MAXIscript® Kit

(Part Number AM1308–AM1326)

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Literature Citation: When describing a procedure for publication using this product, please refer to it as the MAXIscript® Kit.

If a paper that cites one of Ambion's products is published in a research journal, the author(s) may receive a free Ambion T-shirt by sending in the completed form at the back of this protocol, along with a copy of the paper.

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I. Introduction

A. Background

In vitro transcription

SP6, T3 and T7 phage RNA polymerases are 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. The template is then mixed with the corresponding RNA polymerase, rNTPs, and transcription buffer, and the reaction mixture is incubated for 10 min to 1 hr at 37°C. RNA polymerase first binds to its double-stranded DNA promoter, then it separates the two DNA strands, and uses the 3' to 5' strand as a template to synthesize a complementary 5' to 3' at the end of the DNA template (run-off transcription). The initiation of transcription is the rate-limiting step in in vitro transcript reactions; elongation of the transcript is extremely rapid.

The phage RNA polymerases have a high specificity for their respective promoters. Many multi-purpose cloning vectors contain two or more separate phage promoters flanking a multiple cloning site. Because of the high promoter specificity of the RNA polymerases, either strand of the template can be transcribed with virtually no "cross-talk" from the promoter on the opposite strand. The MAXIscript® Kit can also be used to transcribe from DNA templates produced via PCR. In fact, DNA from PCR can be used directly in the MAXIscript Kit without any pretreatment or purification.

RNA probes

RNA transcripts are useful as probes in hybridization reactions because RNA-RNA and RNA-DNA hybrids are more stable than DNA-DNA duplexes in solid support (membrane) hybridizations. Furthermore, single-stranded RNA probes are not depleted by rehybridization to a complementary probe strand. Using the MAXIscript Kit, radiolabeled RNA probes can be synthesized in a 10 min reaction (see Figure 1). RNA probes can be used in Northern and Southern blotting, slot or dot blotting, in situ hybridizations, and nuclease protection assays. The MAXIscript Kit can be used to incorporate virtually any labeled nucleotide into RNA. Traditionally ^{32}P labeled UTP or CTP has been used in the MAXIscript Kit, but other isotopically labeled nucleotides (^{33}P, ^{35}S, ^{3}H) can be used with this kit as well as nonisotopically modified nucleotides. Technical Bulletin #173 contains detailed information about how to use several differently modified nucleotides in the MAXIscript Kit; general information is included in this protocol.

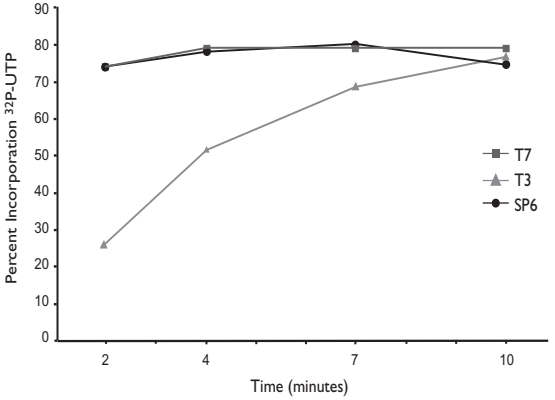


Figure 1. Time Course of MAXIscript® Transcription Reaction Using the 300 bp pTRI-actin Template

Transcription reactions were done under standard MAXIscript® Kit conditions using the indicated RNA polymerases. The total limiting nucleotide concentration (UTP) was 6 µM. At the indicated times, aliquots were TCA precipitated to assess the fraction of [α-^{32}P]UTP incorporated into RNA.

Unlabeled RNA

The MAXIscript Kit can also be used to transcribe unlabeled RNA; on the order of 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 (80–120 µg RNA per 20 µL reaction), we recommend Ambion MEGAscript® Kits.

Capped, fully functional mRNAs can be made by adding cap analog (not supplied with the kit) to MAXIscript reactions. For large scale synthesis of capped RNA, however, we recommend Ambion mMESSAGE mMACHINE® Kits.

B. Reagents Provided with the Kit

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

30 rxns	100 rxns	Component
2 x 30 µL	2 x 100 µL	Enzyme Mix (T3, T7, or SP6)
250 µL	250 µL	10X Transcription Buffer (with DTT)
30 µL	100 µL	ATP Solution (10 mM)
30 µL	100 µL	CTP Solution (10 mM)
30 µL	100 µL	GTP Solution (10 mM)
30 µL	100 µL	UTP Solution (10 mM)
100 µL	100 µL	TURBO DNase (2 U/µL)
1.4 mL	1.4 mL	Gel Loading Buffer II*
1.75 mL	1.75 mL	Nuclease-free Water†
10 µL	10 µL	pTRI-β-actin-Mouse (0.5 mg/mL)

* a 1–2**X** gel loading solution for TBE polyacrylamide and agarose gels
95% Formamide
0.025% xylene cyanol
0.025% bromophenol blue
18 mM EDTA, 0.025% SDS

† Nuclease-free Water can be stored at room temperature, 4°C, or –20°C.

The RNA Polymerase Enzyme mix includes Ribonuclease Inhibitor Protein (RIP) to protect newly transcribed RNA from degradation by RNases that could be inadvertently introduced from sources such as plasmid templates, ungloved fingers, contaminated microcentrifuge rotors, and even airborne dust.

To provide maximum transcription activity, the RNA Polymerase concentration in Ambion MAXIscript kits is optimized empirically as each lot is manufactured. The RNA polymerase concentration will be approximately what is listed in the table below; for actual concentrations, please see the RNA Polymerase tube label.

RNA Polymerase	Concentration
T7	15 U/µL
SP6	20 U/µL
T3	30 U/µL

C. Materials Not Provided with the Kit

- Labeled nucleotide
  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. α^{33}P, α^{35}S, ^{3}H, fluoresceinated, biotinylated, and digoxigenin nucleotides can also be used.
- Trichloroacetic acid: molecular biology grade
- Ethanol: ACS reagent grade
- (optional) 0.5 M EDTA
- (optional) Buffer-saturated phenol:chloroform:IAA
- (optional) Proteinase K (20 mg/mL)

D. Related Products Available from Applied Biosystems

MEGAscript® Kits P/N AM1330–AM1338	High yield transcription kits for production of large amounts of RNA. By employing Ambion's novel, patented MEGAscript® technology, these kits use concentrations of nucleotides that would normally inhibit the RNA polymerases, resulting in ultra high-yield transcription reactions. Kits are available with T7, SP6, and/or T3 RNA polymerase.
mMESSAGE mMACHINE® P/N AM1340, AM1344, AM1348	High yield transcription kits for production of large amounts of capped RNA. These kits employ Ambion's novel, patented MEGAscript® technology, and include cap analog. Kits are available with T7, SP6, and/or T3 RNA polymerase.
mMESSAGE mMACHINE® T7 Ultra Kit P/N AM1345	The mMESSAGE mMACHINE T7 Ultra Kit incorporates Anti-Reverse Cap Analog (ARCA) into Ambion's patented high yield transcription technology to generate RNA transcripts that yield much higher amounts of protein when translated in vitro or in vivo, than messages with traditional cap analog. The increased translation efficiency provided by ARCA is further enhanced by the addition of a poly(A) tail to the transcripts. Experiments comparing ARCA and ARCA/poly(A) tailed transcripts to cap analog and cap analog/poly(A) tailed transcripts show significantly higher levels of protein synthesis with ARCA capped RNA.
NucAway™ Spin Columns P/N AM10070	Guaranteed RNase- and DNase-free, Ambion NucAway Spin Columns provide a fast, efficient way to remove unincorporated nucleotides, and to effect buffer exchange after probe synthesis and other reactions.
Cap Analog & Variants See web or print catalog for P/Ns	Cap analog, m^{7}G(5')ppp(5')G, is used for the synthesis of 5' capped RNA by in vitro transcription. Cap analog is also used as a highly specific inhibitor of the initiation step of protein synthesis. Ambion also offers cap analog variants. All of the Cap Analog products are tested in in vitro transcription, and are certified nuclease-free.
RNA Century™ Marker and RNA Century™-Plus Marker Templates P/N AM7780 & AM7782	Templates for the transcription of 100–500 and 100–1000 nt RNA molecular weight markers. Also available as pre-transcribed biotinylated RNAs (P/N AM7175, AM7180)

