Prime-It Fluor Fluorescence Labeling Kit

Instruction Manual

Catalog #300380

Revision B

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300380-12

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PRIME-IT FLUOR FLUORESCENCE LABELING KIT

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Prime-It Fluor Fluorescence Labeling Kit

Materials Provided

Materials provided[a]	Quantity	Storage temperature
Random 9-mer primers	350 µl of TE buffer[b] of random oligodeoxyribonucleotides	–20°C
5× Nucleotide buffer	350 µl of buffered aqueous solution containing dATP, dGTP and dCTP at 100 µM and dTTP at 20 µM	–20°C
Fluor-12-dUTP	35 µl of 1 mM nucleotide in buffered aqueous solution	–20°C
Exonuclease-free Klenow	175 U in buffered glycerol solution (5 U/µl)	–20°C
Stop mix	100 µl of 0.5 M EDTA (pH 8.0)	–20°C
Control DNA[c]	10 µl of unlabeled cosmid DNA (25 ng/µl)	–20°C

[a] Sufficient reagents are provided for 30 labeling reactions plus 5 control labeling reactions.
[b] See *Preparation of Media and Reagents*.
[c] This is a single-copy gene which hybridizes to the end of the long arm of chromosome 11.

Storage Conditions

All components: –20°C

Revision B

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INTRODUCTION

The Stratagene Prime-It Fluor fluorescence labeling kit generates directly fluoresceinated probes by incorporating fluorescein-linked dUTP into the probe fragments. Denatured DNA template is primed with random 9-mer oligonucleotides, and exonuclease-free Klenow is used to extend the primers by incorporating a mix of nonmodified nucleotides and fluor-12-dUTP. The fluoresceinated probe fragments generated in this way can be used for fluorescence in situ hybridization (FISH) experiments or to detect nucleic acids immobilized on membranes using an antifluorescein antibody-based chemiluminescence nucleic acid detection system.

In Situ Hybridization

In situ hybridization is widely used to localize and map mammalian genes and to detect chromosomal abnormalities.[1,2,3,4] In this technique, labeled DNA is hybridized to chromosomes immobilized on microscope slides. When fluoresceinated nucleotides have been incorporated into the probe fragments, the hybridized probe can be viewed directly using a fluorescence microscope. This eliminates the need for the secondary immunocytochemical detection reagents that are used in other *in situ* hybridization procedures. The direct fluorescence labeling approach reduces background levels and the time and effort involved in the FISH procedure.

Nonradioactive Detection of Nucleic Acids Immobilized on Membranes

DNA probes labeled using the Prime-It Fluor fluorescence labeling kit may also be hybridized to nucleic acids immobilized on membranes by Southern blot, Northern blot, or dot blot analysis. The hybridized probe can then be detected using an antifluorescein antibody-based chemiluminescence nucleic acid detection system. This nonisotopic nucleic acid detection method offers increased safety, alleviates radioactive waste disposal problems, and shortens overall experimental time.

LABELING DNA FOR FLUORESCENCE *IN SITU* HYBRIDIZATION

This protocol is for use with chromosomal hybridization.

Notes *Labeling 25–50 ng of template DNA will produce enough probe to detect a single-copy gene on a single slide of chromosome spreads. The total DNA to be labeled can be calculated according to the number of individual slides which are to be probed. To detect repeat sequences, less than 50 ng of probe DNA/slide is needed to produce a good hybridization signal; the amount required will depend on the number of gene copies.*

For single-copy gene detection, the template should preferably be cosmid or yeast artificial chromosome (YAC) DNA in order to generate sufficient signal; however, templates as small as 15 kb have been used successfully.

Miniprep DNA may be used as a template, provided that an RNase step is included in the preparation protocol.

Labeling Procedure

1. Prepare the reaction buffer by mixing 8 µl of fluor-12-dUTP with 92 µl of 5× nucleotide buffer in a sterile microcentrifuge tube.

