*mir*Vana™ miRNA Probe Construction Kit

(Part Number AM1550)

Protocol

P/N 1550M Revision B **Revision Date: November 7, 2008**

For research use only. Not for use in diagnostic procedures.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the *mir*Vana™ miRNA Probe Construction Kit.

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I. Introduction

A. Product Description and Background Information

Brief product description

Small RNAs, such as microRNA (miRNA) and small interfering RNA (siRNA) are being studied by more and more labs as information about their importance in biological systems emerges. Short RNA probes are essential for the detection of small RNAs by solution or blot hybridization. The *mir*Vana™ miRNA Probe Construction Kit is designed for the rapid preparation of dsDNA templates, and in vitro transcription of these templates to synthesize short (<100 nt) RNA transcripts. If desired, fluorescently-labeled or radiolabeled nucleotides can be incorporated during transcription to make labeled RNA. The kit can also be used for transcription of pre-existing dsDNA templates with a T7 promoter sequence, such as linearized plasmid DNA or PCR products.

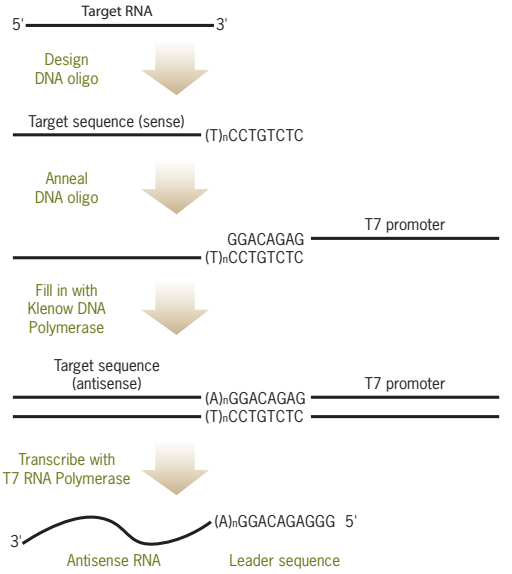
In vitro transcription

T7 phage RNA polymerase is widely used for the in vitro synthesis of RNA transcripts from DNA templates. The template must have a double-stranded 19–23 base promoter upstream of the sequence to be transcribed. To make transcript, the template is mixed with T7 Enzyme Mix, rNTPs, and Transcription Buffer, and the reaction mixture is incubated for 10 min to 1 hr at 37°C. RNA polymerase binds to the dsDNA promoter, which then separates the two DNA strands, and uses the 3' to 5' strand as a template to synthesize a complementary 5' to 3' RNA transcript.

Template preparation

The first step in the *mir*Vana miRNA Probe Construction Kit procedure is to design a DNA oligonucleotide template specific to the sequence you want to detect, which includes an 8 base sequence complementary to the 3' end of the T7 Promoter Primer included in the kit (Figure 1 on page 2). This target-specific oligonucleotide template is then annealed to the supplied T7 Promoter Primer, and is "filled-in" with Exo– Klenow to generate a dsDNA template ready for in vitro transcription without any further treatment or purification. The dsDNA templates prepared with the *mir*Vana miRNA Probe Construction Kit are also compatible with other in vitro transcription kits such as the Ambion® MAXIscript® T7 in vitro Transcription Kit.

Figure 1. Overview of the *mir*Vana™ miRNA Probe Construction Kit Procedure.



Applications

The *mir*Vana miRNA Probe Construction Kit is designed specifically to transcribe short, labeled antisense probes or unlabeled sense RNA. Such transcripts are typically used for the study of small noncoding RNA molecules. Radiolabeled probes prepared with the *mir*Vana miRNA Probe Construction Kit have been successfully used for the sensitive detection of messenger RNAs (mRNAs), small nuclear RNAs (snRNAs), siRNAs, and miRNAs by Northern blot and solution hybridization. Both radiolabeled and nonisotopically labeled probes generated with this kit have been used to study the distribution of miRNA or mRNA in tissues by in situ hybridization. Together with the *mir*Vana miRNA Detection Kit, *mir*Vana miRNA Isolation Kit, *mir*Vana Probe & Marker Kit, and validated FirstChoice® Total RNAs, the *mir*Vana miRNA Probe Construction Kit is part of an optimized system for the study of the biogenesis and functions of small RNAs, such as siRNA and miRNA.

RNA transcripts are useful as probes in hybridization reactions because RNA-RNA and RNA-DNA hybrids are more stable than DNA-DNA duplexes. Furthermore, single-stranded RNA probes are not depleted by rehybridization to a complementary probe strand. In addition to standard detection systems on solid support, i.e. Northern, Southern or dot blot, short antisense RNA probes can be used for in situ hybridization and solution hybridization assays.

Short RNAs prepared by in vitro transcription are also useful in a variety of RNA structure/function experiments. For more information see our web site at:

www.ambion.com/techlib/resources/structure/index.html

Radiolabeled RNA transcripts are also widely used to study protein-RNA interactions. The most common applications include bandshift in nondenaturing gels (EMSA) or in vitro crosslinking with recombinant or native proteins, immunoprecipitation with antibodies specific for the protein of interest, or protein pull-down using recombinant tagged fusion proteins.

Control probe

The Control DNA Primer provided with the kit will generate an antisense RNA probe specific for miR-16 miRNA (Lagos-Quintana et al. 2001). This probe has been successfully used to detect *mir-16* expression in various human and mouse tissues and cell lines by hybridization on solid support (Northern blot), in situ hybridization, and hybridization in solution (*mir*Vana miRNA Detection Kit).

Incorporating labeled nucleotides into RNA transcripts

The *mir*Vana miRNA Probe Construction Kit can be used to incorporate radiolabeled nucleotides as well as many types of nonisotopically modified nucleotides into RNA. Generally [α-32P]UTP or CTP is recommended for synthesis of radioactive probes. Also, α-33P, α-35S, 3H, fluoresceinated, biotinylated, and digoxigenin-labeled nucleotides can be used with the kit.

Unlabeled RNA

The *mir*Vana miRNA Probe Construction Kit can also be used to transcribe unlabeled RNA; between 2–6 µg per reaction can be produced when the maximum concentration of all four ribonucleotides is used in the reaction. For large scale synthesis of RNA (up to 80 µg RNA per 20 µL reaction), we recommend the Ambion MEGAshortscript™ T7 Kit.

B. Reagents Provided with the Kit and Storage

The kit should be stored at –20°C in a non-frost-free freezer.

The *mir*Vana miRNA Probe Construction Kit provides reagents for converting 30 DNA oligonucleotides into transcription templates, and reagents for 30 transcription reactions.