II. MAXIscript® Kit Procedure

A. Transcription Reaction Setup

1. Thaw the frozen reagents

Place the RNA Polymerase on ice, it is stored in glycerol and will not be frozen at –20°C.

Vortex the 10X Transcription Buffer and ribonucleotide solutions until they are completely in solution. Once they are thawed, store the ribonucleotides on ice, but ***keep the 10X Transcription Buffer at room temp***.

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

2. Assemble transcription reaction at room temperature

The spermidine in the 10X Transcription Buffer can coprecipitate the template DNA if the reaction is assembled on ice.

Add the 10X Transcription Buffer after the water and template DNA are already in the tube.

IMPORTANT

See sections II.C. Preparation of Template DNA on page 8 and II.D. Labeled Nucleotide on page 10 for more information.

Component	Type of label		
	Isotopic e.g. ^{32}P	Unlabeled reaction	Nonisotopic e.g. biotin
Nuclease-free Water	to 20 µL	to 20 µL	to 20 µL
DNA template	1 µg	1 µg	1 µg
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	0.6 µL
Labeled UTP*	5 µL†	--	0.4 µL
(T3, T7, or SP6) Enzyme Mix	2 µL	2 µL	2 µL

* The recipe shown uses labeled UTP, if you are using a different labeled nucleotide, substitute it, and its unlabeled counterpart in the component list.

† 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.

3. Mix thoroughly

Gently flick the tube or pipette the mixture up and down gently, and then microfuge tube briefly to collect the reaction mixture at the bottom of the tube.

It is important not to use a large excess of enzyme, as this can cause transcription from inappropriate promoter sites.

4. Incubate 10 min to 1 hr at 37°C

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

Incubate reactions with >10 µM limiting nucleotide for 1 hr. This includes unlabeled, nonisotopically-labeled, and trace-labeled reactions.

The exact incubation temperature is not critical; from room temperature to 40°C, MAXIscript reactions show nearly equivalent yield.

5. (optional) Add 1 µL TURBO DNase, mix well, and incubate at 37°C for 15 min

(optional) Include this DNase digestion to remove the template DNA if that is important for your application.

a. Add 1 µL TURBO DNase 1, and mix well.

b. Incubate at 37°C for 15 min

6. (optional) Add 1 µL of 0.5 M EDTA

(optional) Add 1 µL of 0.5 M EDTA to stop the reaction.

It is important to add EDTA to reactions that will be heated (to inactivate DNase I, for example). EDTA blocks the heat-induced RNA degradation that can occur in Transcription Buffer.

7. After the transcription reaction

Purify the transcript

For some applications it is necessary to have primarily full length probe (e.g. ribonuclease protection assays, mapping studies), 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 on page 17.

Determine amount of radiolabel incorporated

The efficiency of the transcription reaction can be determined for radiolabeled transcripts by measuring the trichloroacetic acid precipitable counts (See section IV.E on page 20).

Remove unincorporated nucleotides

Nonradiolabeled transcripts can be quantitated only after removal of unincorporated ribonucleotides. This can be done by ethanol precipitation or by column purification (described in sections II.B on page 7, and IV.D on page 19).

B. (optional) Removal of Free Nucleotides

There are a number of ways to remove free nucleotides from the transcription reaction, including gel filtration (such as Ambion NucAway™ spin columns, P/N AM10070), gel purification on a denaturing polyacrylamide gel, 2 successive ammonium acetate/ethanol precipitations, or lithium chloride precipitation. Here we provide a procedure for ammonium acetate/ethanol precipitation. Procedures for gel purification and gel filtration are given in sections IV.C on page 17 and IV.D on page 19 respectively.

NOTE

For applications which require primarily full length probe (ribonuclease protection assays, and mapping studies), we recommend gel purifying the probe (described in section IV.C on page 17). Gel purification also removes unincorporated nucleotides.

We generally recommend adding 5 µg of yeast RNA or other carrier (such as Ambion GlycoBlue™ P/N AM9515, AM9516) prior to precipitation of radiolabeled probes since the mass of transcript synthesized can be quite small, usually on the order of 15–50 ng.

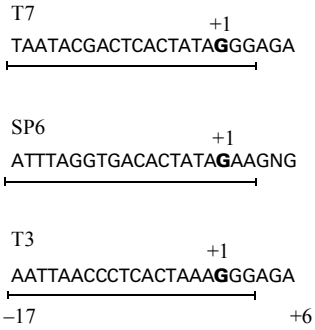
Precipitation with ammonium acetate/ethanol

1. Add 30 µL water to the DNase I-treated transcription reaction to bring the volume to 50 µL.
2. Add 5 µL 5 M Ammonium Acetate and vortex to mix.
3. Add 3 volumes 100% ethanol.
4. Chill the solution at –20°C for 30 minutes or longer.
5. Spin for >15 min at maximum speed in a 4°C microfuge.
6. Carefully discard the supernatant, and wash the pellet once with cold 70% ethanol.
7. For complete removal of unincorporated NTPs, resuspend the pellet in 50 µL water and repeat steps 1–6.
8. Store unlabeled and nonisotopically labeled RNA in 5 µL aliquots at –70°C. The currently used aliquot could be stored at –20°C. The RNA should be stable for at least 6 months at –70°C in the absence of RNase contamination.
9. Isotopically labeled transcripts need not be aliquotted since they are only intact for 2–4 days before radiolysis renders them unusable.

C. Preparation of Template DNA

Linearized plasmid DNA, and PCR products that contain an RNA polymerase promoter site, can be used as templates for in vitro transcription with MAXIscript. In general, any DNA with a promoter site, which is pure enough to be easily digested with restriction enzymes, can be used for in vitro transcription.

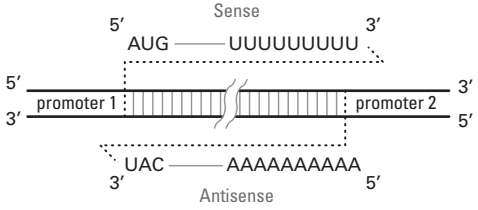
Figure 2. Phage polymerase promoters: minimal sequence requirements



The +1 base (in bold) is the first base incorporated into RNA during transcription. The underline shows the minimum promoter sequence needed for efficient transcription.

