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Just cDNA Double-Stranded cDNA Synthesis Kit

**MATERIALS PROVIDED**

<table>
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<tr>
<td><strong>First-strand reagents</strong></td>
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<tr>
<td>AccuScript reverse transcriptase</td>
<td>15 μl</td>
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<tr>
<td>RNase Block ribonuclease inhibitor (40 U/μl)</td>
<td>200 U</td>
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<tr>
<td>First- and second-strand dNTP mix (10 mM each dNTP)</td>
<td>55 μl</td>
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<tr>
<td>First-strand buffer (10×)</td>
<td>75 μl</td>
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<tr>
<td>Oligo(dT) primer (1.4 μg/μl)</td>
<td>14 μg</td>
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<tr>
<td>Random 9mer primers (0.75 μg/μl)</td>
<td>260 μg</td>
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<td>Test poly(A)+ RNA (0.2 μg/μl)</td>
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<td>DEPC-treated water</td>
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<td>Second-strand buffer (10×)</td>
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<tr>
<td><em>Escherichia coli</em> RNase H (1.5 U/μl)</td>
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<tr>
<td><em>Escherichia coli</em> DNA polymerase I (9.0 U/μl)</td>
<td>500 U</td>
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<tr>
<td>Sodium acetate (3 M)</td>
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<tr>
<td><strong>Blunting reagents</strong></td>
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<tr>
<td>Blunting dNTP mixture (2.5 mM dATP, dGTP, dTTP, and dCTP)</td>
<td>115 μl</td>
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<tr>
<td>Cloned Pfu DNA polymerase (2.5 U/μl)</td>
<td>25 U</td>
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**Caution**  *DO NOT* substitute the reagents listed above with reagents from any other kit. Component substitution may result in lower efficiency cDNA synthesis.

**STORAGE CONDITIONS**

All components: −20°C
**ADDITIONAL MATERIALS REQUIRED**

Certain reagents recommended in this instruction manual are potentially dangerous and present the following hazards: chemical (DEPC, phenol, chloroform, and sodium hydroxide), radioactive ($^{32}$P radioisotope), or physical (high-voltage electrophoresis systems). The researcher is advised to take proper precautions and care with these hazards and to follow the safety recommendations from each respective manufacturer.

**Reagents and Solutions**

Phenol-chloroform [1:1 (v/v)]

*Note*  *Do not use low-pH phenol; this phenol is acidic and may denature the DNA.*

Chloroform [100% (v/v)]
Ethanol [70% and 100% (v/v)]
Sterile distilled water (dH$_2$O)
$\alpha$-$^{32}$P-labeled deoxynucleotide (800 Ci/mmol; $[^{32}$P]dATP, $[^{32}$P]dGTP, $[^{32}$P]dCTP, or $[^{32}$P]dTTP)

**Equipment**

Ribonuclease (RNase)-free microcentrifuge tubes and pipet tips
Portable radiation monitor (Geiger counter)
Water baths (4°, 16°, 42°, and 65°C)
Microcentrifuge
Vacuum centrifuge
Vacuum evaporator
Incubator (37°C)

**NOTICE TO PURCHASER**

This product is for research purposes only and must be used in accordance with NIH guidelines for recombinant DNA.
**INTRODUCTION**

The Just cDNA double-stranded cDNA synthesis kit contains all of the reagents necessary to make double-stranded cDNA from poly(A)+ RNA, including random and oligo(dT) primers. Double-stranded DNA can be used to make non-directional cDNA libraries, to enrich differentially-expressed genes, and as a starting point for subtractive hybridization protocols. Additionally, double-stranded cDNA can be used for the in vitro transcription of the cDNA population.

**cDNA SYNTHESIS**

The yield and length and accuracy of cDNA transcripts are enhanced with the use of AccuScript reverse transcriptase (AccuScript RT) for first-strand synthesis. AccuScript RT is a novel Moloney murine leukemia virus reverse transcriptase (MMLV-RT) derivative combined with a proofreading 3’-5’ exonuclease. AccuScript RT delivers the highest reverse-transcription accuracy while promoting full length cDNA synthesis. AccuScript RT delivers greater than three-fold higher accuracy compared to leading reverse transcriptases, representing a significant advancement in cDNA synthesis accuracy. These advantages make AccuScript RT the enzyme of choice for applications involving the preparation of accurate, full-length, cDNA transcripts, including cDNA library construction.

First-strand cDNA synthesis begins when AccuScript RT, in the presence of nucleotides and buffer, finds a primer that has annealed to an mRNA template. When using oligo(dT) primers, the poly(dT) region binds to the 3’ poly(A) region of the mRNA template, and AccuScript RT begins to synthesize the first-strand cDNA. Alternatively, when using random primers, the primers bind to complementary sites on the mRNA template.

During second-strand synthesis, RNase H nicks the RNA bound to the first-strand cDNA to produce a multitude of fragments, which serve as primers for DNA polymerase I. DNA polymerase I "nick-translates" these RNA fragments into second-strand cDNA. The uneven termini of the double-stranded cDNA are then polished or filled in with cloned Pfu DNA polymerase, yielding blunt-ended double-stranded cDNA.
THE JUST cDNA SYNTHESIS PROTOCOL

**Notes**  
*DO NOT* substitute the reagents in this kit with reagents from any other kit. Component substitution may result in lower efficiency cDNA synthesis.

