USER GUIDE

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Silencer® siRNA Labeling Kit

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life technologies™

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Contents

Silencer® siRNA Labeling Kit

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Introduction

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. It was observed that the use of double-stranded RNA (dsRNA) caused a greater reduction in gene expression than the use of either the sense or anti-sense strands of RNA alone (Fire et al. 1998). 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 into short dsRNAs, which contain a two nucleotide 3' overhang and a 5' phosphate (Ketting et al. 2001). These short dsRNAs, also called short interfering RNA (siRNA), next associate with the RNA-induced silencing complex (RISC) which 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). Better understanding of the RNAi mechanism has led to the advancements which enable RNAi to be used in mammalian cells (Elbashir et al. 2001). Visit **www.lifetechnologies.com/RNAi** for further information.

Product description

The *Silencer*® Cy®3 and fluorescein (FAM™ dye) RNA Labeling Kits enable researchers to label siRNA produced by chemical and enzymatic syntheses. 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 procedure. 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 siRNAs can be used in the analysis of siRNA transfection efficiencies and distribution for pharmacokinetic and metabolism studies. The nucleic acid labeling reagents are designed to covalently attach either Cy®3 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 plate at a final concentration of 100 nM.

Materials provided with the kit

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

Cat. no. AM1632	Cat. no. AM1634	Component	Storage
1.75 mL	1.75 mL	Nuclease-free Water	any temp‡
500 µL	500 µL	10X Labeling Buffer	–20°C
100 µL	100 µL	Reconstitution Solution	–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	–	Cy®3 Labeling Reagent†	–20°C
–	60 µg	FAM™ Labeling Reagent‡	–20°C

† Manufactured for Life Technologies by Mirus Corporation.
‡ Store the Nuclease-free Water at –20°C, 4°C, or room temp.
§ Store at –70°C or below for long term storage (over 6 months).

Excitation and emission wavelengths of dyes

The excitation and emission wavelengths for the dyes used in the *Silencer*® siRNA Labeling Kits are the following:

Cat. no.	Dye	Excitation	Emission
AM1632	Cy®3	550 nm	570 nm
AM1634	FAM™	492 nm	518 nm

Materials not provided with the kit

siRNA to be labeled

siRNA oligonucleotides; either ssRNA, or duplex siRNA can be labeled using the *Silencer*® siRNA Labeling Kit. The siRNA substrate can be made enzymatically or by chemical synthesis. siRNAs produced by enzymatic digestion (e.g. RNase III or Dicer) must be phenol extracted and ethanol precipitated before the labeling reaction. For information on sources of RNA oligonucleotides and siRNA design, see "Related products available from Life Technologies" on page 13.

siRNA must be in a low salt solution or in water for efficient labeling with this kit (see section "siRNA substrate requirements and preparation" on page 7 for more information).

100% ethanol

- 100% ethanol, ACS reagent grade or equivalent: The procedure calls for cold 100% ethanol, so store some ethanol at –20°C.
- 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 tested by either running part of the reaction on a 20% acrylamide gel (instructions in section "Acrylamide gel electrophoresis" on page 18), or by measuring the absorbance of the labeled RNA solution and calculating the labeling efficiency (follow the instructions in section "Measuring base:dye ratio and RNA concentration by spectrophotometry" on page 20.

siRNA labeling procedure

IMPORTANT! Use appropriate RNase control measures.
Treat gloves and work area with an RNase decontamination agent such as RNase*Zap*® Solution, 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.

siRNA substrate requirements and preparation

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.
- For optimal labeling of chemically synthesized siRNAs, anneal the individual siRNAs following the instructions in section "Annealing RNA oligonucleotides to make siRNA" on page 15.
- Duplex siRNA made with the *Silencer*® siRNA Construction Kit will be in Nuclease-free Water at the end of the procedure, and can be used directly in the *Silencer*® siRNA Labeling Kit.

siRNA populations made by enzymatic digestion must be cleaned up before labeling

siRNAs made by enzymatic digestion of larger RNAs, must be cleaned up by phenol chloroform extraction and ethanol precipitation before starting the labeling reaction. Cleanup instructions are provided below.

1. Adjust the volume of the siRNA to ≥100 µL with nuclease-free water, then add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and vortex for several seconds. Centrifuge (≥10,000 x g) at room temp for ~5 min to separate the aqueous and organic phases, then transfer the aqueous phase to a new tube.
2. Add an equal volume of chloroform:isoamyl alcohol (24:1), and vortex for several seconds. Then centrifuge as in the previous step to separate the phases. Transfer the aqueous phase to a fresh tube.