   Note *The reaction buffer may be stored at –20°C for 1 month. Avoid repeated freeze–thaw cycles.*

2. In two separate, sterile microcentrifuge tubes, mix the sample and control reactions as follows:

Sample Reaction

50 ng (1–29 µl) of linearized DNA template
0–28 µl of distilled water (dH_2O)
<u>10 µl of random 9-mer primers</u>
Total reaction volume is 39 µl

Note *This protocol can be used to label up to 500 ng of DNA template. When labeling 200–500 ng of DNA template, use 10 U (2 µl) of exonuclease-free Klenow (see step 5 in this section). For labeling DNA in low-melting-temperature agarose, see Appendix II. For labeling YAC DNA, see Appendix III.*

Control Reaction

50 ng (2 µl) of control DNA template
27 µl of dH_2O
<u>10 µl of random 9-mer primers</u>
Total reaction volume is 39 µl

3. Heat the sample and control reaction tubes to 95–100°C for 5 minutes in boiling water.

4. Briefly centrifuge the sample and control reaction tubes at room temperature and then place the reaction tubes on ice.

5. Add the following reagents to the sample and control reaction tubes:

   10 μl of reaction buffer
   1 μl (5 U) of exonuclease-free Klenow

   Mix the reaction components with a pipet tip.

6. Incubate the sample and control reaction tubes at 37°C for 20–30 minutes.

7. Stop the reactions by adding 2 μl of stop mix to each reaction tube. Store the sample and control reaction tubes at 4°C in the dark.

   Note *Unpurified probes may be used for in situ hybridizations using the method described in Appendix I: In Situ Hybridization.*

Efficiency of Incorporation of Fluorescent Nucleotides into Probe DNA

Using a radiolabeled nucleotide as a tracer, 50 ng of DNA template generates ~10–15 ng of labeled probe in a 20-minute labeling reaction.

When performing *in situ* hybridizations, we recommend using the probe without purification from unincorporated nucleotides. However, a qualitative indication of the efficiency of incorporation of fluoresceinated nucleotide into the probe DNA can be obtained by the method described below. Before analysis of the percentage of incorporation of fluorescent nucleotides into probe DNA, unincorporated nucleotides should be removed.

1. To remove unincorporated nucleotides by precipitation:

   a. Add carrier DNA[||] to a microcentrifuge tube containing fluorescent probe DNA.

   b. Add 1/10 volume of 3 M sodium acetate (final concentration 0.3 M sodium acetate) to the microcentrifuge tube.

[||] Use 1 μg of salmon sperm DNA or, if the labeled DNA is to be used for *in situ* hybridizations, add the appropriate amount of competitor DNA for the hybridization reaction.

c. Add 2.5 volumes of 100% (v/v) ethanol to the microcentrifuge tube and mix well.

d. Incubate the microcentrifuge tube on ice for 20 minutes.

e. Spin the microcentrifuge tube in a microcentrifuge for 20 minutes. Discard the supernatant.

f. Wash the pellet with 70% (v/v) ethanol.

g. Dry the pellet in a vacuum lyophilizer.

h. Resuspend the pellet in 10 μl of TE buffer or, if the probe is to be used undiluted for *in situ* hybridizations, resuspend the pellet in 10 μl of hybridization buffer.

2. Spot 1 μl of labeled, precipitated probe onto a DE81 filter paper disk. Allow the spot to dry for 5–10 minutes. If the probe is resuspended in hybridization buffer, however, it will not dry completely and the fluorescence will be harder to visualize than if the probe is resuspended in TE buffer.

3. Spot 1-μl aliquots of 1:100, 1:250 and 1:500 dilutions of the reaction buffer on a DE81 filter paper disk. Illuminate the DNA with ultraviolet (UV) light (250–320 nm) and compare the intensity of fluorescence of the probe DNA with the dilutions of the reaction buffer. The probe DNA has incorporated sufficient fluor-12-dUTP if it fluoresces at an intensity between that of 1:250 and 1:500 dilutions of the reaction buffer.

For use in FISH, precipitated probe DNA which has been resuspended in TE buffer may need to be resuspended in hybridization buffer by first drying the sample in a vacuum lyophilizer. Probe which has been resuspended in hybridization buffer may be diluted to the appropriate concentration by the addition of more hybridization buffer.