As a nonradioactive alternative for accessing the quality of first- and second-strand products, omit the use of radioactive $[\alpha^{-32}P]dNTP$ in the cDNA synthesis and second-strand reactions. After electrophoresis of the first- and second-strand products, stain the gel using the SYBR® Green nucleic acid gel stain reagent following the manufacturer’s protocols.

The following protocol is optimized for 0.5–5 $\mu$g of poly(A)$^+$ RNA.

**Protocol Guidelines**

- The quality and quantity of the mRNA used is of fundamental importance to the construction of high-quality, representative cDNA (see *Appendix I: Purifying and Quantifying RNA*). The Agilent RNA Isolation Kit uses the guanidine isothiocyanate (GITC)-phenol-chloroform extraction method,$^1$ which quickly produces large amounts of undegraded total RNA. Poly(A)$^+$ RNA can then be isolated using the Agilent Poly(A) Quik mRNA Isolation Kit. The protocol provided is optimized for 0.5–5 $\mu$g of poly(A)$^+$ RNA.

- To relax secondary structure, treatment with methylmercury hydroxide (CH$_3$HgOH) is recommended (see *Appendix II: Treating RNA with Methylmercury Hydroxide*). Alternatively, incubate the RNA at 70°C for 10 minutes, immediately followed by a 2 minute incubation on ice.

- It is imperative to protect the RNA from any contaminating RNases until the first-strand cDNA synthesis is complete. Wear fresh gloves, use newly autoclaved pipet tips, and avoid using pipet tips or microcentrifuge tubes that have been handled without gloves. Ribonuclease A cannot be destroyed by normal autoclaving alone. Baking or DEPC treatment is recommended.

- When removing aliquots of the enzymes used in the cDNA synthesis protocol, flick the bottom of the tube to thoroughly mix the enzyme solution. Do not vortex the enzyme stock tubes.
Control RNA

Perform controls for the first- and second-strand synthesis reactions using the test poly(A)+ RNA provided as template. Synthesizing and analyzing first- and second-strand cDNA from the test poly(A)+ RNA, when compared to the experimental sample(s), indicates that the protocols are being performed correctly and that the sample mRNA is of adequate quality.

When performing the control, simply substitute the sample poly(A)+ RNA with 25 μl of the provided test poly(A)+ RNA (5 μg) in the first-strand synthesis reaction, followed by performing second-strand synthesis. When primed with oligo(dT), the test cDNA first- and second-strand products will migrate as a band at approximately 1.8 kb. When primed with the random primer mix, expect a light smear from about 0–1.8 kb. In either case, the test poly(A)+ will show distinctly different intensity between the first and second strands (see Appendix III: Alkaline Agarose Gels). When using α-32P to access quality, the second strand will generally be only 1/10 to 1/20 the intensity of the first-strand band due to the relative ratio of α-32P to the amount of NTP in the first- or second-strand reaction. When accessing product quality with SYBR Green, the second-strand reaction products will generally be twice the intensity of the first-strand products.

Synthesizing First-Strand cDNA

1. Preheat a 42°C water bath.

2. Thaw the radioactive [α-32P]dNTP and all nonenzymatic first-strand components. Keep the radioactive dNTP on ice for use in step 4 and in the second-strand synthesis. Briefly vortex and spin down the contents of the nonenzymatic tubes. Place the tubes on ice.

   **Note**  AccuScript RT is temperature sensitive and should remain at –20°C until the last moment.

3. In an RNase-free microcentrifuge tube, add the following reagents in order:

   - 5 μl of 10× first-strand buffer
   - 2 μl of first- and second-strand dNTP mix
   - 2 μl of Oligo(dT) primer (1.4 μg/μl) or
   - 3.8 μl of random primer (0.75 μg/μl)
   - X μl of DEPC-treated water
   - 1 μl of RNase Block Ribonuclease Inhibitor (40 U/μl)

   Mix the reaction and then add X μl of poly(A)+ RNA (0.5–5 μg). For the control reaction, prepare the annealing reaction above with 25 μl (5 μg) of test RNA. The total volume of each reaction at this point should be 47 μl. Mix gently.
4. Allow the primer to anneal to the template for 10 minutes at room temperature. During the incubation, aliquot 0.5 μl of the [α-32P]dNTP (800 Ci/mmol) into separate tubes for each first-strand synthesis reaction, including the control reaction.

5. Add 3 μl of AccuScript RT to each first-strand synthesis reaction. The final volume of each first-strand synthesis reaction should now be 50 μl.

6. Mix the samples gently and spin down the contents in a microcentrifuge.

7. Transfer 5 μl of each first-strand synthesis reaction, including the control reaction, to separate tubes containing 0.5 μl of [α-32P]dNTP (800 Ci/mmol). These radioactive samples will be analyzed with the second-strand products.

