 1946	Ambion's NorthernMax™ Kits: NorthernMax™, and NorthernMax™-Gly, combine ultrasensitive, reliable Northern blot protocols with unsurpassed quality control to ensure optimal results in less time.
RPA III™ Cat #1414,1415	Ribonuclease Protection Assay Kit for the detection and quantitation of mRNA. This kit incorporates Ambion's exclusive one-tube format, for fast, sensitive detection of RNA with no proteinase K or phenol extraction steps.
RNase-free Tubes & Tips see Catalog or Web site	Ambion's RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our web site (www.ambion.com) for specific information.

II. siRNA Labeling Protocol

IMPORTANT!
Use appropriate RNase control measures.
Treat gloves and work area with an RNase decontamination agent such as RNase*Zap*®, and always use RNase-free tubes and tips. RNase control is extremely important for working with ssRNA; dsRNA is much less susceptible to degradation by RNases.

A. siRNA Substrate Requirements

siRNA must be in a low salt buffer or water for efficient labeling with this procedure

- Chemically synthesized ssRNA oligonucleotides should be desalted with reverse phase chromatography.
- Duplex siRNA made with the *Silencer* siRNA Construction Kit will be in Nuclease-free Water at the end of the procedure.
- Use the 5X Annealing Buffer provided with the kit following the instructions in section IV.A on page 12 for optimal labeling of duplex siRNA synthesized chemically.

B. Labeling Reaction

1. Add 100 µl Reconstitution Solution to Labeling Reagent and mix well

Using RNase-free barrier tips, add 100 µl of Reconstitution Solution to the dry Cy3 or Fluorescein (FAM) Labeling Reagent.

To ensure that the Labeling Reagent is fully suspended, vortex the tube after adding Reconstitution Solution, then let the mixture sit at room temperature for 5 min and vortex again.

Store reconstituted Labeling Reagent at –20°C in the dark (e.g. wrapped in foil or in a box).

2. Assemble the labeling reaction and mix well

In a sterile, nuclease-free tube, assemble the reagents in the listed order making sure to add the Labeling Reagent last. Mix well by vortexing. Limit exposure of the reaction mixture to light for the entire procedure.

NOTE:
The Labeling Reaction may be scaled up or down depending on the amount of nucleic acid to be labeled. However, the Labeling Reagent should never constitute more than 20% of the total reaction volume.
The time and temperature of incubation, and the amount of Labeling Reagent all influence labeling efficiency (or specific activity).

a. Duplex siRNA (5 µg)

Amount	Component
18.3 µl	Nuclease-free Water
5.0 µl	10X Labeling Buffer
19.2 µl	21 mer duplex siRNA at 20 µM (~5 µg)
7.5 µl	Cy3 or FAM Labeling Reagent

b. Single-stranded siRNA (5 µg)

Amount	Component
22.5 µl	Nuclease-free Water
5.0 µl	10X Labeling Buffer
15 µl	21 mer ssRNA oligonucleotide at 50 µM (~5 µg)
7.5 µl	Cy3 or FAM Labeling Reagent

3. Incubate at 37˚C for 1 hr in the dark

Incubate the reaction mix at a constant temperature of 37˚C for 1 hr in the dark.

C. Ethanol Precipitation of Labeled RNA

IMPORTANT!
Labeled RNA should be kept away from light as much as possible. Since the siRNA will be used in tissue culture, use aseptic technique while handling it. Autoclave any tubes used to hold the siRNA.

We do not observe short term toxicity from transfection of siRNA that has not been ethanol precipitated, however, genetic alterations may occur if cells are exposed to unreacted Labeling Reagent for long periods of time. This ethanol precipitation removes unreacted Labeling Reagent, and we recommend including it.

1. Add 0.1 vol 5 M NaCl, and 2.5 vol 100% ethanol

For the 50 µl reactions described in section *II.B. Labeling Reaction* on page 5, add the following and mix well:

- 5 µl 5M NaCl (0.1 volume)
- 125 µl cold 100% ethanol (2.5 volumes)

2. Place at –20˚C for 30–60 min

After mixing well, place the mixture at –20˚C (or colder) for 30–60 min. The labeled RNA precipitates during this incubation.