Template preparation reagents

Amount	Component	Storage
200 µL	DNA Hybridization Buffer	–20°C
60 µL	T7 Promoter Primer	–20°C
60 µL	10X Klenow Reaction Buffer	–20°C
60 µL	10X dNTP Mix	–20°C
60 µL	Exo– Klenow	–20°C

Transcription reagents

Amount	Component	Storage
60 µL	T7 Enzyme Mix	–20°C
250 µL	10X Transcription Buffer	–20°C
30 µL	ATP Solution (10 mM)	–20°C
30 µL	CTP Solution (10 mM)	–20°C
30 µL	GTP Solution (10 mM)	–20°C
30 µL	UTP Solution (10 mM)	–20°C

Other reagents

Amount	Component	Storage
10 µL	Control DNA Primer (100 µM)	–20°C
45 µL	DNase I	–20°C
1.4 mL	Gel Loading Buffer II	–20°C
8 mL	Probe Elution Buffer	–20°C
1.75 mL	Nuclease-free Water	any temp*

* Store Nuclease-free Water at –20°C, 4°C, or room temp.

C. Materials Not Supplied with the Kit

Gene specific DNA oligonucleotide templates

See section II.A starting on page 6 for complete instructions on designing oligonucleotide templates.

Labeled NTP

Generally [α-^{32}P]UTP or CTP, at about 800 Ci/mmol and 10 mCi/mL or greater in aqueous solution, is recommended for synthesis of radioactive probes. Alternatively, α-^{33}P, α-^{35}S, ^{3}H, fluoresceinated, biotinylated, and digoxigenin nucleotides can be used in the transcription reaction.

General laboratory equipment and supplies

- Constant temperature heat block (37°C and 95–100°C)
- RNase-free 1.5 mL or 0.5 mL polypropylene microfuge tubes, adjustable pipettors and RNase-free tips

D. Related Products Available from Ambion

MAXIscript® Kits P/N 1308–1326	MAXIscript Kits are designed for synthesis of high specific-activity RNA probes with specific activities reaching 1 x 10^9 cpm/µg in just 10 minutes. MAXIscript Kits are available for DNA templates containing T7, T3, and SP6 promoters.
MEGAshortscript™ T7 Kit P/N 1354	The MEGAshortscript Kit gives high yields of in vitro transcription products in the 20–500 nucleotide range. Many modified nucleotides are efficiently incorporated, but the kit is not recommended for the synthesis of high specific-activity radiolabeled probes.
*mir*Vana™ miRNA Detection Kit P/N 1552	The *mir*Vana miRNA Detection Kit provides an extremely sensitive solution hybridization assay capable of detecting attomole amounts of RNA. In addition, it can be used to simultaneously detect several small RNAs such as miRNA and siRNA, or both small RNA and long RNA species in the same sample. For a complete solution for small RNA analysis, use this kit in conjunction with the *mir*Vana miRNA Probe Construction Kit and/or the *mir*Vana Probe & Marker Kit.
*mir*Vana™ Probe & Marker Kit P/N 1554	The *mir*Vana Probe & Marker Kit is an end labeling kit designed for making short radiolabeled probes, and low molecular weight markers for studies involving microRNAs. It can be used with synthetic RNA or DNA oligonucleotides to prepare labeled probes, and the kit also provides reagents to prepare small radiolabeled RNA size markers (Decade™ Markers), and single-nucleotide RNA ladders. Rapid cleanup reagents are included to prepare the reaction products for various downstream application.
PAGE Reagents See web or print catalog for P/Ns	Ambion offers high-quality reagents for polyacrylamide gel electrophoresis (PAGE), including acrylamide solutions for RNA, DNA and protein gels, ultra-pure molecular biology grade urea, and premixed acrylamide/urea solutions for simple preparation of denaturing gels.
miRNA Certified FirstChoice® Total RNA See web or print catalog for P/Ns	All of Ambion's high quality total RNA from normal human, mouse, and rat tissue is prepared by methods that quantitatively recover microRNAs. The entire line of FirstChoice Total RNAs are free of DNA and shown to be intact by stringent quality control standards.

II. miRNA Probe Construction Kit Procedure

A. Design of DNA Oligonucleotide Templates

1. Select the target-specific portion of the template sequence

Template for transcription of antisense RNA probes
The DNA oligonucleotide template should have the same sequence as the target RNA except that U residues are replaced with T's.

Template for transcription of sense RNA
The DNA oligonucleotide template should be the reverse complement of the RNA sequence. (See section A.5 on page 7 for an example.)

2. (optional) Add T residues to the 3' end of the oligonucleotide

Adding T residues to the 3' end of a DNA oligonucleotide template will generate an antisense RNA probe containing a stretch of A residues which cannot be cleaved by RNases A or T1. Such a design allows simultaneous detection of several targets of the same size in solution hybridization assays (e.g. using the *mir*Vana miRNA Detection Kit) by artificially creating size differences between different probes. The addition of 3–5 T residues to the DNA oligonucleotide template sequence is typically sufficient for size resolution on the gels used for multiprobe solution hybridization assays to detect small RNAs.

3. Add the T7 promoter sequence to the 3' end of the oligonucleotide

Add the 8 nt sequence 5'-`CCTGTCTC`-3' to the 3' end of your DNA oligonucleotide template sequence. This sequence is complementary to the T7 Promoter Primer provided with the kit; it allows hybridization of your DNA oligonucleotide template with the T7 Promoter Primer, so that the fill-in reaction will produce a double-strand transcription template containing a T7 promoter.

4. Order oligonucleotide

Order the smallest scale oligonucleotide synthesis scale (e.g. 25 nmol); this will be enough oligonucleotide for hundreds of transcription reactions. Desalting is typically sufficient purification for generating efficient transcription templates.

5. Examples of template design

The examples below illustrate template design for sense-strand RNA, and antisense RNA probe with and without added A residues. Text in **bold** represents sequence complementary to the T7 Promoter Primer which must be added to the template oligonucleotide. Underlined text represents transcript sequence that is not derived from the target RNA.

To synthesize antisense probes	
If target RNA sequence (miR-16, 22 nt) is	5'-UAGCAGCACGUAAAUAUUGGCG-3'
then order the DNA template (mir-16, 30 nt)	5'-TAGCAGCACGTAAATATTGGCG**CCTGTCTC**-3'
and it will generate a mir-16 (32 nt) RNA probe*	3'-AUCGUCGUGCAUUUAUAACCGCGGACAGAGGG-5'
with the protected 22 nt sequence (mir-16)†	3'-AUCGUCGUGCAUUUAUAACCGC-5'

* Note that two extra G's are added during the transcription reaction.
† When used with *mir*Vana miRNA Detection Kit.