LABELING DNA FOR DETECTION OF NUCLEIC ACIDS IMMOBILIZED ON MEMBRANES

Note *Labeling 50–100 ng of template DNA will produce enough probe to hybridize to a 150-cm² blot at a probe concentration of 5–10 ng of starting template/ml of hybridization buffer.*

Labeling Procedure

1. Prepare the reaction buffer by mixing 8 µl of fluor-12-dUTP with 92 µl of 5× nucleotide buffer in a sterile microcentrifuge tube.

   Note *The reaction buffer may be stored at –20°C for 1 month. Avoid repeated freeze–thaw cycles.*

2. In two separate, sterile microcentrifuge tubes, mix the sample and control reactions as follows:

Sample Reaction

50–100 ng (1–29 µl) of DNA template
0–28 µl of dH_2O
<u>10 µl of random 9-mer primers</u>
Total reaction volume is 39 µl

Note *This protocol can be used to label up to 500 ng of DNA template. When labeling 200–500 ng of DNA template, use 10 U (2 µl) of exonuclease-free Klenow (see step 5 in this section).*

Control Reaction

50 ng (2 µl) of control DNA template
27 µl of dH_2O
<u>10 µl of random 9-mer primers</u>
Total reaction volume is 39 µl

3. Heat the sample and control reaction tubes to 95–100°C for 5 minutes in boiling water.

4. Briefly centrifuge the sample and control reaction tubes at room temperature and then place the reaction tubes on ice.

5. Add the following reagents to the sample and control reaction tubes:

   10 µl of the reaction buffer
   1 µl (5 U) of exonuclease-free Klenow

   Mix the reaction components with a pipet tip.

6. Incubate the sample and control reaction tubes at 37°C for 20–30 minutes.

7. Stop the reactions by adding 2 µl of stop mix to each reaction tube. Store the sample and control reaction tubes at 4°C in the dark.

   Note *Labeled probe DNA should be purified from unincorporated nucleotides by ethanol precipitation or a suitable probe purification column. Failure to remove unincorporated nucleotides may lead to increased background.*

The efficiency of incorporation of fluorescent nucleotides into probe DNA can be determined by dot blot analysis of dilutions of the probe as described below.

Dot Blot Analysis of Fluorescent Nucleotide Incorporation

Fluorescent nucleotide incorporation into the probe can be qualitatively analyzed on a dot-blot, using the appropriate antifluorescein antibody-conjugate for detection.

1. To prepare the labeled probe for dot-blot analysis, add TE buffer[§] to make the final concentration of the DNA 0.25 ng of template/µl.

   Example *For 50 ng DNA template, bring the final volume to 200 µl with TE buffer.*

2. Starting with an aliquot of the 0.25 ng/µl probe solution, prepare the following series of serial dilutions:

   Mix 8 µl of dilute probe with 12 µl of TE buffer (= Dilution #1)
   Mix 2 µl of Dilution #1 with 18 µl of TE buffer (= Dilution #2)
   Mix 2 µl of Dilution #2 with 18 µl of TE buffer (= Dilution #3)
   Mix 2 µl of Dilution #3 with 8 µl of TE buffer (= Dilution #4)
   Final dilution 1:1250

3. Spot 1-µl aliquots of each dilution on a nylon membrane and crosslink using UV irradiation at 120,000 µJ/cm^2 of membrane.

   Note *For the Stratalinker UV Crosslinker, use the* autocrosslink *setting.*

4. Process the crosslinked membrane using a chemiluminescence detection system that includes detection by an antifluorescein antibody-conjugate, according to the manufacturer's recommendations.

For most detection reagents, the #2 dilution spot should be visible after a 1-minute exposure to X-ray film and the #4 dilution spot should be visible after a 30-minute exposure to X-ray film. Signal intensity and time needed for detection may vary for different reagent systems.

APPENDIX I: *IN SITU* HYBRIDIZATION

The following is a general procedure for performing FISH.

Note *Prepare the chromosome spreads according to the established protocols.*[5]

Treating the Chromosome Spreads

Day 1

1. Spot each slide with ~150 µl of RNase in 2× SSC buffer (100 µg/ml) (see *Preparation of Media and Reagents*). Cover each slide with a large cover slip and incubate the slides at 37°C for ~1 hour.