8. Incubate the first-strand synthesis reactions, including the control reaction, at 42°C for one hour.

9. Prepare a 16°C water bath for second-strand synthesis. If a water bath with a cooling unit is not available, use a large Styrofoam® container with a lid. Fill the container three-quarters full with water and adjust the temperature to 16°C with ice. Cover the container with a lid.

10. After 1 hour, remove the first-strand synthesis reactions from the 42°C water bath. Place the nonradioactive first-strand synthesis reactions on ice. Store the radioactive first-strand synthesis reactions at −20°C until ready to resolve by electrophoresis on an alkaline agarose gel (see Appendix III: Alkaline Agarose Gels). We recommend electrophoresing each radioactive first-strand reaction alongside the second-strand reaction after blunting and resuspension of the second-strand reaction (see step 17 in Blunting the cDNA Termini).

Notes

If using SYBR Green to access quality, transfer 5 μl of each first-strand synthesis reaction to separate tubes and freeze for gel analysis with the second-strand reaction product.

In general, first-strand synthesis reaction products generated from small amounts of poly(A)+ starting material (i.e., 0.5 μg of poly(A)+) cannot be visualized on a gel.

Synthesizing Second-Strand cDNA

1. Thaw all nonenzymatic second-strand components. Briefly vortex and spin in a microcentrifuge before placing the tubes on ice.

Note

It is important that all reagents be <16°C when the DNA polymerase I is added.
2. Add the following components in order to each 45-μl nonradioactive, first-strand synthesis reaction on ice:

- 20 μl of 10× second-strand buffer
- 6 μl of first- and second-strand dNTP mix
- 114 μl of sterile distilled water
  (DEPC-treated water is not required)
- 2 μl of [α-32P]dNTP (800 Ci/mmol)

**Note**  If using SYBR Green to access quality, do not add [α-32P]dNTP; add two additional microliters of sterile water instead.

3. Add the following enzymes to the second-strand synthesis reactions:

- 2 μl of RNase H (1.5 U/μl)
- 11 μl of DNA polymerase I (9.0 U/μl)

4. Gently vortex the contents of the tubes, spin the reactions in a microcentrifuge, and incubate for 2.5 hours at 16°C. Check the water bath occasionally to ensure that the temperature does not rise above 16°C; temperatures above 16°C can cause the formation of hairpin structures.

5. After second-strand synthesis for 2.5 hours at 16°C, immediately place the reaction tubes on ice.

### Blunting the cDNA Termini

1. Add the following to the second-strand synthesis reactions:

- 23 μl of blunting dNTP mix
- 2 μl of cloned Pfu DNA polymerase (2.5 U/μl)

2. Quickly vortex the reactions and spin in a microcentrifuge. Incubate the reactions at 72°C for 30 minutes. **Do not exceed 30 minutes!**

**Notes**  If combining two cDNA synthesis reactions that have been prepared using different primer types, combine the reactions before proceeding to the next step. Before combining the products, assess the quality of the first- and second-strand products of each synthesis reaction (see Appendix III: Alkaline Agarose Gels).

*If combining two second-strand cDNA synthesis reactions, all reagent volumes used in the purification steps ahead will need to be doubled.*
3. Thaw the 3 M sodium acetate.

**Note**  Since radioactivity can leak out between the lid and body of some microcentrifuge tubes during the vortexing and precipitation steps, wrap a small piece of Parafilm laboratory film around the rim of the microcentrifuge tube to prevent leakage.

4. Remove the reactions from incubation at 72°C and quickly spin the reactions in a microcentrifuge. Then add 200 μl of phenol–chloroform [1:1 (v/v)] and vortex the mixtures.

**Note**  Do not use low-pH phenol; this phenol is acidic and may denature the DNA. The phenol must be equilibrated to pH 7–8.

5. Spin the reactions in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer each upper aqueous layer, containing the cDNA, to new tubes. Be careful to avoid removing any interface that may be present.

6. Add an equal volume of chloroform and vortex the mixture.

7. Spin the reactions in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer each upper aqueous layer, containing the cDNA, to new tubes.

8. Precipitate the cDNA by adding the following to each saved aqueous layer:

   - 20 μl of 3 M sodium acetate
   - 400 μl of 100% (v/v) ethanol

   Vortex the reactions.

9. Precipitate overnight at –20°C.

10. In order to orient the direction of precipitate accumulation, place a mark on the microcentrifuge tube or point the tube hinge away from the center of the microcentrifuge as an indicator of where the pellet will form.

11. Spin in a microcentrifuge at maximum speed for 60 minutes at 4°C.

12. Avoid disturbing the pellet in each tube and carefully remove and discard the radioactive supernatants in a radioactive waste container.

**Note**  The conditions of synthesis and precipitation produce a large white pellet. The pellet accumulates near the bottom of the microcentrifuge tube and may taper up along the marked side of the tube.
13. Gently wash each pellet by adding 500 μl of 70% (v/v) ethanol to the side of each tube away from the precipitate. **Do not mix or vortex!**

14. Spin in a microcentrifuge at maximum speed for 2 minutes at room temperature with the orientation marked as in step 10.