3. Centrifuge at top speed 15–20 min, discard supernatant

a. Pellet the labeled RNA by high speed centrifugation for 15–20 min. Use the top speed compatible with your tubes; this centrifugation should be ≥8,000 x g.

b. Carefully remove the supernatant; avoid disrupting the pellet.

c. A red (Cy3) or green (FAM) siRNA pellet should be visible.

4. Wash pellet with 175 µl 70% ethanol

a. Gently add 175 µl 70% ethanol making sure not to disrupt the pellet, and centrifuge at ≥8,000 x g for 5 min (use the top speed compatible with your tubes).

b. Carefully remove all traces of supernatant with a pipette.

5. Dry at room temp 5–10 min

Dry the RNA at room temperature for 5–10 min. Do not dry the pellet for longer than 5–10 min or it will be difficult to solubilize.

6. Suspend the RNA pellet in Nuclease-free Water or buffer

Resuspend the RNA pellet in Nuclease-free Water or in the buffer of your choice. If desired, resuspend the RNA to its volume before the labeling reaction (e.g. 19.2 µl for duplex siRNA or 15 µl for ssRNA oligonucleotide) to finish with the same nucleic acid concentration.

NOTE:
Labeled ssRNA that will be hybridized to make double-stranded siRNA should be suspended in Nuclease-free Water.

IMPORTANT!
Since the labeled siRNA will be used in tissue culture, filter sterilize buffer before using it to resuspend the labeled siRNA.

A small amount of RNA may be lost during the ethanol precipitation, so if your application requires extremely accurate quantitation of the labeled siRNA, measure the RNA concentration by spectrophotometry. (See section IV.E.2 on page 18.)

III. Troubleshooting

A. Positive Control Reaction

The GAPDH siRNA is a duplex siRNA supplied at 20 µM. It is provided so that users can verify that the kit is working properly.

Also, the GAPDH siRNA has been transfected using Ambion's *Silencer* Transfection Reagents to knock down the expression of GAPDH in several common cell lines from human and mouse.

1. Positive control labeling reaction

a. Label 19.2 µl of the GAPDH siRNA following the instructions for duplex siRNA in section II.B.2.a on page 6.

b. Continue the procedure through the ethanol precipitation in section II.C on page 6.

Resuspend the labeled GAPDH siRNA in 19.2 µl Nuclease-free Water.

2. Analysis of the positive control labeling reaction

Check the labeling of the GAPDH siRNA by spectrophotometry, or by electrophoresis.

By spectrophotometry the base:dye ratio should be 200–300.

By electrophoresis 10–20% of the GAPDH siRNA should be labeled.

a. Analysis of the positive control labeling reaction by spectrophotometry

i. Dilute the labeled GAPDH siRNA solution with 76.8 µl 200 mM MOPS pH 7.5 (adjust the pH with NaOH). This is a 1:5 dilution.

ii. Measure the absorbance of the labeled RNA at 260 nm and at the absorbance maximum for the fluorescent dye (550 nm for Cy3, or 492 nm for FAM). As a baseline, also record the A_{260} and the $A_{550 \text{ or } 492}$ of the 200 mM MOPS used to dilute the labeled siRNA.

iii. Calculate the base:dye ratio using the calculator on our web site at [http://www.ambion.com/techlib/append/base_dye.html], or do the calculatio as described in section IV.E on page 17.

b. Analysis of the positive control labeling reaction by electrophoresis (See section IV.D on page 16 for recipes and gel running instructions.)

i. Put 19.2 µl of unlabeled GAPDH siRNA (supplied with the kit) in a nuclease-free tube, add gel loading buffer, and mix well. This sample will be used for comparison with the labeled GAPDH siRNA.

ii. Add gel loading buffer to the 19.2 µl of labeled GAPDH siRNA from step 1.b on page 8, and mix well.

iii. Load both samples on a 20% acrylamide gel, and run the gel until the bromophenol blue (the faster migrating dye) has migrated about 3/4 of the way through the gel.

iv. Visualize the siRNA by ethidium bromide staining; labeled siRNA will migrate slower in the gel than unlableled siRNA.