To synthesize antisense probes with added A's	
If target RNA sequence (miR-16, 22 nt) is	5'-UAGCAGCACGUAAAUAUUGGCG-3'
then order the DNA template (mir-16 +4, 34 nt)	5'-TAGCAGCACGTAAATATTGGCGTTTT**CCTGTCTC**-3'
and it will generate a mir-16 +4 (36 nt) RNA probe	3'-AUCGUCGUGCAUUUAUAACCGCAAAAGGACAGAGGG-5'
with the protected 26 nt sequence (mir-16 +4)*	3'-AUCGUCGUGCAUUUAUAACCGCAAAA-5'

* The terminal A's, though not complementary to target, are not digested by RNase A or T1.

To synthesize sense-strand RNA	
If target RNA sequence (miR-16, 22 nt) is	5'-UAGCAGCACGUAAAUAUUGGCG-3'
then order the DNA template (mir-16 sense, 30 nt)	5'-CGCCAATATTTACGTGCTGCTA**CCTGTCTC**-3'
it will generate a mir-16 sense RNA (32 nt) transcript*	5'-GGGAGACAGGUAGCAGCACGUAAAUAUUGGCG-3'

* Note that two extra G's are added during the transcription reaction.

B. dsDNA Transcription Template Preparation

To make an efficient transcription template, your target-specific DNA oligonucleotide template must be converted to dsDNA with a T7 promoter at the 5' end. This is accomplished by hybridizing the DNA oligonucleotide to the T7 Promoter Primer provided with kit, and extending the T7 Promoter Primer and template oligonucleotide using Exo– Klenow DNA polymerase.

1. Resuspend the oligonucleotide template to 100 µM

Oligonucleotides are usually supplied dry; centrifuge the tube containing the oligonucleotide for 30 sec to collect the powder at the bottom of the tube. Check the specification sheet supplied with the oligonucleotide to see how much was synthesized, and dissolve the oligonucleotide to 100 µM (100 pmol/µL) in nuclease-free water or 10 mM Tris-HCl pH 7–8, 0.1 mM EDTA.

2. Thaw frozen reagents for template preparation

Thaw the following kit components at room temp, then briefly vortex each before use.

- T7 Promoter Primer
- DNA Hybridization Buffer
- 10X Klenow Reaction Buffer
- 10X dNTP Mix
- Nuclease-free Water

IMPORTANT

Keep the tube of Exo– Klenow at –20°C and do not vortex it.

3. Hybridize your oligonucleotide template to the T7 Promoter Primer

a. In a microfuge tube mix the following:

Amount	Component
2 µL	T7 Promoter Primer
6 µL	DNA Hybridization Buffer
2 µL	oligonucleotide template (100 µM)

b. Heat the mixture to 70°C for 5 min, then leave at room temp for 5 min to allow hybridizaton to occur.

4. Fill in with Klenow DNA polymerase

a. Add the following to the hybridized oligonucleotides:

Amount	Component
2 µL	10X Klenow Reaction Buffer
2 µL	10X dNTP Mix
4 µL	Nuclease-free Water
2 µL	Exo– Klenow

b. Gently mix by pipetting or gentle vortexing. Centrifuge briefly to collect the mixture at the bottom of the tube.

c. Transfer to 37°C incubator and incubate for 30 min.

5. Proceed to transcription, or store the template at –20°C

The template can be used directly in a transcription reaction (see section *C. Transcription* below) or stored at –20°C until it is needed for transcription.

C. Transcription

1. Thaw the frozen transcription reagents

Thaw the following kit components at room temp, then briefly vortex each before use.

- 10X Transcription Buffer
- Nucleotide solutions (ATP, CTP, GTP, and UTP)
- Nuclease-free Water

IMPORTANT

Once thawed, store the NTPs on ice, but keep the 10X Transcription Buffer at room temp.

IMPORTANT

Keep the tube of T7 Enzyme Mix at –20°C and do not vortex it.

2. Assemble the transcription reactions at room temp

Mix the following components in the order shown below. It is important to assemble the reaction ***at room temp*** as components in the 10X Transcription Buffer can precipitate if the reaction is assembled on ice.

Component	Radiolabeled e.g. ^{32}P	Unlabeled reaction	Nonisotopic e.g. biotin
Nuclease-free Water	to 20 µL	to 20 µL	to 20 µL
dsDNA template*	1 µL	1 µL	1 µL
10X Transcription Buffer	2 µL	2 µL	2 µL
10 mM ATP	1 µL	1 µL	1 µL
10 mM CTP	1 µL	1 µL	1 µL
10 mM GTP	1 µL	1 µL	1 µL
10 mM UTP	– –	1 µL	– –
Labeled UTP†	5 µL‡	– –	1 µL**
T7 RNA Polymerase	2 µL	2 µL	2 µL

* Other dsDNA templates with a consensus T7 promoter, such as linearized plasmid DNA, dsDNA prepared by PCR, or two annealed oligonucleotides can also be used in the transcription reaction.

† The reaction shown uses labeled UTP, to use a different labeled nucleotide, substitute the labeled nucleotide for its unlabeled counterpart in the reaction.

‡ In this example, 800 Ci/mmol [α-^{32}P]UTP at a concentration of 10 mCi/ml or 12.5 µM is used, giving a final limiting nucleotide concentration of 3.125 µM.

**In this example, nonisotopically-labeled UTP at a concentration of 10 mM is used, giving a final concentration of 0.5 mM.

3. Mix thoroughly

Gently pipet up and down several times to mix, then centrifuge briefly to collect the reaction mixture at the bottom of the tube.

4. Incubate 10–30 min at 37°C

High specific activity radiolabeling reactions (3–10 µM limiting nucleotide) should typically be incubated for 10 min.

Reactions with >10 µM limiting nucleotide should be incubated for 30 min. This includes unlabeled, trace-labeled, and nonisotopically labeled reactions.

5. Add 1 µL DNase I and incubate 10 min at 37°C

Remove the template DNA by digesting the reaction with DNase I as follows:

a. Add 1 µL DNase I to the transcription reaction, and mix well.

b. Incubate at 37°C for 10 min.

D. After the Transcription Reaction

Storage of transcript

The RNA transcript can be stored at –20°C. Radiolabeled RNA transcripts, however, have a limited shelf life of typically less than 1 week because of radiolytic degradation. Unlabeled transcripts can be stored indefinitely (for storage for longer than ~6 months, consider storing RNA as an ethanol precipitate). The shelf life of RNA transcripts containing modified nucleotides will depend on the stability of the modified moiety.