15. Aspirate the ethanol wash and dry each pellet by vacuum centrifugation.

16. Resuspend each pellet in 9 μl of TE buffer (see *Preparation of Media and Reagents*) and incubate at 4°C for at least 30 minutes to allow the cDNA to resuspend. To ensure that the cDNA is completely in solution, transfer the cDNA to fresh microcentrifuge tubes. Monitor the now empty tubes with a handheld Geiger counter. If the cDNA is in solution, few counts should remain in the empty tubes.

17. **Transfer 1 μl of the sample and control second-strand synthesis reactions to separate tubes.** Run the sample and control first- and second-strand synthesis reactions on an alkaline agarose gel to determine the size range of the cDNA and the presence of any secondary structure (see *Appendix III: Alkaline Agarose Gels*).

**Notes**  
*In general, first- and second-strand synthesis reaction products generated from small amounts of poly(A)+ starting material (i.e., 0.5 μg of poly(A)+) cannot be visualized on a gel.*

*Second-strand synthesis reactions can be stored overnight at −20°C.*

18. We recommend quantitating the cDNA using the PicoGreen® double-stranded DNA quantitation reagent or by performing an ethidium bromide plate assay (see *Appendix IV: Ethidium Bromide Plate Assay*).
**APPENDIX I: PURIFYING AND QUANTIFYING RNA**

**Purifying RNA**

Use the RNA Isolation Kit or the GITC–phenol–chloroform extraction method to isolate total RNA. This method is rapid, yet it produces large amounts of high-quality, undegraded RNA.

Although AccuScript RT is not inhibited by ribosomal RNA (rRNA) and transfer RNA (tRNA) contamination, it is advisable to select the poly(A)$^+$ fraction. The amounts of rRNA and tRNA vastly outnumber the mRNA and will decrease the efficiency of the system. Poly(A)$^+$ RNA is selected on oligo(dT) cellulose columns. Some protocols call for the addition of SDS in the purification steps. Sodium dodecyl sulfate is a powerful enzyme inhibitor and helps prevent degradation of the RNA by RNases, but its presence can also inhibit the enzymes required for cDNA synthesis. If the mRNA intended for use with this kit is suspended in an SDS solution, the RNA must be phenol extracted and ethanol precipitated.

Ribonucleases A and T1 are widely used in almost all molecular biology labs and are nearly indestructible. Ribonucleases are produced by microbes and have also been found in the oils of the skin. Make an effort to use tubes and micropipet tips which have been handled only with gloves. Use freshly autoclaved and baked tips and tubes. Usually these precautions are sufficient, but to be absolutely certain that microcentrifuge tubes and other components intended for use with RNA are not contaminated, the components can be treated with DEPC. Diethylpyrocarbonate is extremely toxic and should be handled with care. Submerge the microcentrifuge tubes in a 0.1% (v/v) DEPC-treated water solution. Leave the beaker of submerged tubes in a fume hood overnight and then dispose of the DEPC-treated water. Autoclave the microcentrifuge tubes for at least 30 minutes. Even though the tubes may still have a sweet DEPC odor, the DEPC is completely inactivated by this procedure. Place the tubes in a drying oven overnight. Equipment which cannot be treated by DEPC can be rinsed in a freshly mixed 3% (v/v) hydrogen peroxide solution, followed by a methanol rinse. Remember, once the RNA is converted to first-strand cDNA, RNases are no longer a concern. Caution should still be exercised in maintaining a sterile, DNase-free environment.

**Quantifying RNA**

RNA can be quantified by measuring the optical density of a dilute RNA solution. The conversion factor for RNA at the wavelength of 260 nm is 40 μg/ml/OD unit as shown in the example below.

Two microliters of a poly(A)$^+$ RNA sample is added to 498 μl of water (e.g., OD$_{260}$ = 0.1). Therefore,

\[
0.1\text{OD unit} \times \left( \frac{500}{2} \text{ dilution factor} \right) \times 40\mu\text{g of RNA/ml} = 1000\mu\text{g of RNA/ml or } 1\mu\text{g of RNA/μl}
\]
If a sample has significant rRNA contamination, the actual amount of mRNA available for cDNA conversion will be overestimated by this procedure.

Secondary structure may be a problem with certain RNAs, particularly plant and tumor mRNAs. These samples can be treated with methylmercury hydroxide (see Appendix II: Treating with Methylmercury Hydroxide). This chemical is extremely toxic and should be used with caution in a fume hood. Alternatively, incubate the RNA at 70°C for 10 minutes, immediately followed by a 2 minute incubation on ice. However, if the RNA contains even a minute amount of RNase, RNase activity will increase by several orders of magnitude with the increased temperature and significantly degrade the RNA.

Formaldehyde RNA Gel Protocol

Additional Reagents Required

10× MOPS buffer
37% formaldehyde solution
Agarose
Formaldehyde gel loading buffer
Size standards
RNA size standards work best
DNA size standards can be used to approximate sizes
If rRNA is present, its intact bands can indicate size and intactness of the sample

Agarose gels [1% (w/v)] usually work well for mixed populations of RNA. If a smaller population of RNA is anticipated, 1.5% (w/v) agarose gels are recommended.

