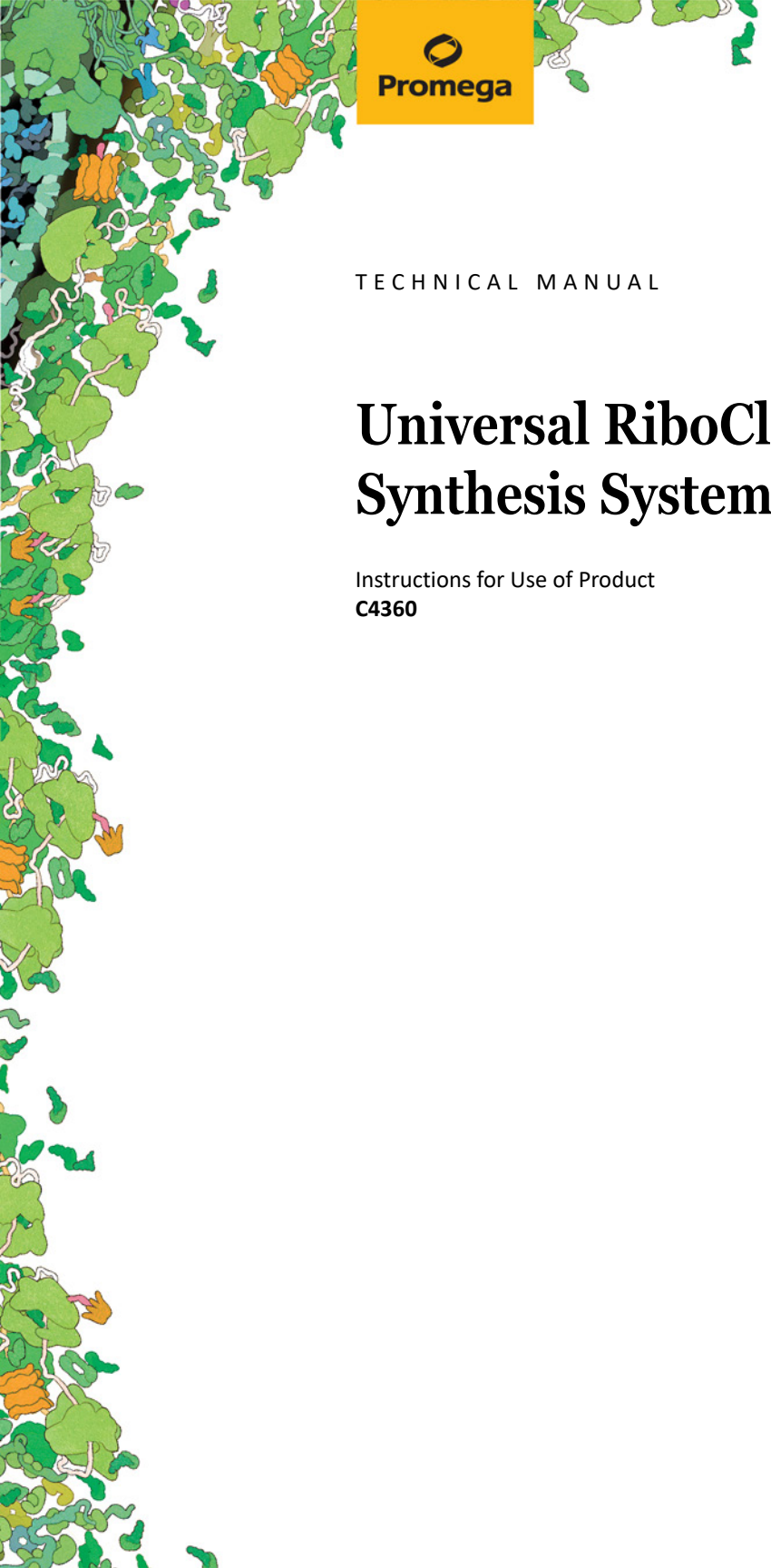

TECHNICAL MANUAL

Universal RiboClone™ cDNA Synthesis System

Instructions for Use of Product
C4360

Revised 4/21
TM038

Universal RiboClone™ cDNA Synthesis System

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Universal RiboClone™ cDNA Synthesis System contains the reagents required for the synthesis of double-stranded cDNA from mRNA and subsequent ligation into a suitable vector. The system is based on the method described by Okayama and Berg (1) with modifications by Gubler and Hoffman (2). First-strand synthesis is driven by AMV (Avian Myeloblastosis Virus) Reverse Transcriptase and either Random Hexameric Primers or an Oligo(dT) Primer, followed directly by second-strand replacement synthesis using RNase H and DNA Polymerase I. After treatment with T4 DNA Polymerase to flush the ends, the double-stranded cDNA molecules are prepared for cloning by size fractionation and the addition of EcoRI Adaptors. The resulting cDNA preparation can then be cloned into a suitable vector.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Universal RiboClone™cDNA Synthesis System	**1 each**	**C4360**

For Research Use Only. Not for Use in Diagnostic Procedures. Each system contains sufficient reagents to process up to 40µg mRNA and to perform 10 adaptor ligation reactions. C4360 consists of three systems. Includes:

Cat.# C4361 cDNA Synthesis Reagents

- 200µl First Strand 5X Buffer
- 20µg Oligo(dT)15 Primer
- 20µg Random Hexameric Primers
- 50µl Sodium Pyrophosphate, 40mM
- 1,000u Recombinant RNasin® Ribonuclease Inhibitor
- 600u AMV Reverse Transcriptase (HC)
- 10µg 1.2kb Kanamycin Positive Control RNA
- 800µl Second Strand 2.5X Buffer
- 20u RNase H
- 500u DNA Polymerase I
- 100u T4 DNA Polymerase
- 1.25ml Nuclease-Free Water

Cat.# C4362 EcoRI Adaptor Ligation Reagents

- 0.5ml T4 DNA Ligase 10X Buffer
- 100u T4 DNA Ligase
- 400µl Acetylated BSA, 1mg/ml
- 100u T4 Polynucleotide Kinase
- 0.5ml Kinase 10X Buffer
- 50µl ATP, 10mM
- 150pmol EcoRI Adaptors

Cat.# C4363 Resin and Spin Columns

- 10 Spin Columns
- 10ml Sephacryl® S-400

Note: Cat.# C4362 contains the BSA needed for second-strand synthesis.