Determination of specific activity

The yield and specific activity of radiolabeled transcripts can be determined by measuring and comparing the total amount of radiolabel present in the reaction mixture and the trichloroacetic acid (TCA) precipitable counts (see section IV.B starting on page 15).

Gel purification

For some applications it is necessary to have primarily full-length probe (e.g. ribonuclease protection assays, mapping studies, and EMSA), and in such cases, we recommend gel purifying the probe. This can be done directly after the DNase I treatment as described in section IV.C starting on page 20.

Removal of unincorporated nucleotides

Unincorporated ribonucleotides can be removed from radiolabeled or nonradiolabeled transcription reaction by ammonium acetate/ethanol precipitation or by column purification (see section IV.D starting on page 22).

III. Troubleshooting

A. Using the Control DNA Primer Supplied with the Kit

Description

The Control DNA Primer provided with the *mir*Vana miRNA Probe Construction Kit can be used to make an antisense mir-16 +4 probe.

Instructions

To make the positive control transcript, use 2 µL of the Control DNA Primer to make a transcription template following the instructions in section II.B starting at step 2 on page 8. Use 1 µL of the resulting dsDNA template in a transcription reaction following the instructions in section II.C starting on page 9.

Expected result

The size of the positive control mir-16 +4 probe is 36 nt. It migrates more slowly than the xylene cyanol dye band in a 15% denaturing polyacrylamide gel.

Approximately 50% of the labeled nucleotide should be incorporated into TCA-precipitable material when fresh [α–^{32}P]UTP is used for transcription in the absence of unlabeled UTP. Under these conditions more than 50% of the transcription products should correspond to the full-length (36 nt) transcript.

The mir-16 +4 probe can be used to detect miR-16 miRNA (22 nt) in human or mouse total RNA samples by Northern blot. When used with the *mir*Vana miRNA Detection Kit, the mir-16 +4 probe will produce a protected fragment 4 bp longer than miR-16 miRNA (26 nt) allowing simultaneous detection of other miRNA of similar length in the same experimental sample. Both radiolabeled and nonisotopically labeled mir-16 +4 probes have been successfully used for in situ hybridization experiments.

B. Low Yield

Limiting nucleotide concentration

For reasons of practicality, the labeled NTP during the transcription step is usually present at a limiting concentration, and is therefore referred to as the "limiting NTP." (Note that the "limiting NTP" can be a mixture of both the labeled and unlabeled form of that NTP.) The greater the concentration of limiting nucleotide, the higher yield (and proportion of full-length transcripts), but if unlabeled nucleotide is used to increase the limiting nucleotide concentration, it will lower the specific activity of the transcript.

In general, probes should be synthesized at the lowest specific activity which will give sufficient sensitivity to detect a particular target. When making probe to detect an unknown amount of target sequence, start

with a maximum specific activity transcription reaction containing no unlabeled limiting nucleotide. If a strong hybridization signal is seen using this probe, the specific activity can be reduced in subsequent experiments by adding unlabeled limiting nucleotide to about 5–10 µM. Reducing probe specific-activity will reduce sensitivity, but these probes will have a longer shelf life (due to less radiolytic decay), and they may also exhibit less nonspecific hybridization.

Modified nucleotides

Labeled nucleotides

Researchers using the *mir*Vana miRNA Probe Construction Kit to incorporate modified nucleotides for nonisotopic detection are usually interested in obtaining the highest possible yield from their reactions. The nucleotide concentration is therefore kept at 0.5 mM (i.e. 1 µL of the 10 mM solutions supplied with the kit in a 20 µL reaction). Modified nucleotides are used at the highest level that does not unduly inhibit either the transcription or subsequent hybridization reactions. Follow the manufacturer's recommendations for amount and proportion of modified nucleotides to be used in transcription reactions. Ambion's Technical Bulletin #173 lists some suppliers of modified nucleotides; it also describes a pilot experiment to empirically determine the amount of a given modified nucleotide that can be used in transcription and nucleic acid hybridization. Technical Bulletin #173 is available on our web site at the following address or by request from our Technical Services Department.

www.ambion.com/techlib/tb/tb_173.html

Other modified nucleotides

Other modified nucleotides may also be used with the *mir*Vana miRNA Probe Construction Kit to prepare RNA with customized nuclease stability, chemical lability, or hybridization efficiency. Using the *mir*Vana miRNA Probe Construction Kit, RNA yield will generally be highest with unmodified nucleotides, and equal or slightly lower using nucleotides with base modifications. RNA yield will be decreased when 2' modified NTPs are incorporated. Yields of 2' N3 RNA will be lowest followed by 2' F RNA and 2' NH_2 RNA. More information about the characteristics and use of modified nucleotides can be found at the following web address:

www.ambion.com/techlib/resources/structure/

GC rich templates

GC rich templates are sometimes difficult to transcribe. There is a report that adding single-stranded binding (SSB) protein (2.6 µg/µg template DNA) can increase the transcription efficiency of a GC rich template (Aziz and Soreq, 1990). Extending the transcription reaction incubation time will also facilitate yield of full-length RNA probe.

C. Premature Termination of Transcription

Limiting nucleotide

Occasionally problems may be encountered in obtaining good yields of full-length transcripts. In general this problem stems from the limiting concentration of labeled nucleotides. Because of the high cost of these nucleotides, transcription reactions are generally run at concentrations of labeled nucleotide well below the K_m of the phage polymerases. Thus, for synthesis of full-length high specific activity probes there is a trade-off between using labeled nucleotide at a low concentration to obtain a high specific activity and adding sufficient amount of the limiting nucleotide to achieve synthesis of reasonable amounts of full-length transcripts (see section B. *Limiting nucleotide concentration* above).

Template sequence

There is also a certain amount of template-specific variation in nucleotide requirements. Some templates may include RNA polymerase pause sites, which can sometimes be overcome by increasing the concentration of the limiting nucleotide to 10–15 µM or by switching to another nucleotide for labeling. Note however that ATP is not incorporated as efficiently as the other three nucleotides and GTP is subject to a higher rate of decomposition during storage.

It has been reported that the presence of the limiting nucleotide in the sequence within 12 bases of the promoter results in increased premature termination (Ling et al. 1989). The 8 nt sequence complementary to the T7 Promoter Primer provided with the kit was designed without U residues in the first 10 nt synthesized. If you use a nucleotide other than UTP for labeling, lowering the temperature of the transcription reaction to 15°C may help to minimize disassembly of the transcription complex and thus maximize synthesis of longer transcripts (Krieg 1990). Incubation time should be increased to 2 hr when reactions are carried out at 15°C.