Orientation

If the RNA produced will be used as a probe to detect mRNA, it is important that mRNA-complementary (antisense) transcripts are synthesized. For plasmid templates, this means that the vector should be linearized using a restriction site in the polylinker on the amino-terminal (5') side of the coding region of the protein; RNA probes should be transcribed towards that site from the RNA phage promoter at the carboxy-terminal side of the coding region (using promoter 2 in the diagram below). For detection of DNA, either strand may be transcribed.



Transcription using the RNA polymerase corresponding to promoter 1 will make sense RNA (the same sequence as the mRNA). If the RNA polymerase for promoter 2 is used, antisense RNA will be transcribed.

Plasmid Templates

DNA should be relatively free of contaminating proteins and RNA. We observe the greatest yields with very clean template preparations. Most commercially available plasmid preparation systems yield DNA that works well in the MAXIscript Kit. Otherwise, a DNA miniprep procedure that generally yields high quality template is presented in section V.B on page 32.

Linearization

Plasmid DNA must be linearized with a restriction enzyme downstream of the insert to be transcribed. Circular plasmid templates will generate extremely long, heterogeneous RNA transcripts because RNA polymerases are very processive. It is generally worthwhile to examine the linearized template DNA on a gel to confirm that cleavage is complete. Since initiation of transcription is one of the limiting steps of in vitro transcription reactions, even a small amount of circular plasmid in a template prep will generate a large proportion of transcript.

Although we routinely use all types of restriction enzymes, there has been one report of low level transcription from the inappropriate template strand in plasmids cut with restriction enzymes leaving 3' overhanging ends (produced by *Kpn* I, *Pst* I, etc.; Schendorn and Mierindorf, 1985).

After linearization

Terminate the restriction digest by adding the following:

- 1/20th volume 0.5 M EDTA
- 1/10th volume of 3 M Na acetate ***or*** 5 M NH_4 acetate
- 2 volumes of ethanol

Mix well and chill at –20°C for at least 15 min. Then pellet the DNA for 15 min in a microcentrifuge at top speed. Remove the supernatant, re-spin the tube for a few seconds, and remove the residual fluid with a very fine-tipped pipet. Resuspend in dH_2O or TE buffer at a concentration of 0.5–1 µg/µL.

Proteinase K treatment

Note that DNA from some miniprep procedures may be contaminated with residual RNase A. Also, restriction enzymes occasionally introduce RNase or other inhibitors of transcription. When transcription from a template is suboptimal, it is often helpful to treat the template DNA with proteinase K (100–200 µg/mL) and 0.5% SDS for 30 min at 50°C, follow this with phenol/chloroform extraction (using an equal volume) and ethanol precipitation.

PCR templates

DNA generated by PCR can be transcribed directly from the PCR provided it contains an RNA Polymerase promoter upstream of the sequence to be transcribed. PCR products should be examined on an agarose gel before use as a template in MAXIscript to estimate concentration, and to verify that the products are unique and the expected size.

D. Labeled Nucleotide

1. Radiolabeled Nucleotides

Which labeled nucleotide?

Generally [α-^{32}P]UTP or CTP at ~400–800 Ci/mmol and ≥10 mCi/mL is used for the synthesis of radioactive RNA probes. ATP is not incorporated as efficiently as the other three nucleotides and GTP appears to be more subject to decomposition during storage (Melton et al. 1984). [α-^{33}P], [α-^{35}S], and ^{3}H nucleotides can also be used.

Concentration

For practical reasons the labeled NTP is usually present at a limiting concentration, and is therefore referred to as the "limiting nucleotide." (Note that the "limiting NTP" can be a mixture of both the labeled and unlabeled form of that NTP). There is a trade-off between synthesis of high specific activity probe and synthesis of full-length probe. The higher the concentration of limiting nucleotide, the higher the 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. This is illustrated in Figure 3 on page 11.

In general, probes should be synthesized at the lowest specific activity which will give the sensitivity required to detect a particular target. When making probe to detect an unknown amount of target sequence, start with a maximum specific activity transcription reaction that does not contain any 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, because fewer ^{32}P-NTPs will be present in each RNA probe molecule, but the probes will have a longer shelf life (due to less radiolytic decay), and they may also exhibit less nonspecific hybridization.

IMPORTANT

The radiolabeled nucleotide can comprise only 25% or less of the total transcription reaction volume because the radiolabeled nucleotide buffer contains stabilizers that may inhibit RNA polymerase.

If full-length transcripts are required, for example in RNase protection assays, they should be isolated by gel purification to achieve maximum sensitivity (see section IV.C on page 17). For Northerns, dot blots, and in situ hybridizations, it is generally not critical that transcripts are full-length.

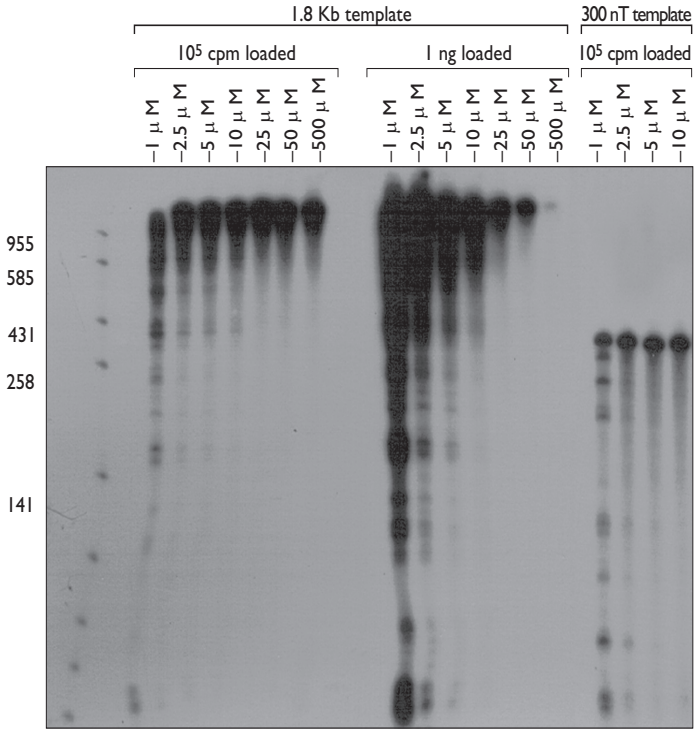


Figure 3. Effect of Limiting Nucleotide Concentration on Transcription

Standard MAXIscript conditions were used to transcribe either the 1.7 kb *Xenopus* elongation factor 1 (Xef1), or the 300 base pTRI-actin control template. All reactions had 1 µM [α-^{32}P]UTP and unlabeled UTP to bring the total UTP concentration to the indicated values. 10^5 cpm of TCA precipitable material, or 1 ng of RNA from each reaction was run on a 4% polyacrylamide/8M urea gel and exposed to X-ray film.

In the left and right panels the concentration of limiting nucleotide is increased from 1 µM to 500 µM. At 1 µM limiting nucleotide, most of the reaction products are prematurely terminated. As the limiting nucleotide concentration increases, the proportion of prematurely terminated transcripts decreases. With the 300 bp template, there was little advantage to adding unlabeled UTP above 2.5 µM. In the middle panel, a constant mass (1 ng) of transcript was loaded in each lane. With 500 µM unlabeled nucleotide, the labeled product is barely visible. These gel lanes clearly illustrate the trade-off between increasing the concentration of limiting nucleotide to obtain full length probe and the consequent decrease in specific activity

To produce transcripts longer than about 400 nt the limiting nucleotide concentration should be raised to 5–25 µM. Of course, supplementing the radiolabeled nucleotide with unlabeled nucleotide decreases the specific activity of the probe, however, the increased sensitivity afforded by longer probes will help to offset the decrease in the specific activity caused by adding unlabeled nucleotide.