Storage Conditions: Store the Positive Control RNA at <–65°C. Store the Sephacryl® S-400 Resin at +2°C to +10°C and Spin Columns at room temperature, +15°C to +30°C. Store all other components at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the T4 DNA Ligase 10X Buffer.

Promega

3. General Considerations

3.A. Methods of cDNA Synthesis

One of the most challenging tasks in molecular biology is the synthesis and cloning of cDNA. A complex series of enzymatic steps is involved in copying mRNA into double-stranded cDNA and subsequently preparing the termini for vector ligation. Many approaches have been used to generate cDNA libraries, and these have tried to preserve as much of the original sequence as possible, to improve cloning efficiency and to facilitate screening and subsequent analysis (3).

Most cDNA molecules produced will lack a few nucleotides corresponding to the 5´end of the mRNA because second-strand replacement only proceeds from 3´-OH RNA primers. These primers are randomly generated by the addition of RNase H and the endogenous RNase H activity of AMV Reverse Transcriptase. The most 5´nick in the mRNA will generally occur several nucleotides from the end, and the remaining RNA oligonucleotide may be too short to remain hybridized. Thus, the 3´→5´exonuclease activity of DNA Polymerase I will remove the last few nucleotides of the cDNA first strand.

However, since all eukaryotic mRNA molecules appear to have 5´-noncoding leader sequences, which commonly range from 40–80 nucleotides (4), it is likely that the vast majority of double-stranded cDNA will contain all of the coding sequences present in the initial cellular mRNA molecules.

If retaining these nucleotides is necessary, alternative procedures involving tailing after first-strand synthesis can be used (3,5). Homopolymeric tailing is best accomplished by adding dG residues (6), and the resulting clones will contain a long stretch (about 20bp) of dG/dC residues. These regions may interfere with screening using GC-rich oligomers, with dideoxy sequencing of clones, and possibly with their expression as part of a fusion protein (6). In addition, these regions can interfere with differential hybridization procedures in the construction of subtraction libraries (7).

3.B. Choice of Primers

The classical method of cDNA synthesis uses the Oligo(dT) Primer to prime first-strand synthesis (Figure 1). This method is suitable in most cases where poly(A)+ RNA of high quality can be prepared from the cell line or tissue of interest. Random Hexameric Primers (hexadeoxyribonucleotides) provide an alternative procedure (Figure 2) by which first-strand cDNA synthesis is initiated from internal sites within the mRNA molecule (8). Random Primers can be used to prime cDNA synthesis from mRNA molecules that do not possess a poly(A)+ tail or for RNA isolated from prokaryotic sources. Random Primers also provide a scheme by which cDNA can be synthesized representing mRNA with strong 5´secondary structure.

3.C. cDNA Cloning

Adaptors eliminate the EcoRI methylation and restriction enzyme steps necessary with conventional linker addition. The adaptor, a duplex DNA molecule, consists of one blunt end (for ligation to the cDNA) and an EcoRI sticky end. This feature of the Universal RiboClone™ System allows cDNA to be cloned into unique EcoRI sites of vectors (Figure 3).

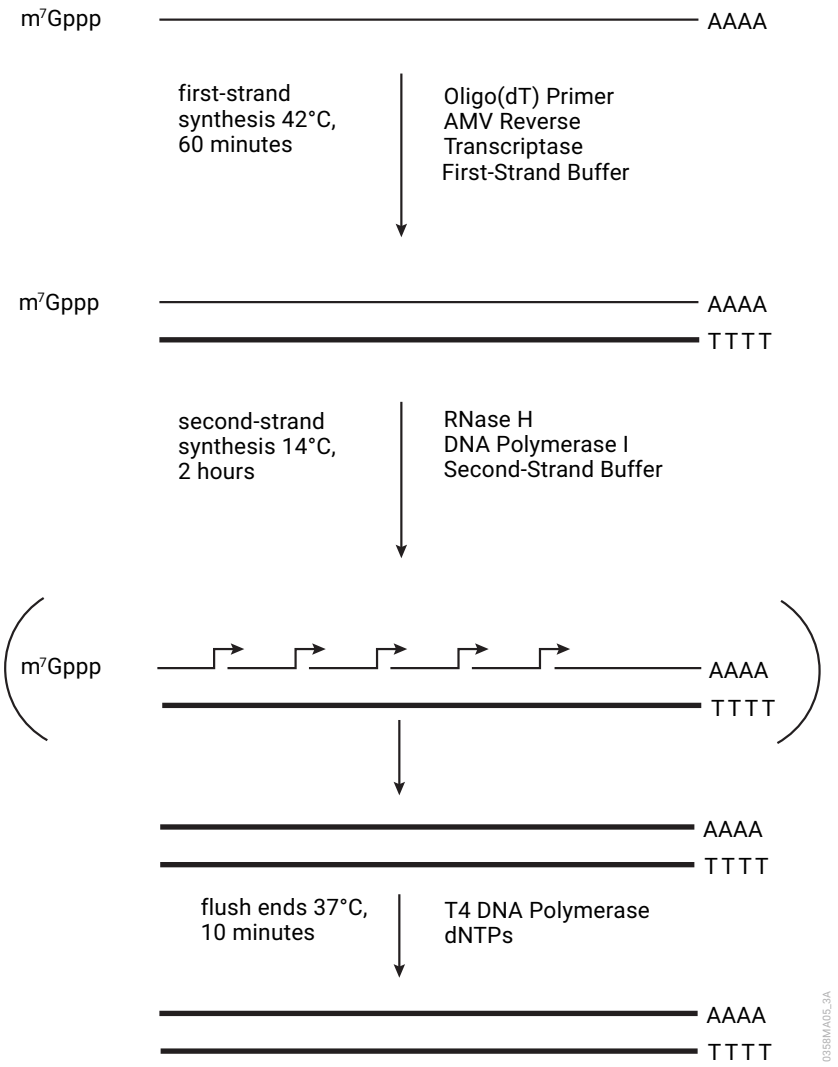

Figure 1. cDNA synthesis using the Universal RiboClone™ cDNA Synthesis System and Oligo(dT) Primer.