DNA oligonucleotide quality

Another possible cause of premature termination of transcription is poor quality DNA oligonucleotide used for the procedure. The *mir*Vana miRNA Probe Construction Kit procedure was designed to accommodate short, inexpensive, desalted DNA oligonucleotides to quickly prepare transcription templates. Occasionally DNA oligonucleotide preps may not contain enough full-length primer due to poor coupling efficiency during DNA synthesis or subsequent degradation by nuclease contamination. The quality of the DNA synthesis can be easily checked by denaturing PAGE analysis (see section IV.G on page 24). If required, DNA oligonucleotides can be gel purified and recovered by ethanol precipitation (see procedures in sections IV.C).

D. Antisense RNA Probe Does Not Hybridize as Expected

1. Check your DNA oligonucleotide design

To prepare an antisense RNA probe complementary to a target RNA, the DNA oligonucleotide template ***must*** have the same sequence as the sense target RNA except that U residues are replaced with T's. The eight nucleotide sequence 5'-CCTGTCTC-3', complementary to the T7 Promoter Primer provided with the kit, must also be added to the 3' ends of the DNA oligonucleotide sequence. See section II.A.5 on page 7 for an example of what oligonucleotide should be synthesized for an example target sequence.

2. Analyze the transcription reaction products

Low yield of full-length probe and poor incorporation of radiolabeled nucleotide may result in poor sensitivity or aberrant results during subsequent target RNA detection experiments. Procedures to troubleshoot and analyze yield and quality of radiolabeled, unlabeled, or nonisotopically labeled transcripts can be found in sections B and C above, and in section IV starting on page 15.

3. Perform the positive control reaction

The mir-16 +4 probe has been successfully used to detect *mir-16* expression in various human and mouse tissues and cell lines. Thus it can be used both as a positive control to confirm that the kit is working properly, and as a positive control for experimental samples (see section III.A).

E. Degradation of RNA During Heating

Heating RNA to temperatures above ~65°C in the presence of divalent cations (especially magnesium, which is a component of the Transcription Buffer) can result in nonenzymatic degradation of the RNA. If the RNA will be heated, it is important to chelate free divalent cations by adding EDTA prior to heating. We recommend adding 1 µL of 0.5 M EDTA per 20 µL transcription reaction.


NOTE

The Gel Loading Buffer II provided with the kit contains EDTA to prevent RNA degradation during the heating step prior to loading the gel.

IV. Additional Procedures

A. TCA Precipitation to Determine Radiolabel Incorporation

1. Dispense 150 µL of carrier DNA or RNA (1 mg/mL) into a nuclease-free 1.5 mL microfuge tube. (Ambion Sheared Salmon Sperm DNA P/N AM9680 can be used for this.)
2. Add 1 µL of the *mir*Vana miRNA Probe Construction reaction and mix thoroughly.
3. Transfer 50 µL of the diluted transcription reaction to aqueous scintillation cocktail and count in a scintillation counter. This will measure the total amount of radiolabel present in the reaction mixture (unincorporated and incorporated counts).
4. Transfer another 50 µL of the mixture to a 12 x 75 mm glass tube, and add 2 mL of cold 10% TCA (trichloroacetic acid). Mix thoroughly and place on ice for 10 min. This will precipitate nucleic acids, but not free nucleotides.
5. Collect the precipitate via vacuum filtration through a Whatman GF/C glass fiber filter (or its equivalent).
6. Rinse the tube twice with 1 mL of 10% TCA and then rinse once with 3–5 mL of 95% ethanol. Pass each of the rinses through the GF/C filter.
7. Place the filter in a scintillation vial, add aqueous scintillation cocktail, and count in a scintillation counter. The number will reflect radiolabel that was incorporated.
8. Multiply the cpm measured in step 7 by 3 to calculate the cpm/µL of probe.
9. Divide the cpm in step 7 by the cpm in step 3 to determine the fraction of label incorporated (multiply by 100 for percent incorporation).

B. Calculating Yield and Specific Activity of Radiolabeled Transcription Reactions

Specific activity is measured in cpm/µg; it reflects the degree to which a molecule is labeled with radioactive nucleotides. The specific activity of RNA transcripts is determined solely by the ratio of ^{32}P-labeled NTP to unlabeled NTP present in the reaction and is, therefore, independent of the mass yield of RNA. High specific activity probes are generally more sensitive than lower specific activity probes.

Specific activity can be calculated in either one of two ways. Table 1 on page 17 was created by calculating specific activity based on the amounts of labeled and unlabeled nucleotides in the transcription reaction, and the specific activity and concentration of the radiolabeled

nucleotide used. Since no data from the actual transcription reaction is needed to do this calculation, it is referred to as "theoretical specific activity." The other way to calculate specific activity also uses the specific activity and concentration of the radiolabeled nucleotide, but instead of *calculating* the amount of radiolabel in the reaction, it is measured directly by scintillation counting. This is referred to as "experimental specific activity" and it is explained in the form of an example in the next section. The results from the two calculations are nearly identical; the slight difference that may be seen between the two results is usually due to pipetting error.

1. Calculating theoretical yield and specific activity

For approximate calculations of theoretical specific activity see web calculator at:

www.ambion.com/techlib/tips/specific_activity_calculator.html

Table 1 on page 17 shows the specific activity of transcription products that would result from using the listed amounts of some of the commonly available radiolabeled nucleotides. In preparing this chart it was assumed that each of the four nucleotides were incorporated in equimolar amounts so that the final fraction of the limiting nucleotide (e.g. U) in the transcript is 0.25. If it is known that the nucleotide composition of your RNA product differs significantly from a 1 to 4 ratio, a correction factor should be applied to reflect the actual proportion of labeled nucleotide. This chart was prepared using a similar calculation to that shown in the next section, and we provide it so that specific activity and mass yield can be determined without going through the whole calculation.

Mass yield

Actual mass yield can be calculated using Table 1 by multiplying the theoretical maximum yield by the percent radiolabel incorporated. Alternatively, mass yield can be determined by dividing the theoretical specific activity shown in the chart by the empirically determined total cpm of the purified product.