3. Finally precipitate the siRNA by adding 0.1 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol (molecular biology grade), and mixing thoroughly. Place the precipitation mixture at –20°C or colder for ≥20 min, then centrifuge at ≥10,000 x g for at least 15 min.
   Remove the supernatant carefully and discard it. To remove the last traces of ethanol, respin the tube briefly and aspirate any remaining supernatant with a fine-tipped pipet.
4. Resuspend the siRNA to 20 µM in Nuclease-free Water.

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 Cy®3 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 temp 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 order shown, 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. 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	Cy®3 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	Cy®3 or FAM™ Labeling Reagent

3. Incubate 1 hr at 37°C in the dark

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

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 this step in your protocol.

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

   For the 50 µL reactions described in section "Labeling reaction" on page 8, add the following and mix well:

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

2. Chill 30–60 min at –20°C

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

   STOPPING POINT. The reaction can be stored overnight or longer at –20°C at this point if desired.

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

   a. Pellet the labeled RNA by centrifugation at ≥8,000 x g for 15–20 min.

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

   c. A red (Cy®3), or green (FAM™ dye) siRNA pellet should be visible.

4. Wash RNA pellet with 175 µL of 70% ethanol

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

   b. Carefully remove supernatant with a pipette. To remove the last traces of solution, respin the tube briefly and discard the supernatant.

5. Dry 5–10 min at room temp

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

6. Resuspend 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 labeled RNA in the same volume it was in before the labeling reaction (e.g. 19.2 µL for duplex siRNA or 15 µL for ssRNA oligonucleotide) to maintain 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 "Measuring base:dye ratio and RNA concentration by spectrophotometry" on page 20.)

Troubleshooting

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 the *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 "Labeling reaction" on page 8.
   b. Continue the procedure through the ethanol precipitation in section "Ethanol precipitation of labeled RNA" on page 9.

      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.
   a. Analysis of the positive control labeling reaction by spectrophotometry
      i. Dilute the labeled GAPDH siRNA solution with 76.8 µL of either 200 mM MOPS pH 7.5 (adjust the pH with NaOH) or 1X Labeling Buffer. 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 (see "Materials provided with the kit" on page 6 for excitation and emission wavelengths). As a baseline, also record the A_{260} and the $A_{abs\ max}$ of the 200 mM MOPS used to dilute the labeled siRNA.
      iii. Calculate the base:dye ratio as described in section "Measuring base:dye ratio and RNA concentration by spectrophotometry" on page 20.

      By spectrophotometry the base:dye ratio should be 200–300 or lower; the lower the base:dye ratio, the more dye molecules are present on the siRNA.

b. Analysis of the positive control labeling reaction by electrophoresis (see section "Acrylamide gel electrophoresis" on page 18 for recipes and gel running instructions).

c. 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.

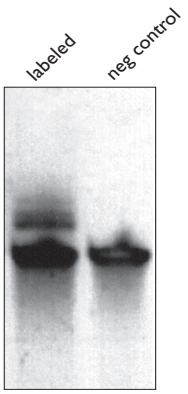
d. Add gel loading buffer to the 19.2 µL of labeled GAPDH siRNA from step 1.b. on page 10, and mix well.

e. 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.

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

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

Figure 1 Acrylamide Gel Analysis of the *Silencer*® siRNA Labeling Kit positive control experiment. The GAPDH siRNA supplied with the kit was labeled with FAM™ dye, 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.



No colored pellet is visible after thanol precipitation

Normally, the siRNA can be visualized at the bottom of the tube after ethanol precipitation (step 3. on page 9) as a red (Cy®3) or green (FAM™ dye) 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 on page 11).

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 following the instructions in section "Annealing RNA oligonucleotides to make siRNA" on page 15, or duplex siRNA can be made with *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 "Ethanol precipitation of labeled RNA" on page 9 for optimal precipitation.

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, or using a spectrophotometer.

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

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 will be used to hold the siRNA and filter sterilize any solutions (other than the Nuclease-free Water supplied with the kit) that will be used to resuspend the labeled siRNA.

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.