3.C. cDNA Cloning (continued)

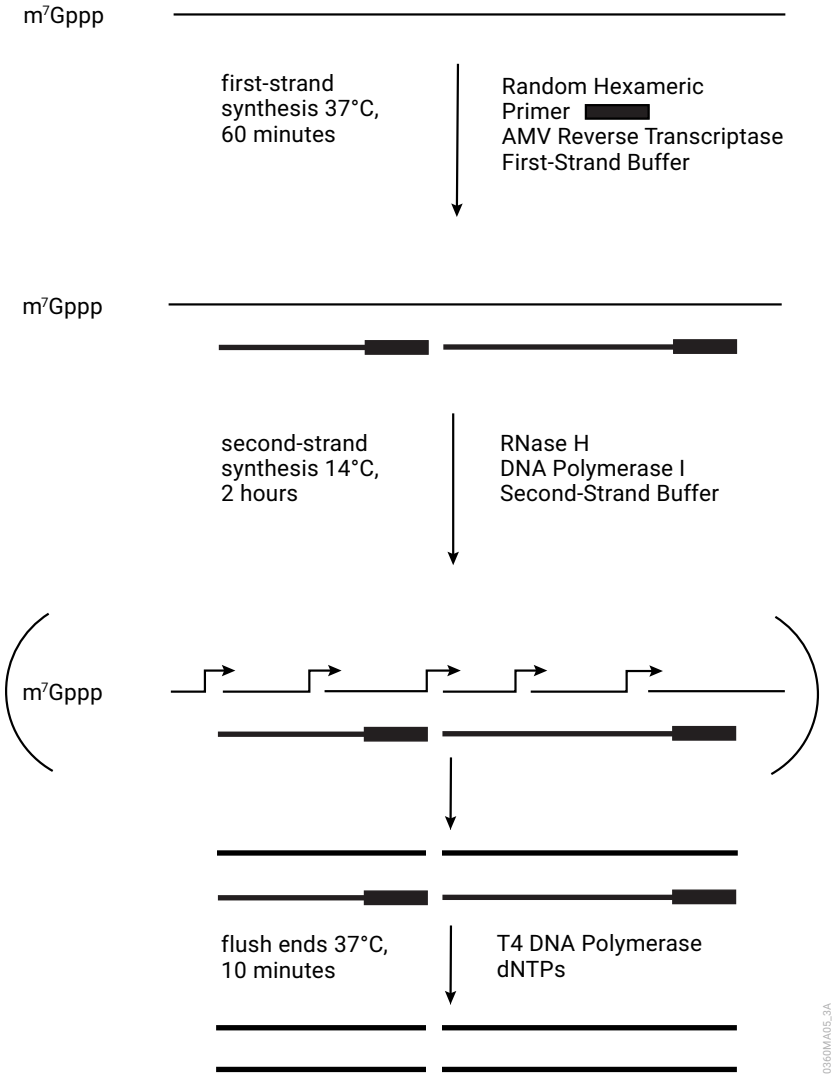

Figure 2. cDNA synthesis using the Universal RiboClone™ cDNA Synthesis System and Random Hexameric Primers.

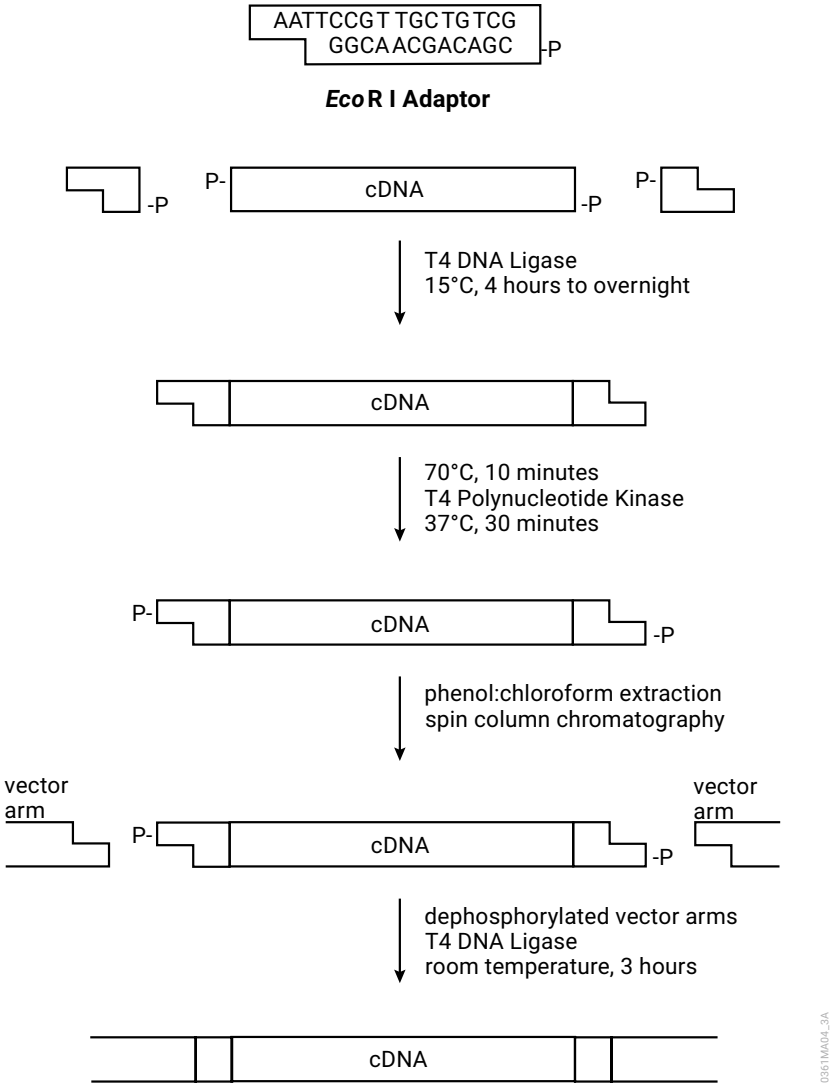

Figure 3. Schematic representation of EcoRI Adaptor ligation.

3.D. Choice of Vector

Many different plasmid and lambda vectors are available for cDNA cloning. The type of vector selected will depend on the possible applications for the cDNA library being constructed and other factors, such as blue-white cloning selection.

3.E. RNA Preparation and Handling

The quality and quantity of cDNA synthesized by any method is critically dependent on the integrity of the mRNA used as the template. Extremely stable RNases are ubiquitous in the laboratory environment (including the surface of fingers of the researcher) and in all cells. Therefore, certain precautions should be taken to eliminate the risk of RNase contamination whenever possible.

