KinaseMax™ Kit

(Part Number AM1520)

Protocol

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I. Introduction

A. Background

The KinaseMax™ Kit provides optimized reagents for the 5' end labeling of nucleic acids using Calf Intestinal Alkaline Phosphatase (CIAP), bacteriophage T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP. The kit is formulated to 5' end label DNA or RNA molecules with terminal mono-, di- or triphosphates. Other nucleic acids such as PCR products, oligonucleotides, restriction-digested plasmid DNA, or complex sample RNA or DNA can also be labeled using the kit. The substrate can be virtually any length starting from as short as a single nucleotide.

PNK catalyzes the transfer of the gamma phosphate from ATP to the 5'-OH of a nucleic acid molecule ([γ-^{32}P]ATP is often used in the reaction). This phosphate transfer is commonly called a ***kinase*** or ***phosphorylation*** reaction. Nucleic acids with a 5'-hydroxyl (OH) group can be added directly to a kinase reaction.

Nucleic acids with a 5'-phosphate should be dephosphorylated before the kinase reaction. The kit includes Calf Intestinal Alkaline Phosphatase and Dephosphorylation Buffer for this reaction. Our novel Phosphatase Removal Reagent (PRR) replaces the organic extraction traditionally used to prepare phosphatase reactions for the kinase step. PRR requires a 3 minute incubation, a quick spin, and the sample is ready to be kinased.

The KinaseMax 5' end labeling kit provides controls and optimized reagents for quick and easy end labeling of DNA or RNA. Additionally, this kit can be used to dephosphorylate nucleic acid, and to phosphorylate nucleic acids using unlabeled ATP. These reactions that do not involve radiolabeling are most commonly used to prepare DNA for cloning, for example, dephosphorylating linearized plasmid, and kinasing (or phosphorylating) oligonucleotides using unlabeled ATP.

B. Reagents Provided with the Kit and Storage

Storage at –20°C should be in a non frost-free freezer.

Amount	Component	Storage
30 µL	T4 Polynucleotide Kinase (10 U/µL)	–20°C
30 µL	Calf Intestine Alkaline Phosphatase (CIAP, 0.1 U/µL)	–20°C
60 µL	10X Kinase Buffer	–20°C
30 µL	10X Dephosphorylation Buffer	–20°C

Amount	Component	Storage
300 µL	Phosphatase Removal Reagent (PRR)	–20°C
10 µL	RNA Transcript Control (0.5 µg/µL)	–20°C
10 µL	pUC19/*Sau*3 A Markers (0.5 mg/mL)	–20°C
30 µL	ATP Solution (10 mM)	–20°C
1.4 mL	Gel Loading Buffer II 1–2X gel loading dye that can be used for denaturing and non-denaturing polyacrylamide gels, and TBE agarose gels 95% formamide 0.025% xylene cyanol and bromophenol blue 18 mM EDTA 0.025% SDS	–20°C
1.0 mL	Water (Nuclease-free)	any temp*

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

The pUC19/*Sau*3 A Markers can be used as a positive control for both the dephosphorylation and kinase reactions. This control has the added advantage of yielding labeled markers which can be used in subsequent experiments. See section IV.A.1 on page 9 for instructions on how to use this component.

C. Materials Not Provided with the Kit

- Radiolabeled ATP—Ambion® recommends using [γ-^{32}P]ATP 7000 Ci/mmol, 150 mCi/mL (available from ICN)
- Gel electrophoresis reagents and equipment

D. Related Products Available from Applied Biosystems

Electrophoresis Reagents See web or print catalog for P/Ns	Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our website (www.ambion.com) for a complete listing as this product line is always growing.
Decade™ Markers P/N AM7778	The Decade Marker System is a set of reagents to prepare radiolabeled low molecular weight RNA markers: from 10–100 nt in 10 nt increments. The user supplies only [γ-^{32}P]ATP to end label a single, gel purified RNA transcript which is then cleaved into the 10 molecular weight markers in a simple 5 minute reaction.
Low Molecular Weight DNA Size Standards see our web or print catalog	pUC19 digested to completion with either *Sau*3A or *Hpa* II. These molecular weight standards can be visualized by ethidium bromide staining or they can be labeled using the KinaseMax™ Kit for visualization by radiolabeling and autoradiography or phosphorimaging.
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.

