Invitrogen™
life technologies

Instruction Manual

cDNA Synthesis System

CAT. NO. 18267-013

Table of Contents

Table of Contents

Figures

Notices to Customer

1

1.1 Important Information

This product is authorized for laboratory research use only. The product has not been qualified or found safe and effective for any human or animal diagnostic or therapeutic application. Uses for other than the labeled intended use may be a violation of applicable law.

1.2 Precautions

Warning: This product contains hazardous reagents. It is the end-user's responsibility to consult the applicable MSDS(s) before using this product. Disposal of waste organics, acids, bases, and radioactive materials must comply with all appropriate federal, state, and local regulations. If you have any questions concerning the hazards associated with this product, please call the Invitrogen Environmental Health and Safety Chemical Emergency hotline at (301) 431-8585.

2

Overview

2.1 The cDNA Synthesis System

The cDNA Synthesis System provides the materials needed to rapidly and reliably synthesize double-stranded cDNA from mRNA. It uses cloned Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase to synthesize first strand cDNA from an mRNA population. Figure 1 is a summary of the two procedures that may be used to prepare double-stranded cDNA for tailing or linker addition. The one-tube system allows second strand cDNA to be synthesized immediately after the first strand reaction, thereby improving the yield of double-stranded cDNA by eliminating extraction and precipitation between steps. Furthermore, the double-stranded cDNA can be cloned into either plasmid or bacteriophage vectors.

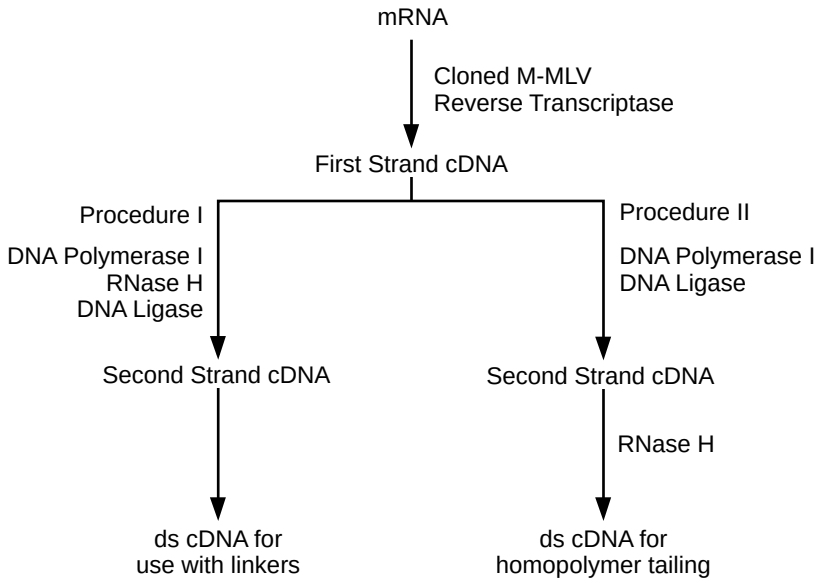

Figure 1. Outline of procedures for the cDNA Synthesis System.

2.2 cDNA Libraries

Bacteria or bacteriophage containing cDNA copies of an mRNA population constitute a cDNA library. A good library should be large enough to contain representatives of all sequences of interest, and the cDNA inserts should be full-length copies of the original mRNA. Sequences at both the 5′ and 3′ ends of mRNAs contain valuable information needed for full understanding of gene expression and processing.

Construction of a cDNA library from mRNAs is an essential step for many studies in molecular biology. A cDNA clone isolated from a cDNA library provides a means of establishing the fully processed form of a message initially transcribed from a genomic sequence containing intervening sequences (introns). It also provides a probe for examining the structure of the gene, determining its location in the genome, or developing *in vitro* or recombinant expression systems. Comparing two libraries constructed from different tissues or different stages of a developmental system can reveal developmentally regulated genes. For these and other types of studies, a high quality library is critical.

Many procedures for synthesizing cDNA have been developed, most attempting to maximize the amount of cDNA produced from a limited amount of mRNA. In most of these procedures, the completeness of the cDNA synthesis is variable and unpredictable. This variation can enter in any of the three steps of cDNA synthesis: mRNA isolation, first strand cDNA synthesis, and second strand cDNA synthesis. The quality of the mRNA preparation, the purity of the enzyme and reagents used in the first strand reaction, and the choice of second strand method affect the quantity of sequence information retained in the library.