Procedures for creating an RNase-free laboratory environment have been described in detail (9). Sterile disposable plasticware should be used whenever possible, and disposable gloves should be worn at all times and changed often.

All glassware, nondisposable plasticware and electrophoresis apparatus used should be kept separately from other labware. Glassware can be soaked and rinsed in a solution of 0.1% diethyl pyrocarbonate (DEPC) and autoclaved.

Proper safety precautions should be taken when using DEPC, which is a powerful acylating agent. This compound should always be used in a fume hood and never added to aqueous solutions containing ammonia, which would result in the formation of ethyl carbamate, a potent carcinogen (10).

Many procedures exist for isolating intact RNA from various cells and tissues. A widely used method that has been successful with RNase-rich sources was first described by Chirgwin *et al.* (11).

The standard method of preparing poly(A)+ RNA has been by chromatography on oligo(dT)-cellulose (12). Even after two cycles of oligo(dT) selection, most preparations still contain varying amounts of ribosomal RNA sequences. In general, these sequences will not interfere with cDNA synthesis using oligo(dT)-containing primers. However, reduced yields of cDNA may be observed with Random Hexameric Primers, and quantitation of the mRNA will be affected.

The PolyATtract® mRNA Isolation Systems use Promega MagneSphere® technology to eliminate the need for oligo(dT) cellulose and its associated problems. With total RNA as the starting material, the poly(A)+ mRNA fraction can be isolated free of all other nucleic acid contamination in approximately 45 minutes. The system uses a biotinylated oligo(dT) primer to hybridize at high efficiency in solution to the 3´ poly(A) region present in most mature eukaryotic mRNA species. The hybrids are captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA is eluted from the solid phase by the simple addition of ribonuclease-free deionized water. This procedure yields mature polyadenylated mRNA after only a single round of magnetic separation. Alternatively, the PolyATtract® System 1000 may be used to isolate mRNA directly from crude cell or tissue lysates and eliminates the need for total RNA isolations.

4. First-Strand Synthesis

The standard reaction described below is performed in a total volume of 25µl, which is recommended for up to 2µg of mRNA. For each additional microgram of mRNA, increase the reaction volume by 10µl. For example, the reaction should be scaled up to 55µl when using 5µg of mRNA. Other components should be scaled up proportionally. After all of the components are added, a 5µl sample is removed to a separate tube containing a small amount of [α-^{32}P]dCTP as a tracer to follow first-strand synthesis. The remainder of the reaction is carried through second-strand synthesis.

4.A. Protocol

Materials to Be Supplied by the User

(Composition of Buffers and Solutions provided in Section 12.)

- EDTA (50mM and 200mM)
- [α-^{32}P]dCTP (>400Ci/mmol, 10mCi/ml)

1. Prepare the mRNA sample and control reactions as described below. The standard reactions described are optimized for 2µg of mRNA template.

   In a sterile, RNase-free microcentrifuge tube, add the appropriate primer to the mRNA or Control RNA sample. For mRNA samples, use 0.5µg primer/µg mRNA in a total volume of up to 15µl in water with either the Oligo(dT) Primer or Random Hexameric Primers.

Do not alter the ratio of primer to mRNA.

mRNA sample

mRNA	2µg
Primer, 0.5mg/ml	2µl
Nuclease-Free Water to final volume	**15µl**

The Control RNA uses a 1:1 ratio of primer to RNA. This ratio has been optimized for the 1.2kb Kanamycin Positive Control RNA and is not the same ratio used for mRNA.

Control reaction

1.2kb Kanamycin Positive Control RNA, 0.5mg/ml	2µl
Primer, 0.5mg/ml	2µl
Nuclease-Free Water	11µl
final volume	**15µl**

4.A. Protocol (continued)

2. Heat to 70°C for 5–10 minutes. Chill the tube on ice for 5 minutes and spin briefly to collect the solution at the bottom of the tube.

3. Add the following components in the order shown.

sample or control reaction	15µl
First Strand 5X Buffer	5µl
RNasin® Ribonuclease Inhibitor	40 units

4. Heat at 42°C (37°C for Random Primers) for 3–5 minutes, then add:

Sodium Pyrophosphate, 40mM	2.5µl
AMV Reverse Transcriptase	30 units
Nuclease-Free Water to final volume	**25µl**

Note: First Strand 5X Buffer and Sodium Pyrophosphate may form a precipitate when frozen. If this occurs, warm the tube to 37°C to dissolve the precipitate before addition.

5. Mix gently by flicking the tube, and transfer 5µl of each reaction mixture to another tube containing 2–5µCi of [α-^{32}P]dCTP (>400Ci/mmol, 10mCi/ml). These are the sample and control tracer reactions, which will be used to measure first-strand synthesis by incorporation and alkaline agarose gel electrophoresis (Sections 6 and 7).

   Note: We recommend using [α-^{32}P]dCTP (aqueous form) with a specific activity of >400Ci/mmol, 10mCi/ml that is less than 1 week old.

6. Incubate both labeled and unlabeled reactions for 60 minutes at 42°C for the Oligo(dT) Primer or 37°C for Random Hexameric Primers.

   Note: The reaction mixture may appear cloudy at this point, but subsequent steps will not be adversely affected.

7. Place reactions on ice.

8. Add 95µl of 50mM EDTA to the tracer reactions (to a total volume of 100µl) and store on ice. The tracer reactions will be used later for incorporation assays (Section 6.A) and gel analysis (Section 7).

9. Proceed to second-strand synthesis (Section 5) using the larger, unlabeled first-strand reactions. No phenol extractions or ethanol precipitations are necessary.

5. Second-Strand Synthesis

The second-strand reaction volume is based on that of the first-strand reaction volume. Maintain a fivefold dilution of the first-strand reaction volume in the second-strand reaction so the component amounts are scaled proportionally.

5.A. Protocol

Materials to Be Supplied by the User

(Composition of Buffers and Solutions are provided in Section 12.)

- EDTA (50mM and 200mM)
- [α-^{32}P]dCTP (>400Ci/mmol, 10mCi/ml)
- TE-saturated phenol:chloroform:isoamyl alcohol
- ammonium acetate, 7.5M (or sodium acetate, 2.5M [pH 5.2])
- ethanol (100% and 70%), –20°C
- TE buffer

1. Add the following components to the first-strand sample and Control RNA reactions in the order shown. Perform this step on ice.