II. KinaseMax Procedure

A. Preparation of Nucleic Acid for use with the KinaseMax Kit

Chemical inhibitors to avoid

The DNA or RNA sample to be labeled must be free of both inorganic phosphate and pyrophosphate because these compounds are inhibitory to T4 Polynucleotide Kinase (PNK). Ammonium ions are strong inhibitors of PNK. Therefore, DNA or RNA should not be precipitated with, or dissolved in buffers containing ammonium salts prior to PNK reactions. Additionally, monovalent cation concentration should be kept low (below 100 mM) since PNK's K_m for ATP is decreased as the monovalent cation concentration is increased. If you are not sure whether your sample meets these criteria, precipitate the nucleic acid with salt and ethanol or isopropanol, and wash the pellet with 70% ethanol before attempting to end-label.

Other common reaction inhibitors

The quality of the nucleic acid preparation can be a significant factor in the performance of the kit. Small RNA or DNA fragments in plasmid minipreps for example, will compete with the intended DNA substrate in the kinase reaction. Furthermore, contaminating bacterial debris can also inhibit the reaction. If you suspect that your sample may contain any of these contaminants, clean up the preparation with a phenol:chloroform:isoamyl alcohol extraction followed by a G-25 spin-column purification (see section *V. Additional Procedures* starting on page 12).

B. Planning the Reaction(s)

1. Is it necessary to do a phosphatase reaction before the kinase reaction?

Calf Intestine Alkaline Phosphatase (CIAP) is used to remove 5'-phosphates from RNA or DNA molecules before 5' end labeling. Nucleic acids that lack a 5'-phosphate, (e.g. oligonucleotides) will not need CIAP treatment before the kinase reaction.

Nucleic acids with a 5'-PO_4 (*do* require CIAP treatment)	Nucleic acids with a 5'-OH (do *not* require CIAP treatment)
DNA cut with restriction enzymes	RNA and DNA oligonucleotides
in vitro transcribed RNA	PCR products
DNA or RNA isolated from an organism	

2. Dephosphorylation and phosphorylation with unlabeled ATP

The KinaseMax Kit can also be used for dephosphorylation and/or phosphorylation with unlabeled ATP for experiments that do not involve radiolabeling. The alkaline phosphatase reaction shown in the next section can be used to dephosphorylate any nucleic acid. The kinase reaction shown in section II.D on page 6, includes component lists for reaction with and without radiolabel.

3. Converting A_{260} units of nucleic acids to micrograms

In general, the following constants can be used to convert A_{260} units to µg:

oligonucleotides (10–40 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

4. Converting micrograms of nucleic acids to picomoles

To convert µg to pmol use the following formula:

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

Example: How many pmol are in 0.1 A_{260} units of a 20 nt oligonucleotide?

$$(30\ \mu g/A_{260})(0.1\ A_{260}) = 3\ \mu g$$

$$\frac{(3 \times 10^{-6}\ g)(1 \times 10^{12}\ pmol/mol)}{(330\ g/mol)(20\ nt)} = 454.5\ pmol$$

Some practical examples:

Nucleic Acid	A_{260} units	µg/mL	pmol
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

5. Amount of radiolabeled nucleotide needed for reaction

A 2–5 fold molar excess of ATP over ends that will be labeled is recommended. If desired, a 1–2 fold molar excess of ATP can be used, but we do not recommend using less an equal molar amount of ATP and ends to be labeled.

To determine pmol/µL of [γ-^{32}P]ATP:

$$pmol/\mu L = \frac{isotope\ conc\ (mCi/mL)}{specific\ activity\ (Ci/mmol)} \times \frac{10^9\ pmol}{mmol} \times \frac{mL}{1000\ \mu L} \times \frac{Ci}{1000\ mCi}$$

C. Alkaline Phosphatase Reaction

1. Assemble the reaction as follows:

There is a large range of RNA or DNA substrate that can be efficiently labeled. Use the amount of material within that range that suits your needs.