The following procedure is for minigels:

1. Melt 1 g of agarose in a solution made with the following:

   10 ml of 10× MOPS buffer
   85 ml of sterile water

2. Allow the melted agarose solution to cool to ~50°C.

3. In a fume hood or in a well-ventilated area, add 5.4 ml of 37% (v/v) formaldehyde.

§ See Preparation of Media and Reagents.
4. Mix the agarose solution by swirling and pour the solution into the gel mold. While the gel is solidifying, dry the RNA samples >2 μl in volume in a vacuum evaporator. Dilute an appropriate amount of 10× MOPS buffer to 1× to be used as running buffer. When the gel is submerged in 1× MOPS running buffer and everything is completely ready, resuspend the dry samples (or RNA in a volume of <2 μl) in 10 μl of formaldehyde gel loading buffer. Heat the sample for 5–10 minutes at 65°C and then load the gel.

5. Run the gel at 5 V/cm. The ethidium bromide (EtBr) will migrate to the negative electrode and the bromophenol blue (BPB) will travel to the positive electrode.

Usually when BPB has run half the distance of the gel, the RNA has migrated sufficiently to allow the examination of the size distribution relative to the standards. Examine the gel under ultraviolet (UV) illumination. Typical eukaryotic RNA has a majority of its size distribution between 400 and 2000 bases.

Northern blots can easily be produced by soaking the gels in two changes of 10× SSC buffer (see Preparation of Media and Reagents) for 20 minutes each time to remove the formaldehyde from the gel and then by blotting the gel either conventionally or by pressure blotting with the PosiBlot 30–30 pressure blotter and Pressure Control Station. The RNA is permanently bound to the nitrocellulose or nylon membrane by conventional baking in an 80°C vacuum oven for 2 hours or by crosslinking for 30 seconds in a Stratalinker UV crosslinker. The blot is probed using conventional methods.
APPENDIX II: TREATING RNA WITH METHYLMERCURY HYDROXIDE

**Warning**  Methylmercury hydroxide is an extremely toxic chemical. Wear gloves and use with caution in a fume hood.

1. Resuspend the mRNA in 20 μl of DEPC-treated water.
2. Incubate at 65°C for 5 minutes.
3. Cool to room temperature.
4. Add 2 μl of 100 mM CH₃HgOH.
5. Incubate at room temperature for 1 minute.
6. Add 4 μl of 700 mM β-mercaptoethanol (see *Preparation of Media and Reagents*).
7. Incubate at room temperature for 5 minutes.
APPENDIX III: ALKALINE AGAROSE GELS

Alkaline agarose gels cause DNA to denature and can be used to identify the presence of a secondary structure called hairpinning. Hairpinning can occur in either the first- or second-strand reactions when the newly polymerized strand "snaps back" on itself and forms an antiparallel double helix.

Denaturing gels such as alkaline agarose gels can reveal this secondary structure and can demonstrate the size range of the first- and second-strand cDNA.

Note The test cDNA sample will migrate as a band at approximately 1.8 kb and will show distinctly different intensity between the first and second strands. When using α-32P to access quality, the second strand will generally be only 1/10 to 1/20 the intensity of the first-strand band due to the relative ratio of α-32P to the amount of NTP in the first- or second-strand reaction. When accessing product quality with SYBR Green, the second-strand reaction products will generally be twice the intensity of the first-strand products.

Alkaline agarose gels differ from conventional gels in the following ways:

1. The absence of any buffering capacity in the "buffer" reduces the speed at which the sample can be run.

2. The thickness of the typical undried agarose gel causes the radioactive emissions to be scattered to a degree which makes a clear autoradiograph difficult to interpret.

The following alternative methods help avoid these complications.

The Slide Technique

The easiest and least expensive method is to use a 5- × 7.5-cm glass slide, position a minigel comb over it with high tension clips and add 10 ml of molten alkaline agarose near the upper center of the slide. The surface tension of the solution will prevent overflow and produce a small, thin gel which can be exposed without further drying. Do not allow the teeth of the comb to overlap the edge of the plate or the surface tension may be broken. To improve the resolution, pat the gel dry with several changes of Whatman® 3MM paper after electrophoresis is complete.

To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.
The Vertical Alkaline Agarose Technique

Vertical alkaline agarose gels can be produced using a vertical gel apparatus with 1.5-mm spacers. Since the alkaline agarose gels do not have sufficient friction to remain bound to ordinary glass, a frosted glass plate or gel bond must be used with the vertical apparatus. The combs normally used for acrylamide can be used with this apparatus, if the outside teeth are wrapped in tape to prevent the comb from sinking more than 1.2 cm into the agarose. The 55°C agarose will solidify almost immediately on contact with the cold glass plates, so it is essential to load the mold rapidly with a 60-ml syringe. The comb should already be in the mold, and if it is necessary to reposition the comb, do it immediately after the gel is poured. In order to reduce the possibility of destroying the wells when pulling out the comb, place the solidified gel in a -20°C freezer for 5 minutes immediately prior to removing the comb. When pulling out the comb, it is essential to avoid a vacuum between the teeth and the well. Vacuum can be detected when the well distorts from its normal square shape. When a vacuum occurs, push the comb to separate the glass plates and break the vacuum. After the samples have been run and the glass plates are ready to be opened, slide the unfrosted glass plate off of the alkaline agarose gel instead of prying the plate away from the gel. Pat the gel dry several times using several pieces of Whatman 3MM paper.