   Note *Do not allow the slides to dry out. Add more RNase under the edges of the cover slip if necessary.*

   Perform subsequent washing, dehydration and denaturation steps in Coplin jars.

2. Rinse the slides briefly in 2× SSC buffer and remove the cover slips.

3. Dehydrate the chromosomes by immersing the slides in a 70, 80 and 100% (v/v) ethanol series at room temperature for 2 minutes each. Allow the slides to air dry.

4. Denature the chromosomes by incubating the slides for 3–4 minutes at 72°C in a solution of 70% formamide and 2× SSC buffer.

   Note *The temperature should not fall below 70°C. Therefore, warm the slides before denaturing, do not treat too many slides at once and monitor the temperature of the formamide solution while denaturing.*

5. Dehydrate the chromosomes by immersing the slides in the following ice-cold ethanol series:

   70% (v/v) ethanol for 2 minutes
   80% (v/v) ethanol for 2 minutes
   100% (v/v) ethanol for 1 minute

6. Immerse the slides in 100% (v/v) ethanol at room temperature for 1 minute and air dry the slides.

Coprecipitating the Probe DNA

1. Ethanol precipitate the fluoresceinated probe DNA with 2–10 μg of carrier DNA (e.g., herring sperm DNA). If required, add 1–5 μg of competitor DNA (e.g., total human DNA) or COT-1® DNA to remove any repeat sequences.

   Note *Sonicate the carrier and competitor DNA until they are ~100–500 bp in length.*

2. Prepare the hybridization buffer (see *Preparation of Media and Reagents*) and preheat the buffer to 42°C. Resuspend the DNA in 30 μl of the preheated hybridization buffer.

3. Incubate the reaction mixture at room temperature for 5–10 minutes to ensure that the DNA is well resuspended.

Denaturing and Preannealing the Probe and Competitor DNA

1. Incubate the DNA for 10–15 minutes at 75°C. Incubate the DNA again at 42°C for 10–30 minutes if repeat sequences are to be eliminated by annealing with competitor DNA.

Hybridizing

1. Pipet the denatured, annealed DNA onto a warmed (37–42°C) slide of denatured chromosomes.

2. Cover the slide with a glass cover slip (22 × 30 mm), eliminating as many air bubbles as possible.

3. Seal the edges of the cover slip with rubber cement and incubate at 37°C in a humid chamber for 16–24 hours, protected from light.

   Note *Prepare the humid chamber as follows: Place dampened paper towels in the bottom of a large petri dish. Then place toothpicks or other supports on the paper towels to prevent the slides from contacting the paper towels directly. Position the slides inside the dish on top of the toothpicks, place the lid on the petri dish, and cover the dish with aluminum foil to protect from light.*

Washing the Slides

Day 2

1. Remove the rubber cement. There is no need to remove the cover slips, because the cover slips will slide off in the first wash step.

   Important *Do not allow the slides to dry out between any of the subsequent steps.*

2. Immerse the slides as follows:

   a. In three washes of 50% formamide and 2× SSC buffer at 45°C for 5 minutes each with agitation.

   b. In three washes of 0.1× SSC buffer at 60°C for 5 minutes each with agitation.

   c. Rinse in 4× SSC buffer and 0.1% Tween® 20 at room temperature.

Counterstaining the Chromosomes

1. Remove any residual traces of the rubber cement from the front of each slide.

2. Spot 25 μl of antifade [25 mg of triethylenediamine/ml of a 1:1 (v/v) glycerol and phosphate-buffered saline (PBS) solution] containing 200 ng/ml of propidium iodide (PI) and/or 20 ng/ml of 4´,6-diamidino-2-phenylindole (DAPI) on the slides.

3. Cover each slide with a glass cover slip (22 x 30mm). Remove any air bubbles from under the cover slip. Turn the slide over and, with the cover slip facing down, press gently to remove the excess antifade. Seal the edges of the cover slip with clear nail polish.

4. Store the slides at 4°C in the dark.

5. View the slides with a fluorescence microscope using a 100× oil-immersion objective, a 450- to 490-nm excitation wavelength and a 520-nm emission wavelength filter.

Appendix II: Labeling DNA Prepared in Low-Melting-Temperature Agarose

The Prime-It Fluor fluorescence labeling kit can be used to label DNA prepared in low-melting-temperature (LMT) agarose.