Table 1. Theoretical Maximum Yield and Specific Activity from Radiolabeled Transcription Reactions

Radiolabeled Nucleotide (e.g. [α-^{32}P]UTP)				unlabeled counterpart (e.g. UTP) concentration	theoretical maximum RNA yield	theoretical specific activity of the reaction products (cpm/µg)
specific activity (Ci/mmol)	concentration (mCi/mL)	volume used	final concentration (in a 20 µL rxn)			
800	10	5 µL	3.125 µM	0 µM	80.1 ng	1.4×10^9
800	10	5 µL	3.125 µM	5 µM	208.3 ng	5.3×10^8
800	10	5 µL	3.125 µM	10 µM	336.5 ng	3.3×10^8
800	10	5 µL	3.125 µM	100 µM	2.6 µg	4.2×10^7
800	10	1 µL	625 nM	500 µM	12.8 µg	1.7×10^6*
800	20	5 µL	6.25 µM	0 µM	160.3 ng	1.4×10^9
800	20	5 µL	6.25 µM	5 µM	288.5 ng	7.6×10^8
800	20	5 µL	6.25 µM	10 µM	416.7 ng	5.3×10^8
800	20	5 µL	6.25 µM	100 µM	2.7 µg	8.1×10^7
800	20	1 µL	1.25 µM	500 µM	12.9 µg	3.4×10^6*
3000	10	5 µL	825 nM	0 µM	21.2 ng	5.2×10^9
3000	10	5 µL	825 nM	3 µM	98.1 ng	1.1×10^9
3000	10	1 µL	165 nM	0 µM	4.2 ng	5.2×10^9
3000	10	1 µL	165 nM	3 µM	81.2 ng	2.7×10^8
6000	40	5 µL	1.68 µM	0 µM	43 ng	1.0×10^{10}
6000	40	5 µL	1.68 µM	3 µM	119.9 ng	3.7×10^9
6000	40	1 µL	335 nM	0 µM	8.6 ng	1.0×10^{10}
6000	40	1 µL	335 nM	3 µM	85.5 ng	1.0×10^9

* trace labeled transcription reaction

2. Calculating experimental yield and specific activity

The actual yield of RNA must be determined experimentally by establishing how much radiolabel (e.g. [α-^{32}P]UTP) was incorporated into transcript product — the ratio of labeled to unlabeled nucleotide (e.g. [α-^{32}P]UTP to UTP) incorporated is assumed to reflect the ratio of labeled to unlabeled nucleotide available in the reaction. The amount of radiolabel incorporated into RNA can be determined by TCA (trichloroacetic acid) precipitation and scintillation counting. Alternatively, an aliquot of product that has been separated from unincorporated nucleotides can be counted directly. Separation of the probe transcript from unincorporated nucleotides can be accomplished with a spin column, precipitation with ammonium acetate and ethanol, or gel purification. (Note that yield based on product that has been gel purified only takes into account full-length probe whereas the other methods will include any prematurely terminated transcripts.) In the following example, TCA precipitation is used to determine the amount of radiolabel incorporated.

Example calculation

Assumptions

A 32 nt probe is synthesized in a 20 µL in vitro transcription reaction. For this example we assume the following:

- The probe sequence is:
  3'-AUCGUCGUGCAUUUAUAACCGCGGACAGAGGG-5'
- Molecular weight of probe is 10303.4 g/mol
- 5 µL of [α–^{32}P]UTP (800 Ci/mmol, 10 mCi/mL) was used in the transcription reaction (3.125 µM final concentration)
- 2 µL of 50 µM unlabeled ("cold") UTP was used in the transcription reaction (5 µM final concentration)

Procedure and data

After DNase treatment and the addition of Gel Loading Buffer, the final volume of the reaction is 42 µL.

A 2 µL aliquot of RNA transcript is diluted into 150 µL of TE containing carrier RNA:

- 50 µL of this dilution is counted directly in a scintillation counter and found to contain 2.6 x 10^6 cpm. This number represents the total counts.
- Another 50 µL of the diluted reaction is TCA precipitated and the precipitate is captured on a filter. The filter is counted and found to have 1.3 x 10^6 cpm. This number represents the TCA precipitable counts.
- The counting efficiency of the ^{32}P isotope in liquid scintillation cocktail is assumed to be 100%. The specific activity of the RNA probe is calculated as follows:

a. What proportion of the UTP was incorporated into RNA (i.e. TCA precipitable material)?

$$\frac{1.3 \times 10^6 \text{ cpm TCA}}{2.6 \times 10^6 \text{ cpm total}} = 50\%$$

(the proportion of [α-^{32}P]UTP incorporated is assumed to reflect the proportion of total [labeled and unlabeled] UTP incorporated.)

b. How many moles of [α-^{32}P]UTP were in the reaction?

This is calculated by converting the volume of [α-^{32}P]UTP added (5 µL) to the number of mCi of ^{32}P added, and then converting the amount of [α-^{32}P]UTP in mCi to a molar amount using the known specific activity and concentration of the [α-^{32}P]UTP (800 Ci/mmol, 10 mCi/mL).

$$\#\ \text{mCi}\ [\alpha\text{-}^{32}\text{P}]\text{UTP in reaction} = \frac{0.005\ \text{mL} \times 10\ \text{mCi}}{\text{mL}} = 0.05\ \text{mCi}$$

$$\#\ \text{mmol}\ [\alpha\text{-}^{32}\text{P}]\text{UTP} = \frac{0.05\ \text{mCi} \times 1\ \text{mmol}}{800\ \text{Ci}} \times \frac{1\ \text{Ci}}{1000\ \text{mCi}}$$

$$= 6.25 \times 10^{-8}\ \text{mmol} = 62.5\ \text{pmol in rxn}$$

c. How many moles of unlabeled UTP were in the reaction?

$$2\ \mu\text{L} \times \frac{50\ \mu\text{mol}}{1000\ \text{mL}} \times \frac{1\ \text{mL}}{1000\ \mu\text{L}} = 1 \times 10^{-4}\ \mu\text{mol} = 100\ \text{pmol}$$

d. How much total UTP was in the reaction?

62.5 pmol [a-^{32}P]UTP + 100 pmol unlabeled UTP

= 162.5 pmol total UTP

$$\frac{162.5\ \text{pmol}}{20\ \mu\text{L}} = 8.13\ \mu\text{M}$$

e. How much total UTP was incorporated into RNA?

162.5 pmol in reaction × 50% incorp'n = 81.3 pmol incorporated

f. What mass amount of RNA was synthesized?

Precise mass amount of RNA synthesized: To precisely determine the mass of RNA, the molecular weight of the RNA product must be known. There is a convenient web based molecular weight calculator at the following address:

www.ambion.com/techlib/misc/RNA_calculator.html

The mass amount of RNA is determined by calculating the molar amount synthesized, then multiplying the moles by the molecular weight of the transcript. The number of moles of product synthesized is calculated by dividing the moles of UTP incorporated by the number of UTP residues in the RNA sequence (7 in this example).

$$\frac{81.3\ \text{pmol}}{7\ \text{nt}} \times 10303.4\ \text{pg/pmol (MW)} \times \frac{1\ \text{ng}}{1000\ \text{pg}} = 119\ \text{ng}$$

Approximate mass amount of RNA synthesized: If the molecular weight is not known, it can be estimated by multiplying the number of residues in the RNA transcript (32 in this example) by the average molecular weight of a nucleotide (320.5 g/mol). This approximation can then be substituted for MW in the equation above.