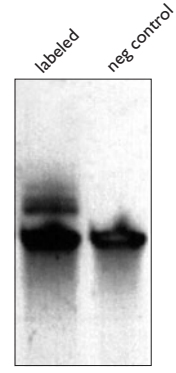

Figure 1. Acrylamide gel analysis of the *Silencer* siRNA Labeling positive control experiment

The GAPDH siRNA supplied with the kit was labeled with FAM, and run on a 20% acrylamide gel. This is the reverse image of the ethidium bromide stained gel. Labeled siRNA runs slower than unlabeled siRNA. Using gel documentation system software to compare the band intensity of labeled and unlabeled GAPDH siRNA reveals that about 45% of the RNA was labeled in this experiment. A fraction of the labeled siRNA contains more than one dye molecule; this produces a pale ladder of bands migrating more slowly than the band representing siRNA labeled with a single dye molecule.

B. No Colored Pellet is Visible after Ethanol Precipitation

Normally, the siRNA can be visualized at the bottom of the tube after ethanol precipitation (step II.C.3 on page 7) as a red (Cy3) or green (FAM) pellet. If a colored pellet is not visible, but the GAPDH siRNA supplied with the kit labels as expected, consider the following troubleshooting suggestions.

1. The siRNA is degraded

Check the integrity of the labeled siRNA by running a 2.5 µg sample of the RNA on a 20% acrylamide gel. Also consider running an equal amount of unlabeled siRNA in an adjacent lane. The labeled siRNA should migrate slower than unlabeled siRNA, but should appear intact (see Figure 1).

2. The labeling was inhibited by salt

The siRNA substrate must be in a low salt buffer or water for efficient labeling. Typically the reverse phase desalting provided by suppliers of RNA oligonucleotides provides RNA that works well in this procedure. To obtain duplex siRNA suitable for use with the *Silencer* siRNA Labeling Kit, chemically synthesized ssRNA oligonucleotides can be annealed in the supplied 5X Annealing Buffer (30 mM HEPES pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate) following the instructions if section IV.A on page 12, or duplex siRNA can be made with Ambion's *Silencer* siRNA Construction Kit (which includes purification reagents and instructions).

3. There is too little RNA to see

Make sure that enough RNA was included in the reaction by checking the A_{260} of the input RNA solution. Also be very careful when removing the supernatant after the ethanol precipitation to avoid dislodging the pellet.

4. The precipitation did not work well

Be sure to follow the recommended conditions, and incubation/centrifugation times, in section *II.C. Ethanol Precipitation of Labeled RNA* on page 6 for optimal precipitation.

C. The siRNA Cannot be Detected in Transfected Cells

1. Transfection of the labeled siRNA was poor

If transfection efficiency is too low, the labeled siRNA will not be detectable in transfected cells. There are many parameters that can affect transfection efficiency, check the literature and any documentation provided with your transfection agent for transfection troubleshooting and optimization suggestions.

2. The siRNA was not adequately labeled

Check whether the siRNA was labeled either by running it on 20% acrylamide gel (section IV.D on page 16), or using a spectrophotometer (section IV.E on page 17).

If labeling efficiency is below 25% the labeled siRNA could be difficult to detect in transfected cells by fluorescent microscopy.

D. Cell Cultures Become Contaminated After Transfection

Since the siRNA will be used in tissue culture, use aseptic technique while handling it. Autoclave any tubes that used to hold the siRNA, and if you use a solution other than the Nuclease-free Water supplied with the kit to resuspend the labeled siRNA, filter sterilize it.