**Note**  To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.
Conventional Submerged Gels

These gels will require drying either by blotting or through the use of a gel dryer.

**Caution**  
Even when multiple layers of absorbent paper are placed under the gel, free nucleotides can easily contaminate the drying apparatus. These gels should be poured as thin as possible and should be dried without heat, if time permits, and should never be dried above 40°C.

Protocol

Additional Reagents Required

- Agarose
- Alkaline buffer (10×)§
- Alkaline agarose 2× loading buffer§

The following formula makes 80 ml of 1% (w/v) alkaline agarose for cDNAs in the 1- to 3-kb size range.

Melt 0.8 g of agarose in 72 ml of water. Allow the agarose to cool to 55°C. During this time, assemble the gel apparatus. Add 8 ml of 10× alkaline buffer to the cooled agarose, swirl to mix, and pour the agarose immediately. If buffer is added before the correct temperature is reached, the agarose may not solidify.

Load the sample in an equal volume of alkaline agarose 2× loading buffer. Run the gel with 1× alkaline buffer at 100 mA and monitor the system for heat. If the apparatus becomes warmer than 37°C, the amperage should be reduced. The migration of the BPB in alkaline agarose is similar to the migration in regular agarose and should be run to at least one-half or three-quarters distance of the gel.

**Note**  
The alkali condition causes the blue dye to fade.

§ See Preparation of Media and Reagents.
APPENDIX IV: ETHIDIUM BROMIDE PLATE ASSAY

An accurate quantitation of DNA can be obtained by UV visualization of samples spotted on EtBr agarose plates. DNA samples of known concentration are prepared for use as comparative standards in this assay.

Preparing the Ethidium Bromide Plates

Note  Prepare the EtBr plates under a fume hood.

Prepare 100 ml of 0.8% (w/v) agarose using Tris-acetate (TAE) buffer (see Preparation of Media and Reagents). Cool the molten agarose to 50°C and then add 10 μl of EtBr stock solution (10 mg/ml). The EtBr stock solution is prepared in dH₂O and is stored in the dark at 4°C. Swirl to mix the EtBr stock solution and pour the solution into 100-mm petri dishes using ~10 ml/plate. Allow the plates to harden and incubate the plates at 37°C to dry, if necessary. These plates may be stored in the dark at 4°C for up to 1 month.

Preparing the Standards

Using a DNA sample of known concentration, make seven serial dilutions in 100 mM EDTA to cover the range from 200 to 10 ng/μl. These standards may be stored at –20°C for 3 months.

Plate Assay for Determination of DNA Concentration

Using a marker, label the petri dish to indicate where the sample and the standards (200, 150, 100, 75, 50, 25, and 10 ng/μl) will be spotted.

Thaw the standards and carefully spot 0.5 μl of each standard onto the surface of a prepared EtBr plate. Be careful not to dig into the surface of the plate. Let capillary action pull the small volume from the pipet tip to the plate surface and do not allow a bubble to form. Change pipet tips between each standard.

After spotting all of the standards, immediately spot 0.5 μl of the cDNA sample onto the plate adjacent to the line of standards. Allow all spots to absorb into the plate for 10–15 minutes at room temperature. Remove the lid and photograph the plate using a UV lightbox. Compare the spotted sample of unknown concentration with the standards.

Do not reuse the plates. Standards and unknowns must be spotted within 10 minutes of each other.
## Troubleshooting