Amount	Component
to 10 µL	Nuclease-free Water
—– µL	0.1–25 pmol DNA or RNA substrate*
1 µL	10X Dephosphorylation Buffer
1 µL	Calf Intestine Alkaline Phosphatase

* Sections II.B.3 and 4 show how to calculate moles from A_{260} units and from mass amount of nucleic acid of a known length. As an example, 25 pmol of RNA is 1 µg of a transcript that is 120 nucleotides long.

2. Incubate 1 hr at 37°C

3. Add 10 µL PRR, and incubate 3 min at RT, with occasional agitation

Vortex the tube of Phosphatase Removal Reagent (PRR) then transfer 10 µL of PRR to the tube with the CIAP reaction.

NOTE

The PRR may become difficult to pipette after multiple uses due to a depletion of fluid in which the resin is suspended. If this is the case, add a volume of Nuclease-free Water equal to approximately 10–20% of the bed volume of the remaining PRR, and vortex thoroughly to recreate a pipettable slurry.

Gently flick tube occasionally during the 3 minute incubation at room temperature (RT).

4. Remove the sample from the PRR and proceed to the Kinase reaction

a. Centrifuge the tube briefly in a microfuge (~15 seconds of ramping up to top speed) to pellet the PRR. Alternatively, you can simply allow the PRR to settle undisturbed on the bench for ~1 minute.

b. Transfer the supernatant containing the CIAP-treated sample to a fresh microfuge tube (its volume will be ~15 µL). It is important to avoid disturbing the pelleted PRR when removing the supernatent. Carryover of PPR could inhibit the kinase reaction.

c. Proceed with the kinase reaction (or other application).

D. Kinase Reaction

1. Assemble the reaction as follows:

There is a large range of RNA or DNA substrate that can be efficiently kinased. Use the amount of material within that range that suits your needs.

Radiolabeled rxn	Unlabeled rxn	Component
to 20 µL	to 20 µL	Nuclease-Free Water
0.1–25 pmol	0.1–100 pmol	DNA or RNA substrate*
1 µL	– –	[γ-^{32}P]ATP†
– –	1 µL	10 mM unlabeled ATP
2 µL	2 µL	10X Kinase Buffer
1 µL	1 µL	T4 Polynucleotide Kinase

* Sections II.B.3 and 4 show how to calculate moles from A_{260} units and from mass amount of nucleic acid of a known length. As an example, 25 pmol of RNA is 1 µg of a transcript that is 120 nucleotides long.

† Use 7,000 Ci/mmol, 150 mCi/mL [γ-^{32}P]ATP; 1 µL of this is approximately equal to 25 pmol.

2. Incubate at 37°C for 1 hr for radiolabeled reactions, or 30 min for unlabeled reactions

Radiolabeled reactions: Incubate 1 hr at 37°C

Unlabeled reactions: Incubate 30 min at 37°C

3. (optional) Stop the reaction

RNA substrates
Add EDTA to 1 mM, and heat to 95°C for 2 minutes.

DNA substrates
Heat to 95°C for 2 minutes.

This heat treatment inactivates the T4 Polynucleotide Kinase.

4. (optional) Purify the reaction products

For many applications the labeled nucleic acid can be used without any purification. However, if it is important to remove free nucleotides, purification by spin-column chromatography (for example using Ambion® NucAway Spin Columns P/N AM10070) or denaturing polyacrylamide electrophoresis (in section V.B starting on page 13) is recommended.