2.3 Choosing a Vector System

Two types of vectors are in general use for cDNA cloning in *E. coli:* plasmids and bacteriophage lambda derivatives. A number of factors affect the choice of vector, which in turn affects the method of cDNA synthesis. In choosing a vector, consider the following:

1. How many clones are needed to provide a reasonable chance of finding the gene of interest?
2. How critical is it to have a complete copy of the mRNA containing the 5′ end of the message?
3. How important is it to obtain a clone that can express a protein product?
4. How familiar and comfortable are you with the manipulation and propagation of plasmids or phage?

The number of clones needed to make a library complete depends upon the relative abundance of the target mRNA in the total mRNA pool (1). Therefore, it is advantageous to use an enriched source of the target mRNA if possible. The enrichment can be biological (as in the case of a developmentally regulated gene) or physical (hybrid selection or immunoprecipitation). The size of a library needed to obtain an mRNA as a cDNA clone generally requires 10^4 to 10^5 recombinants (1). Either plasmids or phage vectors can yield this number of clones; however, the efficiency of cloning with phage vectors is generally higher and requires less mRNA initially.

The method used to synthesize the double-stranded cDNAs will influence the amount of sequence information at the 5′ end of the mRNAs that is preserved in the cDNA library (2). Plasmids are superior to phage if this is an important consideration. If the cDNA library is created in phage (figure 1, Protocol 1), the use of molecular linkers results in loss of sequence information at the 5′ ends of the mRNAs. On the other hand, if cDNAs are cloned into plasmids using homopolymeric tails, the conditions for cDNA synthesis with the cDNA Synthesis System (figure 1, Protocol 2) are designed to conserve all or most of the sequence information at the 5′ termini. In addition, the method used to insert double-stranded cDNAs into a vector can influence the amount of sequence information that becomes established in the library when cloning large inserts. This is discussed later in Section 2.6, *Second Strand Reaction*.

If the cDNA library is to be screened with an antibody, phage vectors provide the best system for regulated expression of antigen and ease of screening (3). Although plasmid-based libraries can be used (4), they are more cumbersome and considerably more tedious to use. Choosing between plasmid and phage vectors

becomes more difficult if a library will be screened with a nucleic acid probe because the technology is equivalent in both systems (5,6). In this case, other considerations will weigh more heavily in the decision.

Cloning into plasmids is generally easier for the novice because it requires less manipulation of the double-stranded cDNA. It also avoids the problems that can be encountered when propagating phage. However, this should not preclude using phage if it is the best choice. Numerous references describe cDNA cloning into phage in detail, and these should be consulted (7,8).

2.4 mRNA Purification

The quality of the mRNA dictates the maximum amount of sequence information preserved during synthesis of double-stranded cDNA. The most prevalent source of ribonuclease (RNase) contamination is the starting material used for the mRNA purification. The endogenous RNases must be inactivated quickly during lysis of the cells. This often is accomplished by using protein denaturants such as phenol, chloroform, sodium dodecyl sulfate, or chaotropic salts (9,10). The rigor of the conditions needed to prevent degradation can vary with the source of the RNA.

It is also possible to introduce RNases into a preparation of RNA by using contaminated reagents, enzymes, or laboratory ware. Microbial contamination of buffers can cause serious problems. We recommend that you maintain sterile stocks and practice sound microbiological technique. A common source of RNase is labware that has been used for RNase digestions and circulated among general laboratory equipment without prior decontamination. All glass and plasticware used for mRNA purification should be dedicated to this purpose to avoid contamination.

After the RNA has been deproteinized and rendered RNase free, it is fractionated by chromatography on a matrix such as oligo(dT) cellulose (11). The RNA that binds to the column is enriched for RNAs containing poly(A) tails. This eliminates rRNA contaminants and partially degraded mRNAs that lack poly (A) at the 3´ terminus. A successful oligo(dT) cellulose purification results in higher yields of first strand cDNA and less spurious copying of nonmessenger RNAs.