<table>
<thead>
<tr>
<th>Observations</th>
<th>Suggestions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor first-strand synthesis</td>
<td>Ensure the reaction components are mixed and soluble. Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible.</td>
</tr>
<tr>
<td></td>
<td>Minute amounts of SDS or lithium in the RNA will inhibit the first-strand synthesis reaction. Do not use these in the RNA preparations. Multiple phenol–chloroform extractions will sometimes remove the inhibitors.</td>
</tr>
<tr>
<td></td>
<td>The reaction may not contain sufficient mRNA. Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation.</td>
</tr>
<tr>
<td></td>
<td>The reaction may contain degraded ( [\alpha-^{32}P]dNTP ). Protect the ( [\alpha-^{32}P]dNTP ) from heat and leave at room temperature for the minimum time required. In addition, ensure that the ( [\alpha-^{32}P]dNTP ) is not contaminated with unlabeled dNTPs, causing poor label incorporation into cDNA, and falsely indicating poor cDNA synthesis.</td>
</tr>
<tr>
<td>Poor second-strand synthesis</td>
<td>Confirm the interpretation of the gel results. Control RNA will show distinctly different intensity between the first and second strand. This is due to the relative amounts of ( \alpha-^{32}P ) to the amount of NTP in the first- or second-strand reaction. Normally, the second strand will have only 1/10 to 1/20 the intensity of the first-strand band.</td>
</tr>
<tr>
<td></td>
<td>Ensure the reaction components are mixed and soluble. Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible.</td>
</tr>
<tr>
<td></td>
<td>Poor first strand synthesis will affect second-strand synthesis. See the previous suggestions for Poor first-strand synthesis.</td>
</tr>
<tr>
<td>No first-strand synthesis, but good second-strand synthesis</td>
<td>Ensure that there is no DNA contamination within the RNA preparation.</td>
</tr>
<tr>
<td>Hairpinning</td>
<td>This can result when incubation temperatures are higher than 16°C. Add second-strand synthesis reaction components to the first-strand reaction mix on ice and then transfer the reaction mixture directly to 16°C for incubation. After incubation, place the samples on ice immediately.</td>
</tr>
<tr>
<td></td>
<td>The reaction may not contain sufficient mRNA. Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation.</td>
</tr>
<tr>
<td></td>
<td>The RNA may have to be treated with methylmercury hydroxide to relax the secondary structure present in some sources of RNA (see Appendix II: Treating with Methylmercury Hydroxide).</td>
</tr>
<tr>
<td></td>
<td>Ensure that the appropriate amount of DNA polymerase is used. Use a calibrated pipet to measure the enzyme. Do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.</td>
</tr>
</tbody>
</table>
## PREPARATION OF MEDIA AND REAGENTS

<table>
<thead>
<tr>
<th><strong>Alkaline Agarose 2× Loading Buffer</strong></th>
<th><strong>10× Alkaline Buffer (per 50 ml)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>200 μl of glycerol</td>
<td>3 ml of 5.0 M NaOH</td>
</tr>
<tr>
<td>750 μl of water</td>
<td>2 ml of 0.5 M EDTA</td>
</tr>
<tr>
<td>46 μl of saturated BPB</td>
<td>45 ml of deionized H₂O</td>
</tr>
<tr>
<td>5 μl of 5 M NaOH</td>
<td></td>
</tr>
</tbody>
</table>

### 10× Alkaline Buffer

<table>
<thead>
<tr>
<th><strong>TE Buffer</strong></th>
<th><strong>1× TAE Buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris-HCl (pH 7.5)</td>
<td>40 mM Tris-acetate</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>1 mM EDTA</td>
</tr>
</tbody>
</table>

### 700 mM β-Mercaptoethanol

<table>
<thead>
<tr>
<th><strong>Formaldehyde Gel Loading Buffer</strong></th>
<th><strong>10× MOPS Buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μl of 14 M β-mercaptoethanol</td>
<td>200 mM 3-([N\text{-morpholino}])propane-sulfonic acid (MOPS)</td>
</tr>
<tr>
<td>95 μl of DEPC-treated water</td>
<td>50 mM sodium acetate</td>
</tr>
<tr>
<td></td>
<td>10 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>Adjust to a final pH of 6.5–7.0 with NaOH</td>
</tr>
<tr>
<td></td>
<td>Do not autoclave</td>
</tr>
</tbody>
</table>

### 1× TAE Buffer

<table>
<thead>
<tr>
<th><strong>10× MOPS Buffer</strong></th>
<th><strong>20× SSC Buffer (per Liter)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mM 3-([N\text{-morpholino}])propane-sulfonic acid (MOPS)</td>
<td>175.3 g of NaCl</td>
</tr>
<tr>
<td>50 mM sodium acetate</td>
<td>88.2 g of sodium citrate</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>800.0 ml of deionized H₂O</td>
</tr>
<tr>
<td>Adjust to a final pH of 6.5–7.0 with NaOH</td>
<td>Adjust to pH 7.0 with a few drops of 10 N NaOH</td>
</tr>
<tr>
<td>Do not autoclave</td>
<td>Add deionized H₂O to a final volume of 1 liter</td>
</tr>
</tbody>
</table>

**Note** The formaldehyde gel loading buffer is not stable and should be made fresh on the day of use.

\[\text{To make saturated BPB, add a small amount of bromophenol blue crystals to water and vortex. Centrifuge the sample briefly and look for the presence of an orange pellet. If a pellet is seen, the solution is saturated. If not, add more crystals and repeat the procedure.}\]

### REFERENCES

**ENDNOTES**

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Styrofoam® is a registered trademark of Dow Chemical Co.  
Whatman® is a registered trademark of Whatman Ltd.

**MSDS INFORMATION**

Material Safety Data Sheets (MSDSs) are provided online at http://www.genomics.agilent.com. MSDS documents are not included with product shipments.
Just cDNA Double-Stranded cDNA Synthesis Kit
Catalog #200453

QUICK-REFERENCE PROTOCOL

Notes As a nonradioactive alternative for accessing the quality of first- and second-strand products, omit the use of radioactive \( \alpha^{32P} \)dNTP. After electrophoresis of the first- and second-strand products, stain the gel using the SYBR Green nucleic acid gel stain following the manufacturer’s protocols.