III. Evaluating Radiolabeled Reactions

It is often necessary to determine the specific activity, or the cpm/µL of KinaseMax reaction products for use in other reactions. Doing the TCA precipitation and scintillation counting described in the next section will tell you how many cpm/µL are in the tube, and what percentage of the radiolabel was incorporated into acid precipitable material. If you intend to purify the labeled nucleic acid by gel electrophoresis (instructions in section V.B starting on page 13) or with a spin column, it may make more sense to simply count a small aliquot of the labeled material after purification to determine how many cpm/µL are present. Dividing the total cpm by the mass amount of sample labeled as described in section *III.B. Determination of Specific Activity* on page 8 will give the specific activity of the reaction products.

A. TCA Precipitation to Determine Radiolabel Incorporation

1. Take 1 µL of the KinaseMax reaction, put it in a fresh microfuge tube, and add 9 µL nuclease free water to make a 1:10 dilution of the reaction.
2. Dispense 198 µL 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.)
3. Add 2 µL of the diluted KinaseMax reaction to the carrier DNA or RNA and mix thoroughly.
4. Measure the total amount of radiolabel in the reaction mixture. Transfer 100 µL of the mixture from step 3 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).
5. Measure TCA-precipitable counts
   - Transfer another 100 µL of the mixture from step 3 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 minutes. This will precipitate nucleic acids, but not free nucleotides.
   - Collect the precipitate via vacuum filtration through a Whatman GF/C glass fiber filter (or its equivalent).
   - 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.
   - 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.
6. Divide the cpm in step 5 by the cpm in step 4 to determine the fraction of label incorporated (multiply by 100 for percent incorporation).

Expected radiolabel incorporation

The percentage incorporation of radiolabel will depend on how much substrate RNA or DNA was present in the reaction. The reaction described in the protocol (section *II.D. Kinase Reaction* on page 6) includes 20 pmoles of [γ-^{32}P]ATP. If the amount of nucleic acid labeled is less than 10 pmoles, the percent incorporation of radiolabel will be low, but a high proportion of the nucleic acid will be labeled. Using larger amounts of substrate will result in higher percent incorporation of radiolabel, but will typically lower the specific activity of the reaction products.

B. Determination of Specific Activity

The specific activity of a labeled nucleic acid reflects the efficiency of the kinase reaction and provides a useful number for subsequent experiments. Specific activity is simply cpm/pmol.

To determine the specific activity of the labeled nucleic acid, calculate the total cpm incorporated in the reaction and divide it by the amount of nucleic acid labeled in pmol.

$$\frac{\text{total cpm incorporated (step III.A.5) x total reaction volume (20) x dilution factor (10)}}{\text{pmol nucleic acid}}$$

Expected specific activity

When [γ-^{32}P]ATP with a specific activity of 7000 Ci/mmol is used for the kinase reaction, the nucleic acid should be labeled to at least 1 x 10^6 cpm/pmol on the reference day stated on the vial of radiolabeled ATP. Table 1 on page 16 can be used to adjust the expected result based on the decay rate of ^{32}P.

IV. Troubleshooting

A. Positive Control Reaction

There are both RNA and DNA positive control templates for end labeling in the KinaseMax Kit. We recommend that you use the RNA Transcript Control if your experiment involves labeling RNA, or the pUC19/*Sau*3 A Markers if your experiment involves labeling DNA.

1. Radiolabel the RNA Transcript Control or the pUC19/*Sau*3 A Markers

Instructions

a. Use 1 µL of the either the RNA Transcript Control (5 pmol) or the pUC19/*Sau*3 A (0.28 pmol) in a standard phosphatase reaction as described in section II.C starting on page 5.

b. After the phosphatase reaction, use all of the material in a standard radiolabeled kinase reaction as described in section II.D starting on page 6.

c. Determine the percent of radiolabel incorporated by the reaction using the TCA precipitation instructions in section III.A starting on page 7

Expected result

Positive control reactions using either the RNA Transcript Control or the pUC19/*Sau*3 A markers should yield labeled nucleic acid with a specific activity of at least 1.0 x 10^6 cpm/pm (use the instructions for calculating specific activity in section III.B on page 8—1 µL of the RNA Transcript Control is 5 pmol, 1 µL of the pUC19/*Sau*3 A provided is 0.28 pmol). This value is based on the reference date for the ^{32}P and should be adjusted accordingly.