   Second-strand sample

first-strand sample reaction	20µl
Second Strand 2.5X Buffer	40µl
Acetylated BSA, 1mg/ml	5µl
DNA Polymerase I	23 units
RNase H	0.8 units
Nuclease-Free Water to final volume	**100µl**

   Second-strand control

first-strand Control RNA reaction	20µl
Second Strand 2.5X Buffer	40µl
Acetylated BSA, 1mg/ml	5µl
DNA Polymerase I	23 units
RNase H	0.8 units
Nuclease-Free Water to final volume	**100µl**

2. Mix gently by flicking the tube. If a tracer reaction for the second-strand sample is desired, remove 10µl to another tube containing 2–5µCi [α-^{32}P]dCTP (>400Ci/mmol, 10mCi/ml).
3. For the second-strand control tracer reaction, add 2–5µCi [α-^{32}P]dCTP directly to the 100µl control reaction.
4. Incubate the reactions at 14°C for 2 hours.

5.A. Protocol (continued)

Note: When cDNA longer than 3kb is desired, increase the second-strand incubation time to 3–4 hours.

5. Heat the unlabeled cDNA sample to 70°C for 10 minutes, collect the contents at the bottom of the tube by brief centrifugation and place on ice.
6. While the unlabeled samples are being heated, add 90µl of 50mM EDTA to the 10µl sample tracer reaction. Add 10µl of 200mM EDTA to the 100µl Control RNA tracer reaction. The tracer reactions will be used later for incorporation assays (Section 6) and gel analysis (Section 7). Store on ice.
7. Add 2 units of T4 DNA Polymerase/µg input RNA to the unlabeled reaction. Incubate at 37°C for 10 minutes.
8. Stop the reaction by adding 10µl of 200mM EDTA and place on ice.
9. To extract the cDNA, add an equal volume of TE-saturated phenol:chloroform:isoamyl alcohol, mix thoroughly, and centrifuge at top speed in a microcentrifuge for 2 minutes at room temperature.
10. Transfer the aqueous phase to a fresh tube, add 0.5 volume of 7.5M ammonium acetate (or 0.1 volume of 2.5M sodium acetate [pH 5.2]), mix and add 2 volumes of cold (–20°C) 100% ethanol. Place at –70°C for 30 minutes and centrifuge at top speed for 5 minutes.
11. Carefully remove the supernatant, add 0.5ml of cold (–20°C) 70% ethanol to the tube with the pellet. Centrifuge again at top speed for 2 minutes.
12. Carefully remove the supernatant and dry the pellet.
13. Dissolve the pellet in 10–50µl of TE buffer. The cDNA is now ready for size fractionation prior to adaptor addition (Section 8).

    Note: The Promega Wizard® DNA Clean-Up System (Cat. # A7280) may be used instead of solvent extraction and ethanol precipitation in Steps 9–13.

6. Incorporation Assays

Trichloroacetic acid (TCA) assays of isotope incorporation are prone to variation (13), and thus some variability can be expected in assays of first- and second-strand conversion. In our experience, cDNA libraries can be successfully constructed from reactions yielding 12–50% first-strand conversion and at least 80% second-strand conversion values.

6.A. TCA Precipitation

Materials to Be Supplied by the User

- 1mg/ml carrier DNA (e.g., herring sperm DNA, Cat.# D1811)
- TCA 5% (w/v), ice-cold
- glass fiber filters (e.g., Whatman® GF-A or GF-C)
- acetone or ethanol, 100%

1. Spot 3µl samples of the diluted first-strand tracer reaction (Section 4, Step 7) and the second-strand tracer reaction (Section 5.A, Step 6) on glass fiber filters and let them air-dry. These samples represent the total cpm in the reactions.

2. Add 3µl samples of the same reactions to tubes containing 100µl of a 1mg/ml solution of carrier DNA and mix. Add 0.5ml of 10% TCA (ice-cold) and vortex. Let sit on ice for 5–30 minutes.
3. Filter the samples through glass fiber filters, washing 3 times each with 5ml of cold 5% TCA. Rinse the filters with 5ml of acetone or ethanol and let air-dry.
4. Count both total and incorporated (TCA-precipitable) samples, either by Cerenkov radiation (without scintillant) or after adding an appropriate scintillation fluid.

6.B. Calculation of First-Strand Yield

The first-strand yield is determined as follows:

$$\frac{\text{incorporated cpm}}{\text{total cpm}} \times 100\% = \%\text{ first-strand incorporation}$$

$$\frac{4\ (\text{nmol dNTP}/\mu\text{l}) \times \text{reaction volume } (\mu\text{l}) \times \%\text{ first-strand incorporation}}{100} = \text{nmol dNTP incorporated}$$

$$\text{nmol dNTP incorporated} \times 330\text{ng/nmol} = \text{ng cDNA synthesized}$$

$$\frac{\text{ng cDNA synthesized}}{\text{ng mRNA in reaction}} \times 100\% = \%\text{ mRNA converted to cDNA}$$

Example: If there were 1,854cpm incorporated and 254,000 total cpm in the tracer reaction, and 2µg RNA was added to the original 25µl reaction, the calculations would be as follows:

$$\frac{1{,}854}{254{,}000} \times 100\% = 0.73\%\text{ first-strand incorporation}$$

$$\frac{4\ (\text{nmol dNTP}/\mu\text{l}) \times 25\mu\text{l} \times 0.73}{100} = 0.73\text{nmol dNTP incorporated}$$

$$0.73\text{nmol dNTP} \times 330\text{ng/nmol} = 240\text{ng cDNA synthesized}$$

$$\frac{240\text{ng cDNA synthesized}}{2{,}000\text{ng mRNA in reaction}} \times 100\% = 12\%\text{ mRNA converted to cDNA}$$

6.B. Calculation of First-Strand Yield (continued)

Of the original 2,000ng of mRNA, 20% (or 5µl/25µl) was removed for the tracer reaction, and 80% (or 20µl/25µl) remained in the main reaction. Therefore, 192ng (0.8 × 240ng) first-strand cDNA was synthesized and 0.58nmol (0.8 × 0.73nmol) dNTPs were incorporporated in the reaction that was carried through to second-strand synthesis.

Note: The minimum quality control specification for first-strand conversion is 12%.