Note that there is a certain amount of template-specific variation in limiting nucleotide concentration requirements. Some templates require more limiting nucleotide than others.

2. Modified nucleotides for non-isotopic labeling

Researchers using MAXIscript 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).Nonisotopic nucleotides are used at the highest level that does not significantly inhibit either the transcription or subsequent hybridization reactions. For all of the nonisotopic nucleotides that we have tested, 25–80% modified nucleotide is ideal. Generally, 25–50% modified nucleotide is used; this means that if, for example you want to have 40% biotin-16-UTP in a transcription reaction, 0.5 mM x 40% or 0.2 mM biotin-16-UTP should be added in addition to 0.3 mM unmodified UTP:

$$\frac{\text{0.3 mM x 20 µL transcription reaction}}{\text{10 mM UTP solution supplied with kit}} = 0.6\ \mu\text{L unmodified UTP solution}$$

To enzymatically incorporate nonisotopic nucleotides:

- Follow the manufacturer's recommendations for amount to be used in transcription.
- For biotin-14-CTP (and biotin-16-UTP), we use 40% biotinylated nucleotide and 60% unmodified CTP (or UTP)
- For digoxigenin-II-UTP, we use 33% digoxigenin nucleotide and 67% unmodified UTP (0.67 µL of the 10 mM solution supplied with the kit)
- For fluorescein-12-UTP, we use 50% fluoresceinated nucleotide and 50% unmodified UTP (0.5 µL of the 10 mM solution supplied with the kit)

Technical Bulletin #173 lists some suppliers of modified nucleotides, and describes a pilot experiment to empirically determine the amount of a given modified nucleotide that can be used in transcription and nucleic acid hybridization. Research at Ambion has shown the above recommendations to give high yields of modified transcripts that are very sensitive in hybridization applications.

III. Troubleshooting

A. Use of the Control Template Supplied with the Kit

The positive control that comes with the MAXIscript kit is a DNA template used to make an antisense mouse ß-actin probe, pTRI-Actin-Mouse. The template is a 250 bp *Kpn*I-*Xba*I fragment of the mouse ß-actin gene subcloned from pAL41 (Alonso et al., 1986). To make the positive control transcript, use 1 µL of control DNA template in a standard MAXIscript reaction. Any of the phage polymerases can be used since pTRI-Actin is a linearized pTRIPLEscript plasmid containing the ß-actin gene fragment under the transcriptional control of tandem SP6, T7, and T3 promoters.

The expected sizes of the transcripts are 334 bases, 304 bases, and 276 bases when pTRI-Actin is transcribed with SP6, T7, and T3 polymerases, respectively. These transcripts migrate above the xylene cyanol dye band (the lighter blue, upper dye band) on a 5% polyacrylamide/8 M urea gel.

Approximately 50% of the labeled nucleotide should be incorporated into TCA-precipitable material. [^{32}P] and [^{35}S] nucleotides deteriorate rapidly after synthesis and may accumulate inhibitors of transcription during storage. Try using a fresh batch of labeled nucleotide if the control DNA does not transcribe efficiently.

B. Premature Termination of Transcription

Occasionally problems may be encountered in obtaining good yields of full-length transcripts, especially for longer transcripts (>500 bases). Because of the high cost of labeled nucleotides, transcription reactions are generally run at concentrations of labeled nucleotide well below the Km 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. There is also a certain amount of template-specific variation in nucleotide requirements. Some templates show the presence of strong 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 (UTP or CTP). 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). Lowering the temperature of the transcription reaction to 15°C or even 4°C may help to minimize disassembly of the transcription complex and thus maximize synthesis of longer transcripts (Krieg, P., 1989). Incubation time should

be increased to 2 hr when reactions are carried out at 15°C or 4°C. Occasionally, by chance, cloned inserts will contain a phage polymerase termination signal. This may sometimes be overcome by increasing the limiting nucleotide concentration. However, in some circumstances it may be necessary to move the cloned insert downstream of a different phage promoter.

C. Transcription from the Wrong Strand

Transcription from the wrong strand can cause background problems with procedures such as ribonuclease protection assays. Linearization of template DNA with an enzyme which leaves a 3' overhang may result in low levels of transcription initiated from the 3' overhang (Schenborn and Mierindorf, 1985). If it is necessary to use a restriction enzyme which leaves a 3' overhang, a brief end-filling reaction with Klenow can be used to blunt the overhang.

Despite the high promoter specificity of the phage polymerases, transcription may originate from the "wrong" promoter, for example T7 polymerase may initiate transcription at a low level from a T3 promoter. This "cross-talk" may result in a low level of transcription of the "wrong" strand from templates with opposable polymerase promoters if linearization of the template was incomplete.

D. Contamination of RNA Probes with Vector Sequences

This can occur if the plasmid is not linearized to completion. Small amounts of supercoiled uncut plasmid can result in the synthesis of significant amounts of vector-complementary probe due to the continuous transcription of the template. It may help to use two different restriction enzymes to linearize the plasmid template.

E. Problems with G-C Rich Templates

G-C rich templates are sometimes difficult to transcribe. There is a report that the addition of single-stranded binding (SSB) protein (2.6 µg/µg template DNA) increased the transcription efficiency of a GC rich template (Aziz and Soreq, 1990).

F. 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) may result in non-enzymatic degradation of the RNA. If the RNA will be heated, it is important to chelate free divalent cations by the addition of EDTA prior to heating. We recommend adding 1 µL of 0.5 M EDTA per 20 µL transcription reaction.

IV. Additional Procedures

A. Reducing Specific Activity for Internal Control Probes

When to decrease specific activity

Commonly used internal controls (ß-actin, GAPDH, cyclophilin, 18S & 28S rRNAs) are typically constituitively expressed housekeeping genes. The former three transcripts are considered moderately abundant. Ribosomal RNAs usually comprise as much as 80% of total cellular RNA.

When doing multiprobe assays, using both an internal control probe and a probe for a less abundant message, the internal control probe should be synthesized to a lower specific activity than the probe for the less abundant target. In this way, a single exposure will show signal from both messages in the linear range of the film.

Setting up low specific activity transcription reactions

To make a low specific activity of probe, add both the labeled ***and*** the unlabeled form of the limiting nucleotide to the MAXIscript reaction. The molar ratio of labeled to unlabeled nucleotide will determine the probe's specific activity. For instance, when transcribing probes for messages that differ 200-fold in abundance, unlabeled UTP should be added to the transcription reaction for the more abundant mRNA at a final molar concentration that is 200-fold greater than the labeled UTP. Table 1 provides a starting point for reducing specific activities of probes to common internal controls, further optimization of probe specific activity may be required to achieve ideal results.