32 nt (length of RNA transcript) × 320.5 g/mol = 10256 g/mol

g. How many cpm were incorporated into the RNA product?

The final reaction volume from which the 2 µL sample was removed to determine label incorporation was 42 µL.

One microliter of the sample was TCA precipitated and found to contain 1.3×10^6 cpm.

So the whole reaction contained the following amount of TCA precipitable material or RNA:

$$42\ \mu L \times (1.3 \times 10^6\ cpm)/\mu L = 55 \times 10^6\ cpm$$

h. Specific activity of the product

The specific activity of the transcript will be the product of the total counts incorporated (g) divided by the mass amount of RNA produced (f).

$$\frac{55 \times 10^6\ cpm}{119\ ng} = 5.29 \times 10^5\ cpm/ng = 5.29 \times 10^8\ cpm/\mu g$$

C. Gel Purification of Probe

For applications where isolation of full-length transcript is important (e.g. ribonuclease protection assays), we recommend gel purifying the probe to separate full-length transcripts from prematurely terminated transcription products as well as from unincorporated nucleotides. Gel purification is not essential if the RNA will be used as a probe for hybridization to target sequences bound to a solid support (e.g. membrane, filter, slides).

1. Separate the transcription products on gel

NOTE

If possible, use a "preparative scale" comb that will form large capacity wells. Alternatively load the material into several smaller wells, e.g. an entire DNase I treated transcription reaction (21 µL plus 21 µL Gel Loading Buffer II) can be loaded in 2 standard 10 X 5 X 0.75 mm wells.

Add an equal volume of Gel Loading Buffer II to the DNase-treated transcription reaction immediately after step II.C.5 or after removal of the free ribonucleotides (see section IV.D).

Heat for 3 min at 95–100°C and load all or part of the transcription reaction into the freshly-rinsed wells of a 0.75 mm thick denaturing polyacrylamide gel (see recipe in section IV.H.2 on page 25). A 12% polyacrylamide gel is typically used for probes 29–45 nt long (the position of bromophenol blue and xylene cyanol in 12% gels corresponds approximately to 15 and 40 nt, respectively). Run the gel at 10–25 mAmp until the bromophenol blue reaches the bottom of the gel.

2. Excise the gel fragment containing the full-length transcript

When the full-length transcript is visualized by autoradiography:

After electrophoresis remove one glass plate from the gel, cover the gel with plastic wrap, and expose it to x-ray film for 30 sec to several minutes; the exposure time will depend on the specific activity of the probe.

The goal is to get an autoradiograph with a faint or "grey" signal so that a small discrete gel fragment can be excised. Glow-in-the-dark stickers are the easiest way to orient the film with the gel to cut out the band. Once exposed, develop the film and identify the full-length labeled transcript; it is usually the most slowly migrating, most intense band on the autoradiograph. Now, align the exposed x-ray film with the gel, and cut out the area of the gel that contains the full-length labeled probe with a razor blade or scalpel, and transfer it to an RNase-free microfuge tube. Remove the smallest possible fragment of gel that contains the full-length probe.

IMPORTANT

If the transcription reaction was not treated with DNase I the most slowly migrating band will correspond to the undigested DNA template. This band is ~20 nt longer than the expected RNA probe and is not radioactive.

When the full-length transcript is visualized by ethidium bromide staining:

After electrophoresis stain the gel for 5–10 min in a 0.5–1 µg/mL solution of ethidium bromide in 1X TBE. Wash the gel 2 times for 2 min in 1X TBE and visualize RNA using a UV transilluminator protected with a plastic wrap. The full-length transcript is usually the most slowly migrating, most intense band on the gel. Excise the smallest gel slice possible containing the full-length transcript with a clean scalpel or razor blade and transfer it to an RNase-free microfuge tube.

3. Elute the RNA from acrylamide gel slice

To elute the full-length transcript, add 100–150 µL Probe Elution Buffer to the gel slice and incubate at 37°C for 30 min. Transfer the Probe Elution Buffer, which contains the eluted RNA, to a clean microfuge tube, and repeat with 50–100 µL of Probe Elution Buffer. Pool the two elution fractions and determine the cpm/µL of the recovered RNA by scintillation counting using 1–2 µL of the eluted material.

With this elution procedure about 50% of the RNA will usually be recovered—enough to set up many hybridization reactions. Since longer RNA molecules elute more slowly from the gel we recommend increasing the elution time for transcripts longer than 40 nt to at least 1 hr. For convenience, or to maximize recovery of RNA from the gel, incubation can be carried out overnight with ~200 µL or more Probe Elution Buffer. The Probe Elution Buffer contains EDTA and SDS which will inactivate low levels of nuclease contamination.

4. Storage of RNA transcript

Eluted RNA can be stored at –20°C in the Probe Elution Buffer up to 10 days. Alternatively probes can be precipitated and resuspended in nuclease-free water or TE. As the Probe Elution Buffer already contains 0.5 M ammonium acetate simply add a carrier, such as yeast RNA or glycogen, and 3 volumes of 100% ethanol and follow steps 4–8 of the procedure described in section IV.D below.

D. Removal of Unincorporated Nucleotides

There are a number of ways to remove free nucleotides from the transcription reaction, including spin columns (such as with the *mir*Vana Probe & Marker Kit), gel purification on a denaturing polyacrylamide gel or two successive ammonium acetate/ethanol precipitations. Here we provide a protocol for ammonium acetate/ethanol precipitation.

IMPORTANT

Always add 5 µg of yeast RNA or other carrier (such as Ambion GlycoBlue™ Coprecipitant) prior to precipitating RNA. Due to the low mass and small size of the transcription products, adding a carrier will greatly improve recovery.

1. Add water to the DNase I-treated transcription reaction to bring the volume to 50 µL.
2. Add 5 µL 5 M ammonium acetate and a carrier reagent (e.g. yeast RNA).
3. Add 3 volumes of 100% ethanol and vortex to mix.
4. Chill the solution at –20°C for 30 min or longer.
5. Spin for >15 min at maximum speed in a 4°C microcentrifuge.
6. Carefully remove the supernatant and discard (dispose of radioactive supernatants appropriately).
7. Wash the pellet by adding 200 µL cold 75% ethanol and repeating steps 5 and 6. Allow to air dry for 5 min.
8. Resuspend the pellet in 20–50 µL of nuclease-free water or TE .
9. For complete removal of unincorporated ribonucleotides repeat steps 1–7.