2. Using the labeled pUC19/*Sau*3 A Markers

Once labeled, the pUC19/*Sau*3 A Markers can be used as a molecular weight marker. For an overnight exposure it is usually sufficient to use 5000 TCA-precipitable cpm of markers per lane. We have used end labeled pUC19/*Sau*3 A markers for 3 months with no visible degradation or smearing of the bands.

B. Troubleshooting Poor Labeling

1. Try the positive control

If the specific activity of your labeled nucleic acid falls substantially below the expected value (adjusted for ^{32}P decay), check that the kit components are functioning properly by using one of the positive controls in a phosphatase reaction followed by a kinase reaction as described in section IV.A on page 9. If your experiment involves labeling RNA,

we recommend doing the reaction with the RNA Transcript Control. If you will be labeling DNA, the pUC19 *Sau*3 A Markers are probably a more pertinent control.

2. The positive control works, but my samples don't label well

a. There is an inhibitor in the nucleic acid substrate

As discussed in section *II.A. Preparation of Nucleic Acid for use with the KinaseMax Kit* on page 3, the quality of the preparation can be a significant factor in the performance of the kit. Consider whether your preparation contains any of the following reaction inhibitors:

- Small RNA or DNA fragments
- bacterial debris
- residual ammonium salts
- inorganic phosphate and/or pyrophosphate
- monovalent cation concentration ≥100 mM

If necessary, clean up the nucleic acid you want to label with a phenol:chloroform:isoamyl alcohol extraction followed by spin-column purification (see section *V. Additional Procedures* starting on page 12).

b. 5' end of RNA is inaccessible because of tertiary structure

In some cases the 5' ends of an RNA molecule may be occluded by its tertiary structure, reducing the efficiency of the phosphatase and kinase reactions. If this is the case try heating the RNA to 90°C for 5 minutes and then placing it immediately on ice just before the reaction. This will help to disrupt any structure in the RNA and potentially make the 5' end more accessible. The RNA is added directly to a tube containing the remaining reagents of the CIAP or Kinase reaction.

C. Troubleshooting Unlabeled KinaseMax Reactions

It is much more difficult to troubleshoot unlabeled phosphatase and kinase reactions because there is no radiolabel incorporation to track the progress of the reaction. Generally, these reactions may be suspect if downstream manipulations fail.

1. Try the positive control

As always, one of the first troubleshooting suggestions is to check that the kit components are functioning properly by using one of the positive controls in a phosphatase reaction followed by a kinase reaction as described in section IV.A on page 9. Use radiolabel in these reactions so that you can tell if they were successful or not. If your experiment involves RNA, do the reaction with the RNA Transcript Control. If you will be working with DNA, the pUC19 *Sau*3 A Markers are probably a more pertinent control.

2. Phosphorylated DNA won't ligate

Try a clean up procedure after the kinase reaction

Generally material can be used directly in a ligation reaction after the kinase step. However, if problems occur with ligation, 2 routes for further purification may help. First, phenol/chloroform extract, ethanol precipitate and wash the DNA (instructions in section V.A on page 12). If this alone does not help, further purification by spin-column chromatography (using Ambion® NucAway™ columns for example) or denaturing polyacrylamide electrophoresis (instructions in section V.B on page 13) should help.

V. Additional Procedures

A. Phenol/Chloroform Extraction

Phenol extraction is a method commonly used for deproteinization of nucleic acids. Most proteins are more soluble in phenol than in aqueous solutions. Conversely, nucleic acids are more soluble in aqueous solutions than in phenol. Centrifugation of the mixture will separate the organic from the aqueous phase; the lower phase is the organic phase and will contain the protein, usually as a white flocculent material at the interface. The upper aqueous phase will contain nucleic acids. Chloroform is mixed with phenol to enhance protein denaturation and phase separation. Chloroform in the phenol also improves its ability to remove lipids; isoamyl alcohol is added to prevent foaming.