6.C. Calculation of Second-Strand Yield

The second-strand yield is calculated in the same manner as the first-strand yield, except that the total dNTP in the reaction will be diminished by that which was incorporated during first-strand synthesis.

$$\frac{\text{incorporated cpm}}{\text{total cpm}} \times 100\% = \%\text{ second-strand incorporation}$$

$$\frac{[0.8\ (\text{nmol dNTP}/\mu\text{l}) \times \text{reaction vol. } (\mu\text{l}) - \text{nmol incorp. in first-strand reaction}] \times \%\text{ second-strand incorporation}}{100} = \text{nmol dNTP incorporated}$$

$$\text{nmol dNTP incorporated} \times 330\text{ng/nmol} = \text{ng second-strand cDNA synthesized}$$

$$\frac{\text{ng second-strand cDNA synthesized}}{\text{ng first-strand synthesized}} \times 100\% = \%\text{ converted to double-stranded cDNA}$$

Example: The first-strand reaction above was used for second-strand synthesis, which resulted in 1,390cpm incorporated out of a total of 235,000cpm.

$$\frac{1{,}390}{235{,}000} \times 100\% = 0.59\%\text{ second-strand incorporation}$$

$$\frac{[0.8\text{nmol}/\mu\text{l} \times 100\mu\text{l} - 0.58\text{nmol first-strand dNTP incorporated}] \times 0.59}{100} = 0.47\text{nmol dNTP incorporated}$$

$$0.47\text{nmol} \times 330\text{ng/nmol} = 155\text{ng second-strand cDNA synthesized}$$

$$\frac{155\text{ng second-strand cDNA}}{192\text{ng first-strand cDNA}} \times 100\% = 81\%\text{ conversion to double-strand cDNA}$$

Notes:

- The minimum quality control specification for second-strand conversion is 80%.
- A low percent conversion for second-strand product may be due to a high percent conversion for first-strand synthesis. If a high yield is achieved for first-strand synthesis (as from extended incubation), the percent converted during second-strand synthesis may be lower, but the amount of DNA should be adequate for most applications.

7. Gel Analysis

The size distribution of cDNA synthesized (usually 350–6,000 bases) in the first- and second-strand reactions should be checked by electrophoresis on a 1.4% alkaline agarose gel (14). The samples used for gel analysis are the first-strand and second-strand tracer reactions. Load approximately equivalent amounts of incorporated cpm for each strand so that comparisons can be made on the same autoradiograph. As a guideline, 5–10µl of the control tracer reaction on a fixed and dried gel results in a dark, sharp signal in a 3–4 hour exposure with intensifying screens.

7.A. Labeling Size Markers

DNA markers that have been ^{32}P-labeled may be used for estimating the sizes of the first- and second-strand products. A convenient way to prepare these markers is to use Lambda DNA/HindIII Markers in a fill-in reaction with Klenow DNA Polymerase.

Materials to Be Supplied by the User

(Composition of Buffers and Solutions provided in Section 12.)

- Klenow DNA Polymerase
- Klenow 10X Buffer
- dATP, dGTP (10mM)
- Lambda DNA/HindIII Markers (Cat.# G1711)
- [α-^{32}P]dCTP (>400Ci/mmol)
- EDTA (200mM)
- sample 2X buffer

1. Assemble the following reaction:

Klenow 10X Buffer	2.5µl
dATP, 0.1mM (1:100 dilution in water of 10mM stock)	0.2mMl
dGTP	0.2mM
[α-^{32}P]dCTP (>400Ci/mmol)	2µCi
Lambda DNA/HindIII Markers	1µg
Klenow DNA Polymerase	1u
Nuclease Free Water to final volume	**25µl**

2. Incubate the reaction for 10 minutes at room temperature. Add 2.5µl of 200mM EDTA to stop the reaction. Add a sample directly to an equal volume of sample 2X buffer and store the remainder at –20°C. A 5µl sample routinely gives a sharp ladder of bands after a 1-hour exposure.

7.B. Electrophoresis

Materials to Be Supplied by the User

(Composition of Buffers and Solutions provided in Section 12.)

- alkaline agarose gel
- alkaline gel running buffer
- sample 2X buffer
- TCA, 7% (w/v)

1. Prepare a 1.4% alkaline agarose gel (see Section 12).
2. Transfer the desired amount of each sample (usually 10,000–50,000 incorporated cpm) to separate tubes, adjust the volumes with TE buffer so that they are equal, and add an equal volume of sample 2X buffer to each.
3. Load the samples and run the gel at up to 5V/cm for the desired time (usually until the tracking dye has migrated about 2/3 of the way through the gel).
4. Soak the gel in several volumes of 7% TCA at room temperature for 30 minutes or until the dye changes from blue to yellow. Dry on a piece of Whatman® 3MM paper, either on a gel dryer or by placing the gel under a weighted stack of paper towels for several hours.
5. Cover the dried gel with plastic wrap and expose to X-ray film at room temperature or at –70°C with an intensifying screen. Alternatively, scan the gel using phosphorimaging analysis.
6. If the Positive Control mRNA was used with the Oligo(dT) Primer, the first- and second-strand reaction products should appear as sharp bands at 1.2kb in the gel. Random priming results in products that are 1.2kb and smaller.

8. EcoRI Adaptor Ligation

8.A. Size Fractionation

Materials to Be Supplied by the User

- spin columns

We recommend size fractionating cDNA samples before ligation with adaptors. Small-sized cDNA, less than 400bp, should be eliminated to ensure successful adaptor addition and cloning. This step can be accomplished by gel filtration using either column fractionation or spin columns. Alternatively, cDNA samples may be fractionated by agarose gel electrophoresis followed by selective elution of particular-sized cDNA molecules (14). We do not recommend precipitating DNA in the presence of ammonium ions after either of these fractionation procedures.

Notes:

- The Sephacryl® S-400 Resin and Spin Columns in the Universal RiboClone™ System are provided for the removal of adaptors. They may also be used for size fractionation of cDNA prior to ligation; however, the quantity of Resin and Spin Columns provided is sufficient for use in a total of 10 size fractionation and adaptor removal reactions. Additional Spin Columns and Resin may be purchased from Promega (see Section 14).
- The size cutoff for the Sephacryl® S-400 Resin is 271bp.

! Do not freeze the Sephacryl® S-400 Resin.

8.B. Ligation of EcoRI Adaptors

1. Set up the following reaction (volumes can be scaled up proportionally, depending upon the amount of cDNA).
2. Incubate at 15°C for 4 hours to overnight.
3. Inactivate the enzyme by heating the reaction to 70°C for 10 minutes.
4. Cool the reaction on ice and proceed to Section 8.C.