Table 1. Lowering Specific Activity of Internal Controls*

Internal Controls	labeled:unlabeled nucleotide	[α-^{32}P]UTP	UTP	specific activity
Ribosomal RNAs (18S and 28S)	1:160,000	0.01 µL	2 µL	2.5 x 10^4 cpm/µg
Moderate Abundance Targets (ß-actin and GAPDH)	1:200	4 µL	1 µL	2 x 10^7 cpm/µg
Low Abundance Targets (cyclophilin)	1:20	4 µL	0.1 µL	2 x 10^8 cpm/µg

* The above examples are for 20 µL transcription reactions where the labeled nucleotide is [a-^{23}P]UTP 800 Ci/mmol, 10 mCi/mL, 12.5 µM, and the stock of unlabeled UTP is 10 mM

Nonisotopically labeled probes

When non-isotopic labeling is used, adjust probe specific activity by titrating non-isotopically labeled with unlabeled probe at ratios of 1:160,000 for ribosomal RNAs, 1:200 for GAPDH and ß-actin, and 1:20 for cyclophilin.

How much low specific activity probe to use

Add the same number of cpm of each probe to hybridization reactions so that all of the full-length probes will be visible in the no target/no RNase control lane.

B. Gel Analysis of Transcription

The best way to analyze the outcome of a transcription reaction is to run of an aliquot of the transcription reaction on a denaturing polyacrylamide gel. Denaturing gels are comprised of 1X TBE, 8 M urea, and polyacrylamide at the percentage appropriate for the size of the RNA.

We suggest the following guidelines for choosing an appropriate acrylamide percentage:

Transcript size	% acrylamide
<30 bases	20%
30–60 bases	15%
61–150 bases	10%
151–500 bases	5%
>500 bases	4%

Solution compositions for denaturing polyacrylamide gels are provided in section *V.A. Reagent Formulations* on page 31.

1. Radiolabeled transcripts

Isotopically labeled transcripts are visualized by exposing the gel to X-ray film since the very small mass amount of RNA produced will not be visible by ethidium bromide staining.

a. Mix 1 µL of the 20 µL transcription reaction with 3–7 µL Gel Loading Buffer II.

b. Heat samples to 95°C for 2 min.

c. Load directly into the freshly rinsed wells of the gel, or store the samples in ice until the gel is loaded.

d. Electrophorese at ~200 volts until the bromophenol blue (the faster migrating dye) approaches the bottom of the gel.
If the unincorporated nucleotides have not already been removed, they will migrate very slightly ahead of the bromophenol blue dye front.

e. Remove one glass plate from the gel, and cover the gel with plastic wrap. Expose the covered gel to X-ray film for ~15 min.

f. Ideally, the transcript will be a single tight intense band migrating at the desired location in the gel. See section *III.B. Premature Termination of Transcription* on page 13, if this is not the case.

2. Unlabeled and non-isotopically labeled transcripts

Because the total yield of RNA produced in nonlimiting rNTP concentration conditions is high, unlabeled and nonisotopically labeled transcripts can be visualized in gels by UV shadowing.

a. Mix 2–4 µL (at least 10%) of the transcription reaction with 4 µL of Gel Loading Buffer II.

b. Heat samples to 95°C for 2 min.

c. Load directly into the freshly rinsed wells of the gel, or store in ice until samples are loaded.

d. Electrophorese at ~200 volts until the bromophenol blue (the faster migrating dye) approaches the bottom of the gel. If the unincorporated nucleotides have not been removed, they will migrate very slightly ahead of the bromophenol blue dye front.

e. Remove one of the glass plates and cover the gel with plastic wrap. Invert and place the gel, plastic wrap side down, on a flat surface and carefully remove the second glass plate. Do not cover the top surface of the gel with plastic wrap.

f. In a darkened room, place the gel with plastic wrap underneath on top of a fluorescent TLC plate (Ambion P/N AM10110) The TLC plate should be "white side up" against the plastic wrap below the gel, shiny side toward the bench. New intensifying screens can also be used for UV shadowing, but the RNA will not be as easily visible as it is with a TLC plate.

IMPORTANT

The xylene cyanol and bromophenol blue dyes also appear as purple bands, do not mistake them for UV shadowed RNA transcript.

g. Visualize the band by shining a hand-held UV light source set on short wavelength: 254 nm (300 nm will not work) on the surface of the gel (UV shadowing). The RNA will appear as a purple band.
The sensitivity limit of UV shadowing is about 0.4 µg. If there is any question about the UV shadowed band, the gel can be soaked in TBE + EtBr and visualized with UV light.

C. Gel Purification of Probe

For applications where isolation of full-length transcripts is important (e.g. ribonuclease protection and S1 nuclease assays), we recommend gel purification of 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). Even truncated transcription products will hybridize and yield a positive signal in these types of experiments.

1. Separate the transcription products on a gel

Add an equal volume of Gel Loading Buffer II to the DNase-treated transcription reaction, heat 3 min at 95°C.

Load all or part of the transcription reaction into the freshly-rinsed wells of a denaturing polyacrylamide gel and run for about 20 min to 1 hr at 100–300 volts until the bromophenol blue approaches the bottom of the gel. (The gel recipe is found in section V.A on page 31.) For this application, it is useful to have a "preparative scale" comb with wide (about 1 cm) teeth that will form large capacity wells.

2. Isolate the gel fragment containing the RNA

Radiolabeled probes

After electrophoresis the gel is covered with plastic wrap and exposed to X-ray film for 30 seconds 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" probe signal so that a small discrete gel fragment can be localized. Glow-in-the-dark stickers are the easiest way to orient the film with the gel to cut out the band. After exposure, the film is developed and the full-length labeled transcript is identified; it is usually the most slowly migrating, most intense band on the autoradiograph. The film is placed on top of the gel and plastic wrap and aligned with the gel via the stickers, then it is lightly taped in place. Next, the gel and film are inverted so that the position of the probe band on the film can be circled with a felt-tip pen on the back of the glass plate. When the gel is turned back over and the film is removed, the area of the gel indicated by the circled region on the glass plate is excised with a razor blade or scalpel, transferred with clean forceps to an RNase-free microfuge tube, and submerged in about 350 µL of an RNase-free solution in an RNase-free microfuge tube. DEPC treated H_2O can be used for the elution, however, we recommend using 0.5 M ammonium acetate/ 1 mM EDTA/ 0.2% SDS (Probe Elution Buffer that is included in Ambion's family of nuclease protection assay kits). The EDTA and SDS will inactivate low levels of nuclease and the salt will precipitate the RNA when 3 volumes of 100% ethanol is added. It is always a good idea to re-expose the gel after cutting out the probe band to be sure that all of the probe was recovered.

Nonisotopically labeled probes

Nonisotopically labeled probes can be visualized by UV shadowing. UV shadowing works best if the gel is removed from both glass plates and enclosed in thin plastic wrap. The wrapped gel should then be laid on a Fluor-coated TLC plate (Ambion P/N AM10110). It may alternatively be possible to use an intensifying screen for visualizing the band, but it will be less sensitive than a Fluor-coated TLC plate.

To see the RNA, short wave UV light is directed onto the gel surface in the dark. There must be at least ~ 0.4 µg of RNA present in the band to use UV shadowing. The RNA will appear as a dark purple or black band. The xylene cyanol and bromophenol blue bands will also be visible when the gel is illuminated with UV light; if the band of interest

comigrates with one of these bands, it may be difficult to distinguish between RNA and dye (consider running a lane of Loading Buffer alone in order to differentiate the dyes from the band of interest).

The full length transcript is usually the most slowly migrating, most intense band on the gel; it should be excised with a clean scalpel and transferred to about 350 µL of an RNase-free solution in an RNase-free microfuge tube. DEPC-treated H_2O can be used for the elution, however, we recommend using 0.5 M NH_4-acetate/1 mM EDTA/0.2% SDS (Probe Elution Buffer that is included in Ambion's family of nuclease protection assay kits). The EDTA and SDS will inactivate low levels of nuclease and the salt will precipitate the RNA when 3 volumes of 100% EtOH is added.