If cell cultures appear to be contaminated as a result of transfection with the labeled siRNA, test each individual component in the kit in your tissue culture system. Also test whether your siRNA alone introduces contamination.

If necessary, the labeled siRNA can be filter sterilized just before preparing transfection agent:siRNA complexes for transfection.

IV. Additional Procedures

A. Annealing RNA Oligonucleotides to Make siRNA

1. Mix equal amounts of each RNA oligonucleotide in 1X siRNA Annealing Buffer

a. In an RNase-free tube combine 50 µM solutions of the sense and antisense RNA oligonucleotides and 5X siRNA Annealing Buffer for a final concentration of 20 µM each RNA strand and 1X siRNA Annealing Buffer (6 mM HEPES pH 7.4, 20 mM potassium acetate, 0.4 mM magnesium acetate).

Example: To make 75 µl of a 20 µM duplex siRNA:

Amount	Component
30 µl	sense RNA oligonucleotide
30 µl	antisense RNA oligonucleotide
15 µl	5X siRNA Annealing Buffer
75 µl	total

b. Vortex to mix, then spin briefly to collect the contents at the bottom of the tube.

2. Incubate 1 min at 90˚C

Heat the mixture to 90˚C for 1 min per 50–100 µl solution in a preheated heat block to denature any secondary structure in the RNA oligonucleotides.

IMPORTANT!

If the volume of the siRNA annealing mixture is >100 µl increase the incubation time at 90˚C proportionally (e.g a 150 µl mixture should be incubated for ~2 min).

3. Incubate 1 hr at 37˚C

The 1 hr 37˚C incubation allows the RNA oligonucleotides to anneal slowly so that they form a perfect duplex siRNA.

4. Store at –20˚C

Once annealed, duplex siRNA is much more nuclease resistant than ssRNA and can be safely stored frozen at –20˚C in a non-frost free freezer for 6 months or longer.

B. Suspension of Dry RNA Oligonucleotides

IMPORTANT!
Treat gloves and surrounding area with an RNase decontamination agent such as RNase*Zap*® prior to starting the work. Use RNase-free tubes and tips for all manipulations.

Oligonucleotides are often supplied dry; briefly centrifuge tubes to ensure that the dried oligonucleotide is at the bottom of the tube. The specification sheet provided by oligonucleotide manufacturers often contains the following information:

- nmoles synthesized
- mass amount synthesized
- OD_{260} units synthesized

The following sets of calculations explain how to make a 50 µM solution of an RNA oligonucleotide for use in the *Silencer* siRNA Labeling procedure.

NOTE:
These calculations can also be done automagically using the calculator on our web site. Find it at http://www.ambion.com/techlib/append/oligo_dilution.html.

Once the ssRNA is in solution, store it at –20°C for up to a few months, or at –80°C for extended periods of time.

Table 1. RNA Oligonucleotide Conversions

Average MW* of ssRNA	# of nt X 320
Average MW of dsRNA	# of nt X 640
MW of 21 mer ssRNA	6.7 µg/nmole
MW of 21 mer dsRNA	13.4 µg/nmole
50 µM solution of 21 mer ssRNA	0.33 µg/µl
20 µM solution of 21 mer dsRNA	0.26 µg/µl
1 A_{260} of 21 mer ssRNA	33 µg/ml
50 µM	0.05 nmoles/µl

* MW = molecular weight

1. Making a 50 µM oligonucleotide solution based on the nmoles synthesized

Calculate the amount of Nuclease-free Water to add as follows:

$$\frac{\text{nmoles synthesized}}{0.05\ \text{nmoles/µl}} = \text{µl for suspension to give a 50 µM solution}$$

Example:

$$\frac{25\ \text{nmoles synthesized}}{0.05\ \text{nmoles/µl}} = \textbf{\textit{500 µl}}\ \text{for suspension to give a 50 µM solution}$$

2. Making a 50 µM oligonucleotide solution based on the mass amount of RNA synthesized

If the specification sheet for the oligonucleotide provides only the mass amount of RNA oligonucleotide synthesized, this is how to calculate the suspension volume necessary to make a 50 µM solution.

a. Determine the molecular weight of the RNA oligonucleotide:

MW = 320 g/mol per base X 21 bases = 6720 g/mole
= 6.7 µg/nmole

b. Calculate the molar amount of RNA synthesized.