8.C. Phosphorylation of DNA

For optimal cloning efficiency, the insert DNA should be phosphorylated and vector DNA should be dephosphorylated. The following protocol should be used for cDNA synthesized with the Oligo(dT) Primer or Random Hexameric Primers.

Materials to Be Supplied by the User

(Composition of Buffers and Solutions provided in Section 12.)

- TE buffer
- TE-saturated phenol:chloroform:isoamyl alcohol

1. Set up the following reaction:

ligation reaction (from Section 8.B)	30µl
Kinase 10X Buffer	8µl
ATP, 0.1mM (1:100 dilution in water of 10mM stock)	2µl
T4 Polynucleotide kinase (5–10u/µl)	10u
Nuclease Free Water to final volume	**40µl**

2. Incubate at 37°C for 30 minutes.
3. Phosphorylated cDNA may be purified prior to ligation with vector. This can be accomplished using a standard DNA cleanup protocol (Steps 4–5) or using a resin-based purification system such as the Wizard® DNA Clean-Up System (Cat.# A7280).

8.C. Phosphorylation of DNA (continued)

4. Add 1 volume of TE-saturated phenol:chloroform. Vortex for 30 seconds and centrifuge at top speed in a microcentrifuge for 3 minutes.
5. Transfer the upper, aqueous phase to a fresh tube. To increase recovery, the phenol:chloroform phase can be re-extracted with a small volume of TE buffer.
6. Proceed to removal of excess adaptors (Section 9).

9. Removal of Excess Adaptors

9.A. Preparation of Spin Columns

Materials to Be Supplied by the User

(Composition of Buffers and Solutions provided in Section 12.)

- TEN buffer

Sephacryl® S-400 Resin is provided in a solution of TEN buffer. It is a good idea to re-equilibrate with this buffer before use.

1. Thoroughly mix the Sephacryl® S-400 slurry and transfer 600µl to a Spin Column. Start the flow of buffer out of the bottom of the column by using a gentle push of air from a Pasteur pipette bulb. Allow the buffer to drain and add an additional 400µl for a total of 1ml.
2. Discard the collected fluid and add 600µl of TEN buffer, allow some to drain, then add an additional 400µl. Allow buffer to drain from the tube. This step should be repeated 2 times.

   Note: Equilibration may also be achieved by repeated centrifugations using smaller volumes of buffer. If this method is used, be careful that the filtrate volume does not touch the frit of the column.
3. Place the column tip into the provided Wash Tube. Put the assembly inside a larger centrifuge tube and centrifuge in a swinging bucket rotor at 800 × *g* for 5 minutes.
4. The bed should have a dry appearance and may pull away from the sides of the tube. The column is now ready for immediate use.

9.B. Spin Column Protocol

The optimal sample volume is 20–60µl. Do not exceed a 60µl sample volume. Spin Columns must be maintained in a vertical position during sample loading and subsequent handling procedures to ensure proper separation.

1. Pipet the reaction mixture from Section 8.C. Hold the pipet tip within a few millimeters of the gel bed, and slowly add the sample dropwise to the center of the dried gel bed. Do not allow the sample to come in direct contact with the sides of the tube.
2. Place the Spin Column into the provided Collection Tube, carefully put this assembly inside a larger tube and centrifuge in a swinging bucket rotor at 800 × *g* for 5 minutes.
3. Remove the assembly, discard the Spin Column and save the Collection Tube. The volume recovered should be approximately equal to the volume that was applied to the Spin Column.
4. The eluted cDNA can either be used directly or precipitated with ethanol to concentrate the sample.

9.C. Concentration of cDNA by Ethanol Precipitation

Materials to Be Supplied by the User

(Composition of Buffers and Solutions provided in Section 12.)

- ammonium acetate, 7.5M (or sodium acetate, 2.5M [pH 5.2])
- ethanol, 100% and 70% (–20°C)
- TE buffer

1. To the sample from Section 9.B, add 0.5 volume of 7.5M ammonium acetate (or 0.1 volume of 2.5M sodium acetate [pH 5.2]) and 2.5 volumes of cold (–20°C) 100% ethanol. Mix and place at –70°C for 30 minutes.
2. Centrifuge at top speed in a microcentrifuge for 15 minutes. Carefully remove and discard the supernatant.
3. Wash the pellet with 1ml of cold (–20°C) 70% ethanol and spin in microcentrifuge at top speed for 5 minutes. Carefully remove and discard the supernatant.
4. Dry the pellet briefly under vacuum. Resuspend the pellet in an appropriate volume of TE buffer for further reactions.

10. Ligation of cDNA Insert to EcoRI-Digested Plasmid Vector

Synthesized cDNA can be cloned into a plasmid vector, which offers versatility and ease of manipulation after cloning. The ratio of insert DNA to vector is critical to successful ligation and should be carefully optimized for each cDNA preparation following the protocol described below. As a general recommendation, an insert:vector molar ratio of 1:3 may be used for dephosphorylated vectors. We do not recommend using phosphorylated vector due to the low cloning efficiency obtained as a result of vector self-ligation.

In the example given below, the molar ratio of plasmid (3kb) to insert DNA is varied from 1:3 to 1:1. A tube containing no insert DNA is also used to determine background levels of religated vector.

10.A. Protocol

1. Set up sample reactions as described. The amounts of insert and vector specified are based on an average cDNA size of 1.5kb. Maintain a constant molar concentration of the vector while adjusting the amount of cDNA.

Sample Reaction: Plasmid Vector

Tube	A	B	C	D
Vector DNA (10ng/µl; 0.005pmol/µl)	2µl	2µl	2µl	2µl
Insert DNA (10ng/µl; 0.01pmol/µl)	0µl	3µl	2µl	1µl
T4 DNA Ligase 10X Buffer	1µl	1µl	1µl	1µl
Nuclease-Free Water	6µl	3µl	4µl	5µl
T4 DNA Ligase	1µl	1µl	1µl	1µl

2. Incubate the reactions at room temperature for 3 hours. Alternatively, the incubation may be performed at 4°C for 16–18 hours.

3. Once optimal ligation conditions have been established, reactions may be scaled up accordingly for library construction.