3. Elution of RNA from acrylamide gel slices

To elute the labeled probe, fragment the gel piece to increase its surface area and incubate the tube at 37°C. After about two hours about 20% of the probe will usually have eluted - enough to set up many hybridization reactions. For the sake of convenience or to maximize recovery of probe from the gel, the incubation can be continued overnight. After overnight incubation, about 95% of the label will typically be eluted. Longer probes elute more slowly. Alternatively, the elution can be done for 1–2 hr at 65°C with intermittent vortexing. This method is not very efficient, but enough probe for several experiments will usually be recovered.

Remove the gel pieces with RNase-free forceps, and determine the amount of radioactive label in a small aliquot of the eluted probe (e.g. 1–2 µL) by scintillation counting. Store the probe at –20°C.

D. Spin Column Preparation and Use

Unincorporated labeled nucleotides can be removed by size exclusion chromatography on RNase-free Sephadex G-25 or G-50 spin columns. The following is a protocol for the preparation and use of spin columns:

1. Resuspend and equilibrate Sephadex G-25 or G-50 with 2 volumes of TE (10 mM Tris-HCL, pH 8.0, 1 mM EDTA), then wash with several volumes of TE.
2. Place the resuspended and washed resin in 1.5 volumes of TE in a glass bottle and autoclave. Store at 4°C until use.
3. Rinse a 1–3 mL spin column thoroughly with distilled water; frits may be pre-installed, or made by plugging the bottom of a 1 mL syringe with a support such as siliconized glass beads.
4. Pipet 1–3 mL of the prepared, well mixed resin into the washed spin column. Place the column in a 15 mL plastic centrifuge tube and spin at 2,000 rpm for 10 minutes in a centrifuge with a swinging-bucket rotor.

5. Place the end of the spin column containing the spun resin into an appropriate microfuge tube (typically, 0.5 mL) and insert the assembly into a new 15 mL centrifuge tube.
6. Load 20–100 µL of the sample onto the center of the resin bed (dilute sample with nuclease-free water or TE Buffer if necessary), and spin at 2,000 rpm for 10 minutes. The eluate collected in the microfuge tube should be approximately the same volume as the sample loaded onto the column, and it will contain about 75% of the nucleic acid applied to the column.

IMPORTANT

It is important that the centrifugation conditions for column packing and sample purification be identical; varying them could lead to either incomplete recovery or dilution of the sample. The spin column can be tested by loading 100 µL of TE onto it and centrifuging: 100 µL of eluate should be recovered. If recovery is much greater or less than 100 µL, the column is not equilibrated and should be tested again.

E. 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's Sheared Salmon Sperm DNA P/N AM9680 can be used for this.)
2. Add 2 µL of the MAXIscript reaction and mix thoroughly.
3. Transfer 50 µL of the diluted MAXIscript 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 1.5 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).

F. 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 more sensitive than lower specific activity probes.

Specific activity can be calculated in either of two ways. The chart shown below 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 results is usually due to pipetting error.

1. Calculating theoretical yield and specific activity

Table 2 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 4 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 composition of the RNA product differs significantly from a 1:4 ratio, a correction factor should be applied to reflect the actual proportion of labeled nucleotide. This chart was prepared using a calculation similar to that shown in the next section, and we have provided 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 2 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 table by the empirically determined total cpm of the purified product.

Table 2. Theoretical Yield and Specific Activity from Radiolabeled Transcription Reactions

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

2. Calculating experimental yield and specific activity

To calculate the actual yield of RNA, first establish 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 trichloroacetic acid (TCA) 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 lithium chloride; 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 will be used to determine the amount of radiolabel incorporated.

Consider the following example:
20 µL in vitro transcription reaction with:

- 5 µL of [α^{32}-P]UTP (800 Ci/mmol, 10 mCi/mL)
- 2 µL of 50 µM unlabeled ("cold") UTP

At the end of the synthesis reaction, 1 µL of DNase I is added; after incubation, 21 µL of gel loading buffer is added; the final volume is now 42 µL.

A 2 µL aliquot of the final reaction is removed and diluted into 198 µL of TE containing 100 µg of carrier RNA;

- 100 µL of this dilution is counted directly in a scintillation counter and found to contain 2.6×10^6 cpm.
- The remaining 100 µL of the diluted reaction is TCA precipitated and the precipitate is captured on a filter. The filter is counted and found to have 1.3×10^6 cpm. This represents the amount of ^{32}P incorporated into RNA
- 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).

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

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

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

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

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

d. How much total UTP was in the reaction?

62.5 pmol [α^{32}-P]UTP + 100 pmol unlabeled UTP = 162.5 pmol total UTP

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

e. How much total UTP was incorporated into RNA?

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

f. What mass amount of RNA was synthesized?

If we assume that the RNA synthesized contained equal molar amounts of all 4 ribonucleotides (ATP, CTP, GTP, and UTP), and it is known that the amount of total UTP in the reaction is limiting, then the amount of the other 3 nucleotides incorporated will equal the amount of UTP incorporated. Therefore, 81.3 pmol of each ribonucleotide was incorporated. The sum of the molecular weights of the 4 ribonucleotides is about 1282 g/mol. (The average molecular weight of a nucleotide in RNA is 320.5 g/mol.)

(If the nucleotide composition of the RNA product is known to contain non-equimolar amounts of all 4 ribonucleotides, a correction factor can be applied to more accurately reflect the amount of product synthesized.)

$$ng\ synthesized = \frac{1282 \times 10^{12}\ pg}{10^{12}\ pmol} \times 81.3\ pmol = 1.04 \times 10^{5}\ pg = 104\ ng$$

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.

1 µL 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:

$$42\ \mu L \times 1.3 \times 10^{6}\ cpm/\mu L = 55 \times 10^{6}\ cpm = 5.5 \times 10^{7}\ cpm$$

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*).

$$\frac{5.5 \times 10^7 \text{ cpm}}{104 \text{ ng}} = 5.29 \times 10^5 \text{ cpm/ng} = 5.29 \times 10^8 \text{ cpm/µg}$$

G. Calculating Yield of Nonistopically Labeled Transcription Reactions

Comparison to standards

The rate of incorporation of a given modified nucleotide can be indirectly monitored by doing spot detection assays of probes prepared at different times, or by comparison to known standards. Yield can also be inferred by comparison of signal generated from standards and newly-labeled probe. Detection methods for nonisotopic labels are specific to the label used.

Measuring yield by absorbance at 260 nm

IMPORTANT

Some nonisotopic labels such as digoxigenin interfere with quantitation by absorbance at 260 nm. Transcripts with these labels should be quantitated by comparison to known standards, or by ethidium bromide spot assay.