2.5 cDNA Synthesis: The First Strand Reaction

The classical conditions for the reverse transcription of RNA derive entirely from work with the Avian Myeloblastosis Viral (AMV) reverse transcriptase. This enzyme has been used extensively and its reaction parameters exhaustively documented (12). Available preparations of this enzyme are of variable quality, causing fluctuations in the quantity and quality of the first strand cDNA. The source of this variation is probably due to the level of contaminating RNase. As the RNase contamination becomes more severe, the first strand cDNAs will be shorter because the RNA template is degraded during first strand synthesis. In cases of extreme contamination, the yield of first strand cDNA will also decline as the size of the cDNA drops below the threshold of acid insolubility.

An array of modifications and additions to the first strand reaction have been reported to improve the quality and yield of cDNA; some of these are attempts to reduce the effect of RNase contamination. The cDNA Synthesis System relies on a cloned enzyme, M-MLV reverse transcriptase. Cloned and overexpressed in *E. coli* (13), M-MLV reverse transcriptase is rigorously purified and does not require additional components to synthesize high quality first strand cDNA.

2.6 cDNA Synthesis: The Second Strand Reaction

Many procedures for second strand synthesis have been developed. The main features of three commonly used methods are discussed briefly to facilitate your

understanding of the second strand conditions in the cDNA Synthesis System.

2.6.1 Hairpin-Primed Synthesis

The earliest procedure (14) for priming second strand synthesis relied on the ability of AMV reverse transcriptase to randomly initiate the formation of hairpin loops at the 3´ end of the first strand cDNA (figure 2). The mechanism for generation of the hairpin is believed to depend upon the action of the endogenous RNase H activity of AMV reverse transcriptase: as the RNA template is being reverse transcribed, it is also being degraded by the RNase H. When the RNA at the 5´ end of the message has been degraded, the exposed, single-stranded cDNA at the 3´ end can fold back upon itself in a transient manner and provide a priming point for the reverse transcriptase to initiate second strand synthesis. Although this procedure is simple to perform, it is the most unpredictable because the priming event occurs randomly along the mRNA template. Furthermore, the 3´ hairpin is stabilized by elongation