The most rigorous way to perform a phenol/chloroform extraction is to first extract with buffer saturated phenol (P/N AM9710, AM9712), followed with a phenol:chloroform:isoamyl alcohol extraction (Ambion® P/N AM9730, AM9732), and finally, to extract the sample with chloroform:isoamyl alcohol. Instructions to prepare these reagents can be found in *Current Protocols in Molecular Biology* (Ausubel et al., eds.). Each of these extractions is done as follows:

1. Adjust the volume of the sample to 100–200 µL with nuclease-free water or TE.
2. Add an equal volume of organic solvent solution, vortex for 2 minutes to mix thoroughly.
3. Spin at top speed in a room temperature microfuge 2 minutes.
4. Recover the aqueous phase by removing it to a new tube.
5. After the organic extractions are complete, the nucleic acid can be purified away from low molecular weight RNA and/or DNA fragments by passing it through a spin column. We recommend Ambion NucAway™ Spin Columns (P/N AM10070) for this purpose.

B. Gel Purification

1. Gel preparation

Gel-purification is a reliable and inexpensive method to purify the labeled nucleic acids away from free [γ-^{32}P]ATP. We recommend using denaturing (8 M urea) polyacrylamide gels at the appropriate percentage of polyacrylamide for the size of the nucleic acid being purified.

a. Acrylamide percentage

Size of molecule	% Acrylamide
< 30 bases	20%
30–60 bases	15%
61–150 bases	10%
151–500 bases	5%
>500 bases	4%

b. Denaturing acrylamide gel mix

The following recipe makes 15 mL of gel solution, which is enough for one 13 x 15 cm x 0.75 mm gel

- 7.2 g urea (high quality)
- 1.5 mL 10X TBE
- 40% acrylamide (19:1 acrylamide:bis acrylamide) and water according to the percent acrylamide desired:

Percent acrylamide	Volume of 40% acryl soln	Water
20%	7.5 mL	2.5 mL
15%	5.6 mL	4.4 mL
10%	3.75 mL	6.25 mL
5%	1.9 mL	8.1 mL
4%	1.5 mL	8.5 mL

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

- 120 µL 10% ammonium persulfate
- 16 µL TEMED

Mix briefly after adding the last 2 ingredients, which will catalyze polymerization, then pour the gel immediately.

c. Gel set up

Use 1X TBE as the gel running buffer. Follow the manufacturers instructions for the details of attaching gels to the running apparatus.

2. 10X TBE

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

IMPORTANT

Do not treat TBE with diethylpyrocarbonate (DEPC).

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

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

Alternatively, 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.

3. Loading samples and electrophoresis conditions

- Rinse the urea from the wells just before loading the samples.
- Add an equal volume of Gel Loading Buffer II to the kinase reaction mixture, heat the tube at 95°C for 2 minutes, and then immediately load onto gel.
- Gels should be run at about 20 V/cm gel length; for a 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.

4. Recovering the sample from the gel

a. After electrophoresis, cover the gel (adhered to one of the glass plates) with plastic wrap and expose to X-ray film for about 1–10 minutes.

After electrophoresis cover the gel with plastic wrap and expose it 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" signal so that a small discrete gel fragment can be localized. Fluorescent stickers are the easiest way to orient the film with the gel to cut out the band. Alternatively, ink "spiked" with ^{32}P or ^{35}S isotopes can be used to make asymmetric marks on the surface of the plastic wrap.

b. Align the film with the gel, and excise the band of interest.

After exposure, develop the film and identify the full-length labeled molecule; it is usually the most slowly migrating, most intense band on the autoradiograph. Place the film on top of the gel and plastic wrap, align it with the gel via the fluorescent stickers, and lightly tape it in place. Next, invert the gel and film and circle the position of the band of interest on the film with a felt-tip pen on the back of the glass plate. Then, turn the gel back over and remove the film.

Finally, excise the area of the gel indicated by the circled region on the glass plate with a razor blade or scalpel, and transfer it to a microfuge tube containing about 350 µL of elution buffer.