The molar amount of RNA synthesized is the mass amount divided by the molecular weight.

$$\#\text{ nmoles synthesized} = \frac{\mu\text{g synthesized}}{6.7\ \mu\text{g/nmole}}$$

c. Calculate the amount of Nuclease-free Water for suspension as in section B.1 on page 13

$$\frac{\text{nmoles synthesized}}{0.05\ \text{nmoles/µl}} = \mu\text{l for suspension to give a 50 µM solution}$$

d. Example:

$$\frac{167.5\ \mu\text{g synthesized}}{6.7\ \mu\text{g/nmole}} = 25\ \text{nmoles synthesized}$$

$$\frac{25\ \text{nmoles}}{0.05\ \text{nmoles/µl}} = \textbf{\textit{500 µl}}\ \text{for suspension to give a 50 µM solution}$$

C. Calculating the Volume of ssRNA or dsRNA Needed for the Procedure

1. Calculating the volume of a molar solution of *duplex* siRNA needed for 5 µg

The *Silencer* siRNA Labeling procedure uses 5 µg of siRNA. The following calculation shows how to determine what volume of a ***duplex*** siRNA solution of known molarity contains 5 µg of RNA.

a. Calculate the concentration of the RNA oligonucleotide solution

concentration = (MW) X (molarity of the solution)

b. Divide the mass amount desired by the concentration of the solution

c. Example: 20 µM solution

MW = 640 g/mol per base X 21 bases = 13440 g/mole

$= 1.344 \times 10^4$ µg/µmole

20 µM solution = 20 µmoles/L

concentration = 1.344×10^4 µg/µmole X 20 µmoles/L = 2.6×10^5 µg/L

$$\frac{5\ \mu g}{2.6 \times 10^5\ \mu g/L} = 1.92 \times 10^{-5}\ L =$$ ***19.2 µl*** of a 20 µM solution is 5 µg of RNA

2. Calculating the volume of a molar solution of RNA oligonucleotide needed for 5 µg

The *Silencer* siRNA Labeling procedure uses 5 µg of RNA. The following calculation shows how to determine what volume of a ***single-strand*** RNA oligonucleotide solution of known molarity contains 5 µg of RNA.

a. Calculate the concentration of the RNA oligonucleotide solution

concentration = (MW) X (molarity of the solution)

b. Divide the mass amount desired by the concentration of the solution

c. Example: 50 µM solution

MW = 320 g/mol per base X 21 bases = 6720 g/mole

= 6720 µg/µmole

50 µM solution = 50 µmoles/L

concentration = 6720 µg/µmole X 50 µmoles/L = 3.36×10^5 µg/L

$$\frac{5\ \mu g}{3.36 \times 10^5\ \mu g/L} = 1.49 \times 10^{-5}\ L =$$ ***15 µl*** of a 50 µM solution is 5 µg of RNA

D. Acrylamide Gel Electrophoresis

1. 6X non-denaturing gel loading buffer

Concentration	Component	for 10 ml
37%	glycerol (100%)	3.7 ml
0.025%	bromophenyl blue	2.5 mg
0.025%	xylene cyanol	2.5 mg
20 mM	1 M Tris-HCl, pH 8	200 µl
5 mM	500 mM EDTA	100 µl
	nuclease-free water	to 10 ml

Alternatively, Ambion offers an all-purpose Gel Loading Solution for native gels, Cat #8556; this 10X solution is rigorously tested for nuclease contamination and functionality.

2. 10X TBE

TBE is generally used at 1X final concentration for preparing gels and/or for gel running buffer.