After removing unincorporated nucleotides from the reaction, the RNA transcript can be quantitated by measuring A_{260} units in the following manner:

1. Zero spectrophotometer at 260 nm with a quartz cuvette filled with 500 µL water.
2. Add 5 µL or 25% of the transcription reaction to the cuvette, cover top with parafilm, and mix by inverting the cuvette several times.
3. Multiply reading by the dilution factor of 100 to give A_{260}, and then by 40 to give µg/mL.
   In general, the following constants can be used to convert A_{260} units to µg:

oligonucleotides (between 10 and 40 bases)	30 µg/mL/A_{260} unit
single-stranded nucleic acids > 40 bases	40 µg/mL/A_{260} unit
double-stranded nucleic acids	50 µg/mL/A_{260} unit

To convert µg to pmol use the following formula:

$$\frac{(\mu g \times 10^{-6})(1 \times 10^{12} \text{ pmol/mol})}{(330 \text{ g/mol})(\#\text{ of bases})} = \text{pmol of nucleic acid}$$

Example: How many pmol of a 300 nucleotide long RNA transcript were produced in a 20 µL MAXIscript reaction? Five microliters was diluted to 500 µL in water, and the A_{260} read 0.1.

$$(100 - \text{dilution factor})(40\ \mu g/mL/A_{260})(0.1\ A_{260}) = 400\ \mu g/mL$$

$$(400\ \mu g/mL)(0.02\ mL) = 8\ \mu g$$

$$\frac{(8 \times 10^{-6}\ g)(1 \times 10^{12}\ pmol/mol)}{(330\ g/mol)(300\ nt)} = 80.8\ pmol$$

Some practical examples:

Nucleic Acid	A_{260} units	µg/mL	pmol/mL
20-mer oligonucleotide	1	30	4545
tRNA (76 bases)	1	40	1600
PSTV RNA (359 bases)	1	40	338
STNV RNA (1300 bases)	1	40	93
pUC19 (2686 base pairs)	1	50	28
pBR322 (4363 base pairs)	1	50	17
M13 (6407 bases)	1	40	19

Ethidium bromide spot assay

Another technique that can be used to quantitate RNA samples is an ethidium bromide spot assay. Simply make a standard curve of various dilutions of an RNA of known concentration and compare the ethidium fluorescence of the unknown sample to that from the standard curve.

Make a standard curve with several 2-fold dilutions of an RNA solution of known concentration. Using 2 µg/mL ethidium bromide as the diluent, start at about 80 ng/µL RNA, and make several 2-fold dilutions, ending about 1.25 ng/µL RNA. Make a few dilutions of the unknown RNA as well. The final concentration of ethidium bromide in all the samples should be 1 µg/mL. Spot 2 µL of the RNA standards and the unknown RNA dilutions onto plastic wrap placed on a UV transilluminator. Compare the fluorescence of the RNAs to estimate the concentration of the unknown RNA sample. Make sure that the unknown sample dilutions are in the linear range of ethidium bromide fluorescence. This assay will detect as little as 5 ng of RNA with an error of about 2-fold.

H. Using RNA Probes for Nuclease Protection Assays

For nuclease protection assays, we recommend using between 2–8 x 10^4 cpm (or 0.2 and 1 ng) of a high specific activity 300 nucleotide-long RNA probe per 20 µg of sample total RNA or 0.6 µg of poly (A+) RNA in the hybridization reaction. This achieves an adequate excess of probe over target for even mid-level constituitively expressed mRNAs such as ß-actin. (Ribosomal RNA requires a much greater mass amount of probe, see section *IV.A. How much low specific*

activity probe to use on page 15). For a probe that is 600 nucleotides long, twice as many cpm (twice as much mass) is required to maintain the same molar concentration; for a probe that is 150 nucleotides long, half as much should be used. Likewise if 40 µg total RNA or 1.2 µg poly (A+) RNA sample is used, twice as much probe cpm (mass) is required; for 10 µg total RNA or 0.3 µg poly (A+) RNA sample, half as much probe cpm (mass) is necessary.

I. Using RNA Probes for Blot Hybridizations

The final concentration of the RNA probe in a Northern, Southern, dot blot or colony hybridization buffer should be 1 x 10^6 cpm/mL or approximately 0.1 nM for a high specific activity probe. For a 300 nucleotide-long RNA, this is equivalent to 10 ng/mL.

In traditional formamide and aqueous hybridization buffers, RNA-RNA hybrids are substantially more stable than DNA-RNA hybrids which are more stable than DNA-DNA hybrids. Consequently, when using RNA probes hybridization and washing conditions need to be more stringent than those that are appropriate for DNA probes (see below, and Casey and Davidson, 1977).

1. Southern blots

Hybridization

Although suitable recipes for prehybridization and hybridization solutions can be found in most manuals of basic molecular biology techniques, we recommend using the Ambion ULTRAhyb® Ultrasensitive Hybridization Buffer (P/N AM8670). ULTRAhyb increases the sensitivity of blot hybridization by 10–100 fold over commonly used hybridization buffers. Instructions for using ULTRAhyb are provided with the product. Do not use the procedure that follows with ULTRAhyb.

If you prefer to make your own hybridization buffer, a typical prehybridization buffer follows.

Southern blot prehybridization solution

Amount	Component
50 %	formamide
6 X	SSPE
5 X	Denhardt's solution
0.5 %	SDS
100 µg/mL	sheared, denatured, heterologous DNA

50X Denhardt's Solution

Amount	Component
10 g	Ficoll 400
10 g	bovine serum albumin
10 g	polyvinylpyrrolidone
to 1 L	water

20X SSPE

Amount	Component
175 g	NaCl
7.4 g	Na_2EDTA
28 g	NaH_2PO_4
pH to 7.4	with NaOH

Hybridize in the same solution as the prehybridization except reduce or eliminate the concentration of Denhardt's Solution in the hybridization buffer.

Using the Southern blot prehybridization solution described above, prehybridize (30 min–1 hr), add probe, and hybridize overnight at 68°C.

Washes

a. Do 2 low stringency washes in 2X SSPE/0.5% SDS for 15 min each.

b. Do 1 high stringency wash in 0.1X SSPE/0.5% SDS for 30 min to 1 hr at 50–60°C.

2. Northern blots

Hybridization

Although suitable recipes for prehybridization and hybridization solutions can be found in most manuals of basic molecular biology techniques, we recommend using the Ambion ULTRAhyb® Ultrasensitive Hybridization Buffer (P/N AM8670). ULTRAhyb increases the sensitivity of blot hybridization by 10–100 fold over traditional hybridization buffers. Instructions for using ULTRAhyb are provided with the product. Do not use the procedure that follows with ULTRAhyb.

If you prefer to prepare your own hybridization buffer, a typical prehybridization and hybridization buffer is shown below:

Northern blot prehybridization/hybridization solution

Amount	Component
50 %	formamide
5 X	SSPE
2 X	Denhardt's solution
0.1 %	SDS
100 µg/mL	heterologous RNA

The same solution can be used for prehybridization and hybridization.

Using the Northern blot prehybridization/hybridization solution described above, prehybridize (30 min to 1 hr) and hybridize (overnight) at 68°C with RNA probes, and 42°C with DNA probes.

Washes

a. Do 3 low stringency washes in 1X SSPE, 0.5% SDS for 15 min each at room temperature.

b. Do 1–2 high stringency washes in 0.1X–0.2X SSPE/0.1% SDS for 20 min to 1 hr at 55–65°C.

If high background is a problem, the stringency of the wash conditions should be increased by decreasing the salt concentration and/or increasing the temperature of the last wash.