Elution buffer

Concentration	Component
0.5 M	ammonium acetate
0.1 mM	EDTA
0.1 %	SDS

It is always a good idea to re-expose the gel after cutting out the band to be sure that the entire band was recovered.

c. Elute the nucleic acid from the gel into elution buffer for 1 hr–overnight at room temperature

The tube should be left at room temperature overnight for maximal recovery of nucleic acid (usually about 95% can be recovered).

d. Clean up the eluted nucleic acid by phenol:chloroform extraction and ethanol precipitation

After the overnight elution, transfer the elution solution to a fresh microfuge tube and add an equal volume of water-saturated phenol. Vortex the tube and centrifuge at room temperature in a microfuge for 5 minutes to separate the phases. Carefully withdraw the aqueous phase (top layer), transfer it to a clean tube, and add 3 volumes of ethanol. It may be desirable to add a coprecipitant to the precipitation such as linear acrylamide (Ambion P/N AM9520), glycogen (Ambion P/N AM9510) or GlycoBlue™ (Ambion P/N AM9515, AM9516). Vortex the mixture thoroughly, incubate for 15–30 minutes at –20°C, and centrifuge at 4°C for ≥15 minutes. Finally, remove the supernatant, dry the pellet and resuspend it in an appropriate volume of nuclease-free water.

VI. Appendix

A. ^{32}P Decay Calculation

Once you establish the cpm/µL of your labeled nucleic acid solution, the following formula can be used to calculate its cpm/µL on any day after the initial measurement:

$$\frac{\text{original cpm/µL}}{2^{(\text{days after original reading}/14.3)}}$$

B. ^{32}P Decay Data Table

To use the table, select the number of days after the reference date on the vial of [γ-^{32}P]ATP from either the top of the table, the left hand side of the table, or the sum of the two. The value in the chart which corresponds to the number of days after the reference date is the proportion of radioactivity left; multiply this factor by the cpm on the reference date to calculate the current cpm.

Example: To find the proportion of label left after 6.5 days, look for the number at the intersection of the number 5 on the left hand side, and the number 1.5 from the top of the chart (because 5+1.5=6.5), to find the factor 0.73. So, if there were 10^7 cpm on the reference date, there will be 10^7 X 0.73 or 7.3 x 10^6 cpm 6.5 days later.

Table 1. ^{32}P Decay Table

Days	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
0	1.000	0.976	0.953	0.930	0.908	0.886	0.865	0.844	0.824	0.804
5	0.785	0.766	0.748	0.730	0.712	0.695	0.678	0.662	0.646	0.631
10	0.616	0.601	0.587	0.573	0.559	0.545	0.532	0.520	0.507	0.495
15	0.483	0.472	0.460	0.449	0.438	0.428	0.418	0.408	0.398	0.388
20	0.379	0.370	0.361	0.353	0.344	0.336	0.328	0.320	0.312	0.305
25	0.297	0.290	0.283	0.277	0.270	0.264	0.257	0.251	0.245	0.239
30	0.233	0.228	0.222	0.217	0.212	0.207	0.202	0.197	0.192	0.188
35	0.183	0.179	0.174	0.170	0.166	0.162	0.158	0.155	0.151	0.147
40	0.144	0.140	0.137	0.134	0.130	0.127	0.124	0.121	0.118	0.116
45	0.113	0.110	0.107	0.105	0.102	0.100	0.098	0.095	0.093	0.091
50	0.088	0.086	0.084	0.082	0.080	0.078	0.077	0.075	0.073	0.071
55	0.069	0.068	0.066	0.065	0.063	0.062	0.060	0.059	0.057	0.056
60	0.054	0.053	0.052	0.051	0.049	0.048	0.047	0.046	0.045	0.044

C. 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.

D. Quality Control

Functional Analysis:

The RNA Transcript Control and the pUC19/*Sau*3 A Markers are radiolabeled as described in section *IV.A. Positive Control Reaction* starting on page 9.

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.