A Supplemental Information

Related products available from Life Technologies

Silencer® siRNAs	*Silencer*® Pre-designed siRNAs, Validated siRNAs, and siRNA Libraries are designed with the most rigorously tested siRNA design algorithm in the industry. *Silencer*® siRNAs are available for >100,000 human, mouse, and rat targets from our searchable online database. Because of their carefully optimized design, *Silencer*® siRNAs are very effective, and they are guaranteed to reduce target mRNA levels by 70% or more. Furthermore, their exceptional potency means that *Silencer*® siRNAs effectively induce RNAi at very low concentrations, minimizing off-target effects.
Silencer® siRNA Construction Kit Cat. no. AM1620	The *Silencer*® siRNA Construction Kit 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).
Silencer® siRNA Transfection II Kit Cat. no. AM1631	The *Silencer*® siRNA Transfection II Kit contains both siPORT™ *NeoFX™* and siPORT™ *Amine* Transfection Agents in addition to a well-characterized siRNA targeting human, mouse, and rat GAPDH. This kit is ideal for developing an optimal transfection protocol for your cells. Also included are a highly validated non-targeting negative control siRNA and a detailed Protocol.
RNase-free Tubes & Tips	RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free.
RNase*Zap*® Solution Cat. nos. AM9780, AM9782, AM9784	RNase*Zap*® RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*® Solution.

B Additional procedures

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.

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

Amount	Component
30 µL	sense RNA oligonucleotide (50 µM)
30 µL	antisense RNA oligonucleotide (50 µM)
15 µL	5X siRNA Annealing Buffer
75 µL	total volume

   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 incubation at 37°C 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.

Suspension of dry RNA oligonucleotides

IMPORTANT! Treat gloves and surrounding area with an RNase decontamination agent such as RNaseZap® prior to starting the procedure. 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 Kit procedure.

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 A260 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:

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

   Example:

   $$\frac{25\ \text{nmoles synthesized}}{0.05\ \text{nmoles/µL}} = 500\ \text{µL for suspension to make a 50 µM solution}$$

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:

   $$\text{MW} = 320\ \text{g/mol per base} \times 21\ \text{bases} = 6720\ \text{g/mole} = 6.7\ \text{µg/nmole}$$

   b. Calculate the molar amount of RNA synthesized.

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

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

c. Calculate the amount of Nuclease-free Water for suspension as in section "Suspension of dry RNA oligonucleotides" on page 15

$$\frac{\text{nmoles synthesized}}{0.05\ \text{nmoles}/\mu\text{L}} = \mu\text{L for suspension to make a 50 } \mu\text{M solution}$$

Example:

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

$$\frac{25\ \text{nmoles}}{0.05\ \text{nmoles}/\mu\text{L}} = 500\ \mu\text{L for suspension to make a 50 } \mu\text{M solution}$$

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 Kit 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

      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

      $$\frac{5\ \mu\text{g}}{2.6 \times 10^5\ \mu\text{g/L}} = 1.92 \times 10^{-5}\ \text{L}$$

      = 19.2 µL of a 20 µM sol'n contains 5 µg RNA

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

   The *Silencer* siRNA Labeling Kit 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

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 sol'n contains 5 µg RNA

Acrylamide gel electrophoresis

1. 6X non-denaturing gel loading buffer

Concentration	Component	for 10 mL
37%	glycerol (100%)	3.7 mL
0.025%	bromophenol 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, Life Technologies offers an all-purpose Gel Loading Solution for native gels, Cat. no. AM8556; 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.

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

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

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

Alternatively, Life Technologies offers nuclease-free solutions of 10X TBE (Cat. nos. AM9863, AM9865) and ready-to-resuspend powdered 10X TBE packets (Cat. no. AM9864). Both are made from of ultrapure molecular biology grade reagents.

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. Cat. no. AM9022)
to 15 mL	distilled deionized 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 the gel mix 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.

Measuring base:dye ratio and RNA concentration by spectrophotometry

1. Measuring absorbance of RNA and dye
   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.

	Absorbance maximum	Extinction coefficient	Dye correction factor	MW_{base}
Cy®3	550	150,000	0.08	1296
FAM™dye	492	30,000	0.32	1006
21-mer $ssRNA_{base}$	260	9,700	––	320
21-mer $dsRNA_{base}$	260	8,000	––	320

   c. Measure the absorbance of the diluted RNA at 260 nm, and at the maximum absorbance wavelength for the dye (A_{dye}).

2. Calculating the base:dye ratio and concentration of the nucleic acid

   With the absorbance values obtained above, calculate the base:dye ratio and nucleic acid concentration by the following method:

   Follow the equations below:

   a. Since Cy®3 and FAM™ dyes 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 the above table.

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

   b. Calculate the ratio of bases to dye molecules using the following equation and extinction coefficients from the above table:

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

   c. Using values from the above table and the corrected absorbance of the labeled RNA from step 2.a. 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.

C

Safety

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:
Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.).
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/ 29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

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Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**

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