The following protocol is optimized for 0.5–5 µg of poly(A)+ RNA.

Synthesizing First-Strand cDNA

♦ Add the following components in order to an RNase-free microcentrifuge tube while mixing gently:

- 5 µl of 10× first-strand buffer
- 2 µl of first- and second-strand dNTP mix
- 2 µl of Oligo(dT) primer (1.4 µg/µl) or 3.8 µl of random primer (0.75 µg/µl)
- X µl of DEPC-treated water
- 1 µl of RNase Block Ribonuclease Inhibitor (40 U/µl)

♦ Mix the reaction and then add X µl of poly(A)+ RNA (0.5–5 µg). For the control reaction, prepare the reaction above with 25 µl (5 µg) of the test RNA provided. The total volume of each reaction at this point should be 47 µl. Mix gently. Incubate for 10 minutes at room temperature.

♦ Add 3 µl of AccuScript RT to each reaction. The final volume should now be 50 µl. Mix the samples gently and spin down the contents in a microcentrifuge.

♦ If using \( \alpha^{32P} \)dNTP to access reaction product quality, transfer 5 µl of each first-strand reaction, including the control reaction, to separate tubes containing 0.5 µl of \( \alpha^{32P} \)dNTP (800 Ci/mmol).

♦ Incubate all first-strand reactions at 42°C for 1 hour.

♦ After 1 hour, remove the reactions from the 42°C water bath. Place the nonradioactive first-strand synthesis reactions on ice. Store the radioactive first-strand synthesis reactions at –20°C until ready to resolve by electrophoresis on an alkaline agarose gel. If using SYBR Green to access quality, transfer 5 µl of each reaction to separate tubes and freeze at –20°C until ready for gel analysis.

Synthesizing Second-Strand cDNA

♦ Add the following components in order to each 45-µl nonradioactive first-strand synthesis reaction on ice. It is important that all reagents be <16°C.

- 20 µl of 10× second-strand buffer
- 6 µl of first- and second-strand dNTP mix
- 114 µl of sterile distilled water (DEPC-treated water is not required)
- 2 µl of \( \alpha^{32P} \)dNTP (800 Ci/mmol)
- 2 µl of RNase H (1.5 U/µl)
- 11 µl of DNA polymerase I (9.0 U/µl)

Note If using SYBR Green to access quality, add two additional microliters of sterile water in place of \( \alpha^{32P} \)dNTP.

♦ Gently vortex the contents of the tubes, spin the reactions in a microcentrifuge, and incubate for 2.5 hours at 16°C. Ensure that the temperature does not rise above 16°C.

♦ After the incubation, immediately place the reaction tubes on ice.
Blunting the cDNA Termini

♦ Add the following to the second-strand synthesis reactions:
  - 23 µl of blunting dNTP mix
  - 2 µl of cloned *Pfu* DNA polymerase (2.5 U/µl)

♦ Quickly vortex the reactions and spin in a microcentrifuge. Incubate the reactions at 72°C for 30 minutes. Do not exceed 30 minutes!!

  **Note** If combining two cDNA synthesis reactions that have been prepared using different primer types, combine the reactions before proceeding to the next step. All reagent volumes used in the purification steps ahead will need to be doubled.

♦ After incubation at 72°C, quickly spin in a microcentrifuge. Add 200 µl of phenol–chloroform [1:1 (v/v)] to each tube and vortex the mixtures.

  **Note** Do not use low-pH phenol; this phenol is acidic and may denature the DNA. The phenol must be equilibrated to pH 7–8.

♦ Spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Carefully transfer each upper aqueous layer, containing the cDNA, to new tubes. Add an equal volume of chloroform to each tube and vortex the mixtures.

♦ Spin at maximum speed for 2 minutes at room temperature and transfer each upper aqueous layer, containing the cDNA, to new tubes.

♦ Precipitate the cDNA by adding the following to each saved aqueous layer:
  - 20 µl of 3 M sodium acetate (thawed)
  - 400 µl of 100% (v/v) ethanol

♦ Vortex the reactions. Precipitate overnight at –20°C.

♦ Spin in a microcentrifuge at maximum speed for 60 minutes at 4°C. Carefully remove and discard each supernatant.

♦ Gently wash each pellet by adding 500 µl of 70% (v/v) ethanol. **Do not mix or vortex!** Spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Aspirate the ethanol wash and dry each pellet by vacuum centrifugation.

♦ Resuspend each pellet in 9 µl of TE buffer and incubate at 4°C for at least 30 minutes to allow the cDNA to resuspend.

♦ Run the sample and control first- and second-strand synthesis reactions on an alkaline agarose gel. It is important to determine the size range of the cDNA and the presence of any secondary structure (see Appendix III: Alkaline Agarose Gels).

  **Note** Second-strand synthesis reactions can be stored overnight at –20°C.

♦ Quantitate the cDNA using the PicoGreen double-stranded DNA quantitation reagent or by performing an ethidium bromide plate assay (see Appendix IV: Ethidium Bromide Plate Assay).