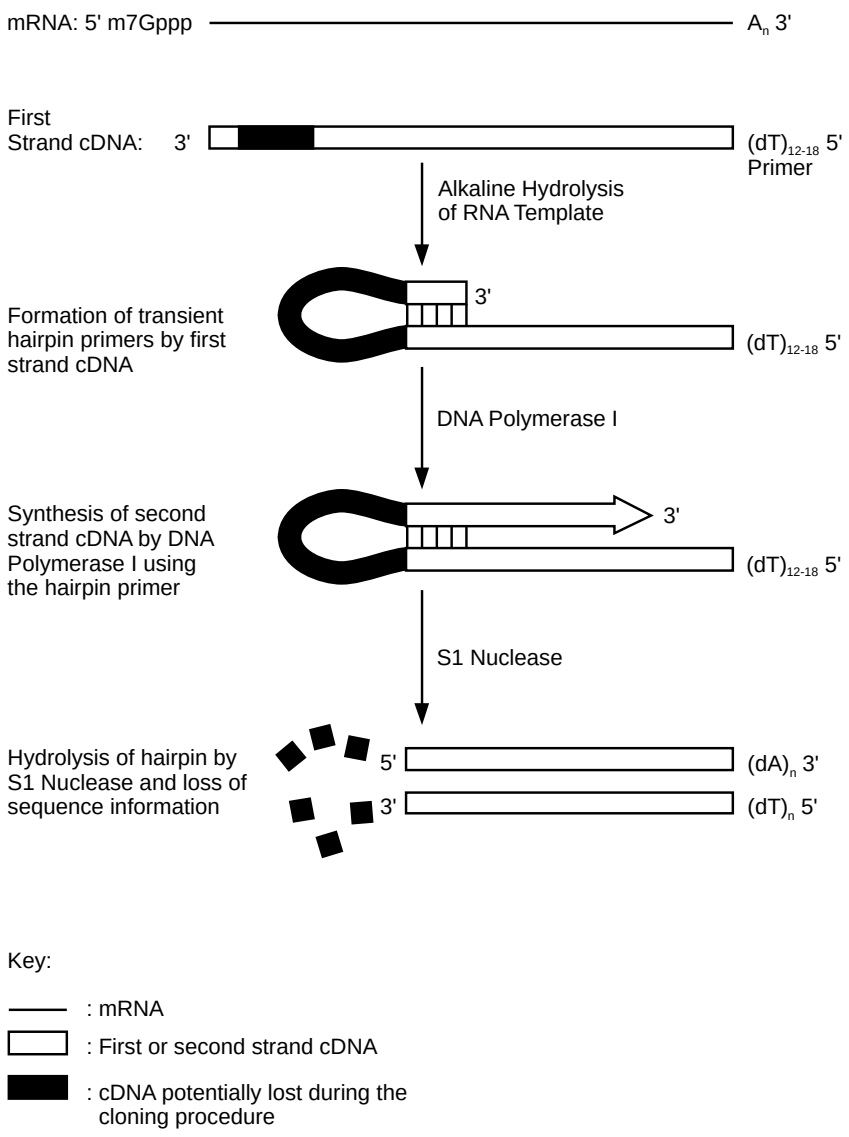

Figure 2. Hairpin-primed cDNA synthesis.

and must be cleaved with S1 nuclease to make it amenable to cloning. This is yet another point at which sequence information can be lost from the cDNA population.

2.6.2 Okayama and Berg Procedure

A major improvement in double-stranded cDNA synthesis was based upon an innovative method for generating primers for second strand synthesis (15). Rather than relying upon reverse transcriptase to initiate from a hairpin primer, the mRNA is used to prime synthesis by *E. coli* DNA polymerase I after the mRNA is nicked randomly. The nicks are introduced by the action of *E. coli* RNase H, and the RNA is replaced in a nick translation reaction catalyzed by *E. coli* DNA polymerase I. Okayama and Berg exploited this methodology and designed a specialized, two-part plasmid cloning vector to preserve as much sequence information at the 5´ end of the RNA as possible. Although their technique is elegant, it is relatively complex and can be applied readily only to plasmid cloning vectors. This excludes a large group of researchers that would like to clone into bacteriophage lambda.

2.6.3 Gubler and Hoffman Procedure

It is possible to combine the classical oligo(dT) primed synthesis of first strand with the Okayama and Berg second strand reaction conditions to produce a method that is more versatile than either alone (figure 3; 16). The Gubler and Hoffman method does not require the elaborate two-part cloning vector of Okayama and Berg or the