Concentration	Component	for 1 L
0.9 M	Tris base	109 g
0.9 M	Boric Acid	55 g
20 mM	0.5 M EDTA solution	40 ml

IMPORTANT!
Do not treat TBE with diethylpyrocarbonate (DEPC).

Dissolve with stirring in about 850 ml nuclease-free water. Adjust the final volume to 1 L.

Alternatively, Ambion offers 10X TBE as a ready-to-resuspend mixture of ultrapure molecular biology grade reagents (Ambion Cat #9863). Each packet makes 1 L of 10X TBE.

3. 20% Non-denaturing Acrylamide Gel Mix

We suggest running gels that are approximately 15 cm long to adequately resolve the labeled and unlabeled RNA. 15 ml is enough gel solution for one 13 x 15 cm x 0.75 mm gel

for 15ml	Component
1.5 ml	10X TBE
7.5 ml	40% acrylamide (acryl: bis-acryl = 19:1) (e.g. Ambion Cat #9022)
to 15 ml	high quality water

Stir at room temperature to thoroughly mix, then add:

120 µl 10% ammonium persulfate
16 µl TEMED

Mix briefly after adding the last 2 ingredients, which will catalyze polymerization, then pour gel immediately. (It is not necessary to treat with diethylpyrocarbonate)

4. Gel set up and sample loading

- Follow the manufacturers instructions for the details of attaching gels to the running apparatus.
- Use 1X TBE as the gel running buffer.
- Add non-denaturing gel loading buffer to the samples to 1X, mix well and load the samples in the gel wells.

5. Electrophoresis conditions

Run gels at ~250 Volts constant voltage until the bromophenol blue (the faster-migrating dye) has moved about 3/4 of the length of the gel.

6. Stain the gel in 0.5–1 µg/ml ethidium bromide in water or 1X TBE

Stain the nucleic acids in the gel by soaking for 5–10 min in 0.5–1 µg/ml ethidium bromide in water or 1X TBE. Visualize the gel on a UV transilluminator.

E. Measuring Base:Dye Ratio and RNA Concentration by Spectrophotometry

Table 2. Reference values for spectrophotometry calculations[21]

	Absorbance maximum	Extinction coefficient	Dye correction factor	MW_{base}
Cy3	550	150,000	0.08	1296
FAM	492	30,000	0.32	1006
21-mer ssRNA$_{base}$	260	9,700	—	320
21-mer dsRNA$_{base}$	260	8,000	—	320

1. Measuring the base:dye ratio

a. Dilute the labeled RNA 5–10 fold in 200 mM MOPS pH 7.5 (adjust the pH with NaOH).

b. Blank the spectrophotometer with the 200 mM MOPS at 260 nm, and at the maximum absorbance wavelength for the dye (A_{dye}): 550 nm for Cy3 or 492 nm for FAM.

c. Measure the absorbance of the diluted RNA 260 nm, and at the maximum absorbance wavelength for the dye (A_{dye}): 550 nm for Cy3 or 492 nm for FAM. With these absorbance values in hand, use the convenient base:dye ratio calculator on our web site to do the math. Find it at http://www.ambion.com/techlib/append/base_dye.html.

d. Since Cy3 and FAM absorb some light at 260 nm (as well as at their absorbance maxima), remove their contribution to the A_{260} reading with the following calculation and the appropriate dye correction factor from Table 2

$$A_{base} = (A_{260} \times \text{dilution factor}) - (A_{dye} \times \text{dilution factor} \times \text{dye correction factor})$$

e. Calculate the ratio of bases to dye molecules using the following equation:

$$\text{base:dye} = \frac{(A_{base} \times \text{extinction coefficient}_{dye})}{(A_{dye} \times \text{extinction coefficent}_{base})}$$

2. Calculating the concentration of the nucleic acid:

Using values from Table 2 and the corrected absorbance of the labeled RNA from step 1.d above, the concentration of the labeled RNA can be calculated using the following equation:

$$\text{mg/ml RNA} = \frac{(A_{base} \times MW_{base})}{(\text{extinction coefficent}_{base} \times \text{path length in cm})}$$

NOTE:
Most spectrophotometers have a 1 cm path length; if you don't know the path length for the spectrophotometer, assume that it is 1 cm.