1. Electrophorese the DNA sample in a LMT agarose gel containing 0.05 μl/ml of ethidium bromide (EtBr) .

2. Visualize the DNA on a long-wave UV box and cut out the desired DNA band.

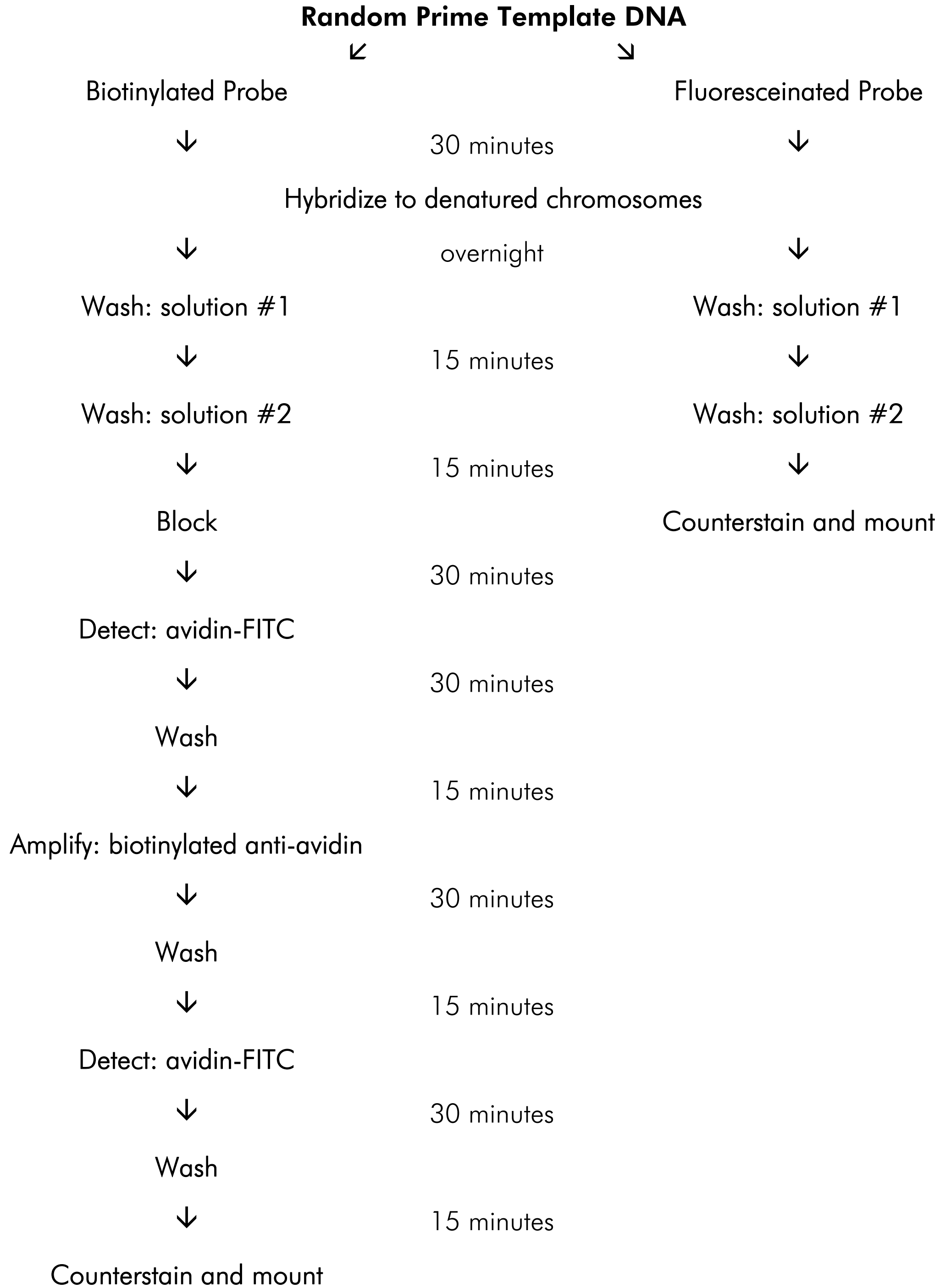
3. Place the gel slice in a preweighed microcentrifuge tube and weigh the gel.