Storage

Unlabeled RNA should be stored in aliquots at –70°C. The currently used aliquot can be stored at –20°C. The RNA should be stable for at least 6 months at –70°C in the absence of RNase contamination.

Isotopically labeled RNA should be stored at –20°C until use. Due to high specific activity of the probe, radiolytic degradation will occur. It is therefore advisable to use the probe within 1 week after synthesis, or to gel purify the remaining full-length probe a second time just before use.

E. Calculating Yield of Nonradiolabeled Transcription Reactions

Specific activity

There is no reasonably easy way to directly measure the specific activity of transcripts labeled with modified nucleotides without a standard. The rate of incorporation of a given modified nucleotide between experiments can be indirectly monitored by doing spot detection assays of probes prepared at different times.

Measuring yield by absorbance at 260 nm

After removing unincorporated nucleotides from the reaction, the RNA transcript can be quantitated by A_{260} spectrophotometry. Below are two methods to calculate concentration by spectrophotometry, an approximate method and a more precise method.

Dilute the sample 1:50 to 1:500 in water to bring the concentration into the linear range of the spectrophotometer. Quantitate by measuring A_{260} units. Multiply the reading by the dilution factor to give A_{260}.

The RNA concentration can be calculated with either of the following equations:

$$C\ (\text{mol/L}) = A_{260} / (\varepsilon \times l)$$

$$C\ (\mu\text{g/mL}) = \frac{A_{260}}{\varepsilon \times l} \times M \times 1000 = \frac{1000 \times M}{\varepsilon \times l} \times A_{260}$$

Where:

- ε = extinction coefficient ($L \times mol^{-1} \times cm^{-1}$)
- l = path length (cm): All modern spectrophotometers have a path length of 1 cm.
- M = molecular weight (g/mol)
- C = concentration

Precise quantitation

For accurate quantitation of small RNA transcript, we recommend using the exact molecular weight and extinction coefficient for each RNA sequence. A web-based calculator for calculating molecular weights and extinction coefficients is available at the following address:

www.ambion.com/techlib/misc/RNA_calculator.html

Approximate quantitation

Because for short oligonucleotides (<80 nt), $1000 \times M/\varepsilon \times l \approx 33$, the approximate concentration can be determined with the following formula:

$C\ (\mu g/mL) \approx 33 \times A_{260}$

[Use $C\ (\mu g/mL) \approx 40 \times A_{260}$ for long ssRNA, and
$C\ (\mu g/mL) \approx 50 \times A_{260}$ for long dsDNA.]

Denaturing gel analysis

The yield of a transcription reaction can be estimated by running ~10% of the reaction on a denaturing polyacrylamide gel (see section IV.F on page 24) and comparing the ethidium bromide staining of the transcript with various dilutions of an RNA oligonucleotide of known concentration.

F. Gel Analysis of Transcription Products

The best way to analyze the outcome of a transcription reaction is to run an aliquot of the reaction on a denaturing 12% polyacrylamide gel (see H.2 on page 25 for recipe).

1. Mix an aliquot of the transcription reaction (1–5% for radiolabeled transcripts, at least 10% for unlabeled or nonisotopically labeled transcripts) with 5 µL of Gel Loading Buffer II.
2. Heat sample for 3 min at 95–100°C.
3. Load the sample on a denaturing 12% polyacrylamide gel and electrophorese at 20–25 mAmp.
4. Stop electrophoresis when the bromophenol blue dye front has migrated to the bottom of the gel (the position of bromophenol blue and xylene cyanol in 12% gels corresponds approximately to 15 and 40 nt, respectively).
5. Expose to x-ray film for one to several min for radiolabeled transcripts or stain the gel as described in section IV.G, steps 5–7, for nonradioactive transcription reactions.

G. Gel Analysis of DNA Oligonucleotides

The quality of DNA oligonucleotide synthesis can be assessed by gel electrophoresis using a denaturing 12% polyacrylamide gel (see section IV.H.2 on page for recipe).

1. Mix 1–2 µL of a 100 µM oligonucleotide solution with 5 µL Gel Loading Buffer II.
2. Heat for 3 min at 95–100°C.
3. Load the sample on a denaturing 12% polyacrylamide gel and electrophorese at 20–25 mAmp.
4. Stop electrophoresis when the bromophenol blue dye front has migrated to the bottom of the gel (the position of bromophenol blue and xylene cyanol in 12% gels corresponds approximately to 15 and 40 nt, respectively).
5. Stain the gel for 5 min in a 0.5–1 µg/mL solution of ethidium bromide in 1X TBE.
6. Wash the gel for 2–5 min in 1X TBE.
7. Visualize the DNA oligonucleotide using a UV transilluminator.

H. Additional Recipes

1. 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, Ambion offers nuclease-free solutions of 10X TBE (P/N AM9863, AM9865) and ready-to-resuspend powdered 10X TBE packets (P/N AM9864). Both are made from of ultrapure molecular biology grade reagents.

2. Denaturing acrylamide gel mixes

The following instructions are to prepare 15 mL of gel mix with the indicated percentage of acrylamide and 8 M urea. 15 mL is enough gel solution for one 13 x 15 cm x 0.75 mm gel. Ambion offers reagents for acrylamide gel preparation; see our latest catalog or our website for specific information.

Amount		
12% gel	15% gel	Component
7.2 g	7.2 g	Urea
1.5 mL	1.5 mL	10X TBE
4.5 mL	5.6 mL	40% acrylamide (acrylamide: bis acrylamide = 19:1)
to 15 mL	to 15 mL	Nuclease-free water
Stir to mix, then add:		
75 µL	75 µL	10% ammonium persulfate
15 µL	15 µL	TEMED
Mix briefly after adding the last 2 ingredients, and pour gel immediately.		

V. Appendix

A. References

Aziz RB and Soreq H (1990) Improving poor in vitro transcription from G, C-rich genes. *Nucl. Acids Res*. **18**: 3418.

Krieg P (1990) Improved synthesis of full-length RNA probe at reduced incubation temperatures. *Nucl. Acids Res*. **18**: 6463.

Lagos-Quintana M, Rauhut R, Lendeckel W, and Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. *Science* **294(5543)**:853-858.

Ling M, Risman SS, Klement JF, McGraw N, and McAllister WT (1989) Abortive Initiation by Bacteriophage T3 and T7 RNA Polymerases under Conditions of Limiting Substrate. *Nucl. Acids Res.* **17**: 1605–1618.

B. Quality Control

Functional Testing

The Control DNA Primer is used in an [α-^{32}P] labeled *mir*Vana miRNA Probe Construction Kit reaction following the instructions in section II starting on page 6.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with super-coiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

C. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.