J. Synthesis of Capped RNA Transcripts

Most mRNA molecules in vivo have a 5' 7-methyl guanosine residue or cap, which plays an important role in the initiation of translation. Uncapped molecules are degraded very rapidly after microinjection into *Xenopus* oocytes. Uncapped mRNAs are translated less efficiently than capped molecules in cell-free translation systems, although under low salt conditions cap dependence is greatly reduced. The Reticulocyte Lysate Kit from Ambion includes a low salt buffer for the translation of uncapped mRNAs in vitro. Capped mRNAs can be readily synthesized in vitro by addition of cap analog to the transcription reaction. The level of GTP is reduced to 50 µM when synthesizing capped mRNAs.

Thaw reagent solutions and place on ice. Assemble the following components in the indicated order at room temperature.

Amount	Component
to 50 µL	RNase-free dH_2O
2.5 µg	template DNA (linearized)
2.5 µL	10 mM ATP
2.5 µL	10 mM CTP
2.5 µL	10 mM UTP
2.5 µL	1 mM GTP (1/10 dilution of 10 mM stock)
2.5 µL	10 mM cap analog — $m^7G(5')ppp(5')G$
5.0 µL	10X Transcription Buffer
5.0 µL	(T3, T7, or SP6) Enzyme Mix

Incubate at 37°C for 1 hr.

V. Appendix

A. Reagent Formulations

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 mix

5% acrylamide /8M urea gel

15 mL is enough gel solution for one 13 x 15 cm x 0.75 mm gel

a. Mix the following:

for 15mL	Component
7.2 g	Urea (high quality) (Ambion P/N AM9902)
1.5 mL	10X TBE
1.9 mL	40% Acrylamide (19 acryl:1 bis-acryl) (Ambion P/N AM9022, AM9024)
to 15 mL	ddH_2O

b. Stir at room temperature until the urea is completely dissolved, then add:

120 µL	10% ammonium persulfate
16 µL	TEMED

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

Gel set up

- Follow the manufacturers instructions for the details of attaching gels to the running apparatus.
- Use 1X TBE as the gel running buffer.
- It is very important to rinse the wells of urea-containing gels immediately before loading the samples.

Electrophoresis conditions

Gels should be run at about 20 V/cm gel length; for 13 cm long gel this will be about 250 V. Alternatively, denaturing acrylamide gels of this size can be run at ~25 mAmp, constant current.

3. RNase-free water

a. Add DEPC to 0.05% to double-distilled, deionized water (i.e. add 0.5 mL per liter of water).

b. Stir well, incubate several hours to overnight at 37°C or 42°C.

c. Autoclave 2 L or smaller volumes for at least 45 min. The scent of DEPC should be either not detectable or only very slightly detectable.

B. Miniprep for Isolating Transcription-quality Plasmid DNA

Generally, the cleaner the template DNA, the greater the yield of the transcription reaction. The following miniprep protocol yields high quality transcription template. This protocol is derived from a published procedure (Molecular Cloning, A Laboratory Manual), but differs in that the phenol/chloroform extraction is done after linearization of the plasmid with restriction enzyme(s), and proteinase K treatment (Step 2). In this way, any possible ribonuclease contamination from the restriction enzyme is eliminated without an additional proteinase K or phenol/chloroform extraction step. If you have difficulty getting good restriction digestion of your plasmid prep, it may be necessary to include a phenol/chloroform extraction before the ethanol precipitation at Step 5.

Solution I

Amount	Component
50 mM	glucose
10 mM	EDTA, pH 8
25 mM	Tris-HCl, pH 8

Autoclave for 15 min. Store at 4°C in small aliquots.

Solution II (make fresh)

Amount	Component
0.2 N	NaOH
1 %	SDS

Solution III

for 100 mL	Component
60 mL	5 M potassium acetate
11.5 mL	glacial acetic acid
28.5 mL	water (distilled deionized)

Store at room temperature.

1. **Pellet cells**

Centrifuge a 1.5 mL bacterial culture (grown overnight) for about 30 seconds; pour off supernatant, respin briefly (about 5 seconds), and remove residual supernatant via aspiration.

2. **Resuspend pellet in 110 µL Solution I, vortex**

Add 110 µL of Solution 1 and vortex vigorously to resuspend the pellet. Check for complete resuspension of pellet by inverting the tube and confirming that the solution is homogenous.

3. **Add 220 µL Solution II, incubate 1 min on ice**

Add 220 µL of Solution II and invert the tube several times to mix. Incubate the tube on ice for at least 1 min.

4. **Add 165 µL Solution III, incubate 5 min on ice, centrifuge 5 min**

Add 165 µL of Solution III and vortex medium-fast for 10 seconds. Incubate the tube on ice for 5 min.

Centrifuge for 5 min at maximum speed: this spin should be done at 4°C if possible. Most of the proteins, genomic DNA, and other cellular components will pellet during this spin.

5. **Add supernatant to a fresh tube with 1 mL ethanol, incubate 5 min on ice, centrifuge 5 min**

Add the supernatant to a fresh tube containing 1 mL of 100% ethanol, and invert several times to mix. Incubate the mixture for 5 min on ice to precipitate the plasmid DNA and some of the RNA.

Centrifuge for 5 min at maximum speed at 4°C if possible. This will pellet the plasmid DNA. Pour off the supernatant, centrifuge briefly, and aspirate off any residual supernatant.

6. **Resuspend in ~50 µL TE containing RNase, incubate 5 min at 37°C**

Resuspend the DNA pellet in ~50 µL TE (10 mM Tris HCl, pH 8 and 1 mM EDTA).

Add 0.5 U or 1 µg RNase A or use 1 µL of Ambion's RNase Cocktail. Vortex vigorously, incubate about 5 minutes at 37–42°C, and revortex to thoroughly solubilize the pellet.

7. Digest with appropriate restriction enzyme

Digest with an enzyme that will linearize the plasmid so that the polymerase promoter site will be upstream of the sequence you want to transcribe. The volume of the restriction digest should be about 2–3 times the volume of plasmid DNA used. Follow the recommendations of the restriction enzyme supplier for buffer composition, units of enzyme to use, and incubation conditions.

8. Treat with Proteinase K and SDS

Add SDS to a final concentration of 0.5% (usually a 10 to 20% SDS stock solution is used). Add 50–100 µg/mL Proteinase K (final concentration). Mix well by inversion, and incubate at 50°C for at least 30 min.

9. Phenol/chloroform extract and ethanol precipitate

Add an equal volume of phenol/chloroform or phenol/chloroform/IAA, vortex vigorously, centrifuge ~1 min at room temp.

Remove the aqueous (top) phase to fresh tube, add 1/10th volume of 5 M ammonium acetate (RNase-free), add 2 volumes ethanol, incubate at least 15 min at –20°C.

10. Pellet DNA

Pellet the DNA by centrifuging at top speed for 15 min. After the spin, discard the supernatant, re-spin briefly and remove any residual supernatant.

Resuspend the DNA in 10–20 µL nuclease-free water per 1.5 mL culture. Vortex until the pellet has completely dissolved.

11. Gel analysis

Assess the DNA by running an aliquot on an agarose gel in the presence of ethidium bromide. Estimate the concentration of the DNA by comparison to a known quantity of similar-sized DNA run on the same gel.

C. References

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D. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

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

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

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 glasses, 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.

E. Quality Control

Functional Analysis

A 20 µL probe synthesis reaction with pTRI-actin-mouse as template gave Š 50% incorporation of [α-^{32}P]UTP when the reaction contained 3.1 µM UTP. Approximately 90% of the transcripts were full-length.

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 supercoiled 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.