4. Add 3 ml of dH_2O/g of LMT agarose. Heat at 65°C for 5 minutes to melt the gel. Mix the sample thoroughly and then store the tubes at –20°C until ready to perform a labeling reaction.

Appendix III: Yeast Artificial Chromosome (YAC) Templates

Yeast artificial chromosome templates should be pretreated with DNase I before labeling by random priming. The optimal final concentration of DNase I is determined empirically for each YAC template. A dilution range of 1:500-1:2000 of 1 mg/μl of DNase I for 1 hour at 14°C is normally utilized for 25–50 ng of template DNA. A smear of DNA below the full-length YAC band should be seen when a sample of the digested DNA is run on an agarose gel.

APPENDIX IV: THE FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) FOR BIOTINYLATED AND FLUORESCEINATED PROBES



TROUBLESHOOTING

Chromosomal In Situ Hybridization

Observation	Suggestion(s)
No signal from the clone, but the control produces a signal	Check the concentration of the template DNA Check the labeling efficiency Increase the amount of probe or decrease the amount of competitor DNA
No signal from the clone and the control does not produce a signal	Check the chromosome preparation protocol and the quality of the chromosome spreads Check the temperatures of the following: the chromosome denaturing solution, the hybridization incubation, and the wash solutions Lower the concentration of the PI counterstain
Low signal-to-noise ratio	Check the quality of the chromosome spreads Vary the amount of probe and/or competitor DNA Check the hybridization and wash temperatures
Rubber cement is hard to remove	Make sure the paper towels in the hybridization chamber are always soaked in enough water Dip the slides in wash solution to help remove the rubber cement
Chromosomes are not in focus	Check to see if there is more than one coverslip on the slide Remove the excess antifade by using a tissue or cotton swab to press gently on the coverslip

Detection of Nucleic Acids Immobilized on Membranes

Observation	Suggestion(s)
Weak signal	Low probe concentration due to insufficient template in the labeling reaction Repeat the labeling reaction using twice as much template, while keeping all the other reaction components constant (Do not forget to boil the probe prior to addition to the hybridization solution.)
Poor incorporation of the fluor-12-dUTP into the probe	Incorporation can be checked by dot blot analysis. If incorporation is inadequate, allow the labeling reaction to proceed for 30 minutes instead of for 20 minutes

Preparation of Media and Reagents

TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA	**20× SSC Buffer (per Liter)** 175.3 g of NaCl 88.2 g of sodium citrate 800 ml of water 10 N NaOH Adjust to pH 7.0 with a few drops of a 10 N NaOH Adjust volume to 1 liter with water
Hybridization Buffer 50% formamide 10% dextran sulfate 4× SSC buffer	

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Endnotes

Tween® is a registered trademark of ICI Americas, Inc.
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MSDS Information

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

STRATAGENE

An Agilent Technologies Division

Prime-It Fluor Fluorescence Labeling Kit

Catalog #300380

QUICK-REFERENCE PROTOCOL

- Add the following to a sterile microcentrifuge tube:

  50–100 ng of DNA
  0–28 μl of dH_2O
  <u>10 μl of random 9-mer primers</u>
  Total reaction volume is 39 μl

- Add the following to a sterile microcentrifuge tube:

  50 ng of control DNA template
  27 μl of dH_2O
  <u>10 μl of random 9-mer primers</u>
  Total reaction volume is 39 μl

- Mix and heat the reaction tubes to 95–100°C for 5 minutes
- Briefly centrifuge the reaction tubes and then place the tubes on ice.
- Add the following to each reaction tube:

  10 μl of the reaction buffer
  (prepared by mixing 8 μl of fluor-12-dUTP nucleotide and 92 μl of 5× nucleotide buffer)
  1 μl (5 U) of exonuclease-free Klenow

- Incubate the reaction tubes at 37°C for 20–30 minutes
- Add 2 μl of stop mix to each reaction tube
- Store the reaction tubes at 4°C in the dark
- The probe may be used unpurified for *in situ* hybridizations, but should be purified for detection of nucleic acids immobilized on membranes