   Note: Synthesized cDNA can also be cloned into bacteriophage lambda vectors. These vectors may be preferred when high-efficiency cloning is desired. If substituting a lambda vector for plasmid in the sample reaction, the molar ratio of lambda arms (43kb) to DNA insert is varied from 3.6:1 to 1.2:1 (e.g., use the same volumes as for Plasmid Vector, but use 0.5µg/µl; 0.018pmol/µl lambda vector).

11. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms	Causes and Comments
Low yield of first- and second-strand cDNA, or no cDNA synthesized	RNase contamination in synthesis reaction. Check control reaction for yield. Using the Oligo(dT) Primer, the Positive Control RNA should give at least 12% first-strand conversion, 80% second-strand conversion and clear bands of 1.2kb on alkaline agarose gels. Follow guidelines in Section 3.E to minimize RNase contamination.
	Degraded mRNA template. Check mRNA preparation by electrophoresis using either denaturing or native conditions. If total RNA is run on a gel, bands corresponding to 28S and 18S ribosomal RNA should be apparent, and 28S RNA should stain with approximately twice the intensity of 18S RNA. Procedures outlined in Section 3.E should be followed to ensure the isolation of undegraded mRNA template.
	mRNA template has high degree of secondary structure. Heat mRNA to 70°C for 5–10 minutes, then place immediately on ice to ensure denaturation of template.
	Incorrect primer:RNA ratio. Use 0.5µg primer/µg mRNA for the Oligo(dT) or Random Hexameric Primers and mRNA samples. The control RNA uses a 1:1 ratio of primer:RNA.
	Inhibitors in mRNA preparation. Inhibitors such as SDS, EDTA, polysaccharides or salts may affect first-strand synthesis. Add a small amount of the mRNA preparation to the Positive Control RNA first-strand reaction to check for inhibition of synthesis.
	A low percent conversion for second-strand product may be due to a high percent conversion for first-strand synthesis. If a high yield is achieved for first-strand synthesis (as from extended incubation), the percent converted during second-strand synthesis may be lower, but the amount of DNA should be adequate for most applications.

11. Troubleshooting (continued)

Symptoms	Causes and Comments
Low cloning efficiency	Poor ligation of adaptors to cDNA. Check ligation reactions with pure DNA preparations (e.g., purified plasmid DNA or Lambda DNA/HindIII Markers). Longer incubation time at a lower temperature may increase ligation efficiency (e.g., overnight at 4°C). Hairpin priming. First-strand synthesis should be performed in the presence of sodium pyrophosphate to suppress formation of hairpins.
	Insufficient phosphorylation of ligated EcoRI Adaptors. The efficiency of the kinase reaction may be monitored using [γ-^{32}P]ATP.
	Do not dissolve or precipitate DNA in the presence of ammonium ions after fractionation. Ammonium ions inhibit T4 polynucleotide kinase.
	Poor yield of cDNA from Sephacryl® S-400 columns. Resin may not be sufficiently equilibrated. Wash column three times with TEN buffer before use.
	Ratio of cDNA to vector not optimal for ligation. Determine optimal conditions for ligation by adjusting the amount of cDNA in small-scale reactions while maintaining a constant concentration of vector.
	Poor ligation of cDNA to vector. Cloning vector should be dephosphorylated for optimal results.

12. Composition of Buffers and Solutions

alkaline gel running buffer

30mM	NaOH
1mM	EDTA

Prepare new buffer for each use.

alkaline agarose gel

1.4%	agarose
50mM	NaOH
1mM	EDTA

Equilibrate solidified gel for at least 30 minutes in alkaline gel running buffer prior to electrophoresis.

First Strand 5X Buffer

250mM	Tris-HCl (pH 8.3 at 42°C)
250mM	KCl
50mM	$MgCl_2$
2.5mM	spermidine
50mM	DTT
5mM	each dATP, dCTP, dGTP, dTTP

Kinase 10X Buffer

700mM	Tris-HCl (pH 7.6)
100mM	$MgCl_2$
50mM	DTT

Klenow 10X Buffer

50mM	Tris-HCl (pH 7.2)
100mM	$MgSO_4$
1mM	DTT

sample 2X buffer

20mM	NaOH
20%	glycerol
0.025%	bromophenol blue (added fresh for each use)

Second Strand 2.5X Buffer

100mM	Tris-HCl (pH 7.2)
225mM	KCl
7.5mM	$MgCl_2$
7.5mM	DTT

TE buffer

10mM	Tris-HCl (pH 8.0)
1mM	EDTA

TEN buffer

100mM	NaCl in TE buffer

TE-saturated phenol: chloroform:isoamyl alcohol

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

T4 DNA Ligase 10X Buffer

300mM	Tris-HCl (pH 7.8)
100mM	$MgCl_2$
100mM	DTT
5mM	ATP

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14. Related Products

cDNA Synthesis and Cloning Reagents

Product	Size	Cat.#
Oligo(dT)15 Primer	20µg	C1101
Random Primers*	20µg	C1181
EcoRI Adaptors	150pmol	C1291
Spin Columns	10 each	C1281
Sephacryl® S-400*	10ml	V3181
1.2kb Kanamycin Positive Control RNA*	5µg	C1381

*For Laboratory Use

Product	Size	Cat.#
PolyATract® mRNA Isolation System I	3 isolations	Z5210
PolyATtract® mRNA Isolation System II	10 isolations	Z5200

For Laboratory Use. These systems contain sufficient reagents to perform three separate mRNA isolations, each from 1–5mg of total RNA. System II includes the Magnetic Separation Stand.

Product	Size	Cat.#
PolyATtract® mRNA Isolation System III	15 isolations	Z5300
PolyATtract® mRNA Isolation System IV	15 isolations	Z5310

For Laboratory Use. These systems contain sufficient reagents to perform fifteen separate mRNA isolations, each from approximately 100–1,000µg of total RNA. System III includes the Magnetic Separation Stand.

Product	Size	Cat.#
PolyATtract® System 1000	Scalable	Z5400
PolyATtract® System 1000 with Magnetic Separation Stand	Scalable	Z5420

For Laboratory Use. These systems contain sufficient reagents to isolate poly(A) RNA from up to 2g of tissue.

Product	Size	Cat.#
PolyATtract® System 1000 Magnetic Separation Stand	1 each	Z5410

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15. Summary of Changes

The following changes were made to the 4/21 revision of this document:

1. Corrected the reaction components listed in Step 4, Section 4.A.
2. Updated the cover page.