V. Appendix

A. References

Byrom M, Pallotta V, Brown D, Ford LP (2002) RNAi in mammalian cells: visualizing siRNA and analyzing induction of RNAi. *Ambion TechNotes* **9(3):** 6–8.

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

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391:** 806–811.

Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev.* **15(20):** 2654–2659.

B. *Silencer* siRNA Labeling Kit Specifications

Kit Contents:

Cat #1632	Cat #1634	Component	Storage
500 µl	500 µl	10X Labeling Buffer	–20˚C
100 µl	100 µl	Reconstitution Solution	–20˚C
1.75 ml	1.75 ml	Nuclease-free Water	–20˚C
500 µl	500 µl	5M NaCl	–20˚C
500 µl	500 µl	5X siRNA Annealing Buffer	–20˚C
40 µl	40 µl	GAPDH siRNA	–20˚C*
40 µg	– –	Cy3 Labeling Reagent	–20˚C
– –	60 µg	Fluorescein Labeling Reagent	–20˚C

* ≤–70˚C for long term storage (over 6 months)

Quality control:

Functional Testing

The GAPDH siRNA is tested and determined to knock down the expression of GAPDH in HeLa cells. The labeling kit is tested and determined to label the GAPDH siRNA efficiently.

Nuclease testing

The Cy3 and Fluorescein Labeling Reagents are tested for RNase using Ambion's RNaseAlert assay, the remainder of the kit components are tested in Ambion's rigorous nuclease assays described below.

RNase activity
None detected after incubation with ^{32}P-labeled RNA; analyzed by PAGE.

Non-specific endonuclease/nickase activity
None detected after incubation with supercoiled plasmid DNA; analyzed on 1% agarose.

Exonuclease activity
None detected after incubation with ^{32}P-labeled *Sau*3A fragments of pUC19; analyzed by PAGE.

Safety information:

A material safety data sheet is provided for the Reconstitution Solution which contains DMSO. None of the other kit components are thought to represent a health hazard.

C. Dimethyl Sulfoxide Material Safety Data Sheet

Physical Data	
Synonyms	DMSO, Methyl sulfoxide, Sulfinylbis {Methane}
Appearance and Odor	Colorless, hygroscopic
Boiling Point	n/a
Specific Gravity (H_20 = 1)	1.10
CAS#	67-68-5
Fire and Explosion Hazard Data	
Flash Point	89°C
Flammable Limits in Air (% by volume)	Combustible, moderate fire hazard when exposed to heat or flame.
Extinguishing Media	CO_2, Foam, Dry Chemical
Special Fire Fighting	Wear self-contained breathing apparatus and protective equipment as needed for surrounding fire.
Health Hazard Data	
Effects of Overexposure	Irritating to eyes, respiratory system and skin, readily absorbed into the skin. ***Wear gloves at all times.***
Emergency First Aid Procedures	Wash affected area with plenty of soap and water. Induce vomiting if swallowed. Irrigate eyes for at least 15 minutes. See physician, treat symptomatically.
Reactivity Data	
Stability	Stable
Incompatibility	Strong oxidizing agents, alkalies
Hazardous Decomposition Products	Possible toxic fumes of SO_2
Hazardous Polymerization	Will not occur.
Conditions to Avoid	Heat, flames, ignition sources
Spill or Leak Procedures	
If released or spilled	***Do not use combustible materials to absorb spill.*** Absorb on sand and place in open container for disposal. Ventilate area & wash spill site.
Waste Disposal Method	Dispose according to federal, state, and local regulations.

Special Protection Information

Respiratory Protection	Use NIOSH approved respirator as needed.
Ventilation	Lab hood as appropriate.
Protective Gloves	Neoprene or PVC
Eye Protection	Use full eye protection-goggles as standard precautionary measures.
Handling procedures	Follow routine safe handling procedures.