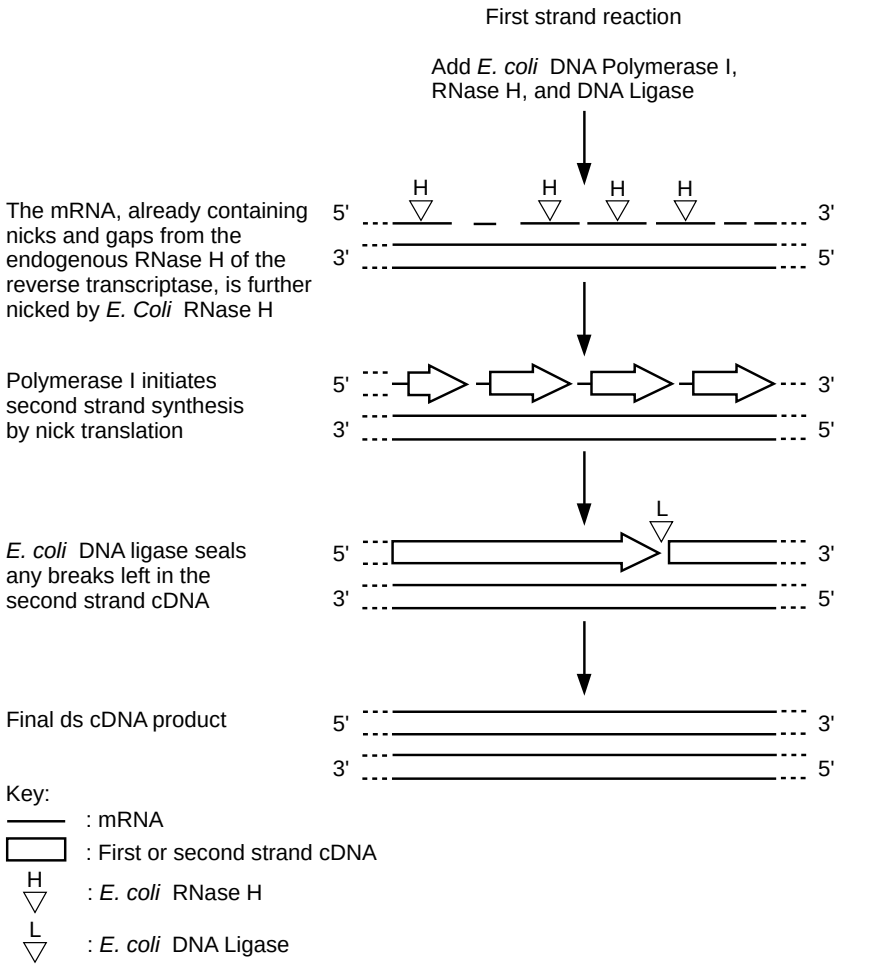

Figure 3. Second strand synthesis.

use of nuclease S1. Although some sequence information at the 5′ end of the RNA is usually lost by this method, the amount is minor compared to hairpin-primed/S1-digested second strand synthesis.

2.7 cDNA Synthesis System: Modification of the Gubler and Hoffman Procedure

A one-tube double-stranded cDNA synthesis system has been designed using M-MLV reverse transcriptase for first strand synthesis. Based on the Gubler and Hoffman procedure, the one-tube concept minimizes manipulations and improves the chances of constructing a library successfully. Using model RNAs as templates, we have optimized the reaction conditions of the cDNA Synthesis System to maximize length and yield of double-stranded cDNAs.

2.7.1 First Strand Reaction

The conditions for the first strand reaction are similar to the conditions first recommended for the use of M-MLV reverse transcriptase (17). However, actinomycin D (an inhibitor of second strand synthesis) is *not* added to the reaction. Using the conditions described in this manual, M-MLV reverse transcriptase does not hydrolyze sufficient mRNA to allow significant hairpin formation at the 3′ end of the first strand cDNA. Furthermore M-MLV reverse transcriptase does not use hairpins efficiently as a primer for initiating second strand synthesis. These properties of M-MLV reverse transcriptase are important for two reasons: second strand synthesis can be performed in the same tube as the first strand reaction without resorting to an intermediate extraction and precipitation, and the amount of sequence information lost from the library because of hairpin-primed second strand synthesis is minimized.

2.7.2 Second Strand Reaction

M-MLV reverse transcriptase contains an endogenous RNase H activity (18). This activity initially generates oligomers of 20 to 30 nucleotides on model RNA:DNA substrates, and with extended incubations, smaller digestion products 2 to 20 nucleotides long begin to appear (18). Thus, it is likely that the RNA portion of the RNA:cDNA duplex is both nicked and gapped following first strand synthesis. However, under the conditions that we describe for first strand synthesis, a significant number of the 5′-most RNA oligomers survive the RNase H activity of the M-MLV reverse transcriptase and serve as primers for near full-length second strand cDNAs synthesized by *E. coli* DNA polymerase I.

Second strand synthesis with *E. coli* DNA polymerase I can be performed with or without *E. coli* RNase H: the amount of sequence information retained at the end of the double-stranded cDNA that corresponds to the 5′ end of the message is affected by this choice. This is due to the multiple activities of the polymerase I. In addition to its polymerase activity, polymerase I is both a 5′ and 3′ exonuclease (19). The 5′ exonuclease activity allows polymerase I to replace a DNA strand in the nick translation reaction, degrading the strand in the 5′ to 3′ direction while the polymerase activity replaces the strand in the same direction. The 5′ exonuclease also functions as an RNase H exonuclease (19) and can degrade the RNA portion of an RNA:DNA hybrid. However, the blunt end of an RNA:DNA hybrid is not degraded by the RNase H activity of polymerase I.

The cDNA Synthesis System can be used to produce double-stranded cDNA by two procedures. In *Protocol 1* (figure 4), *E. coli* RNase H is included in the second strand reaction. The *E. coli* RNase H is an endonuclease (20) that will attack the RNA:DNA hybrid in a random fashion, producing nicks in addition to those produced by the reverse transcriptase. If enough additional nicks are introduced into the RNA at the 5′ end of the message before second strand synthesis commences, fragments may be produced that are too short to remain hybridized; thus, they will not be able to serve as primers. In addition, the 5′-most RNA oligomer that primes second strand DNA synthesis will continue to be degraded until only two ribonucleotides remain at the 5′ end of the second strand DNA (2). These are substrates for

Overview

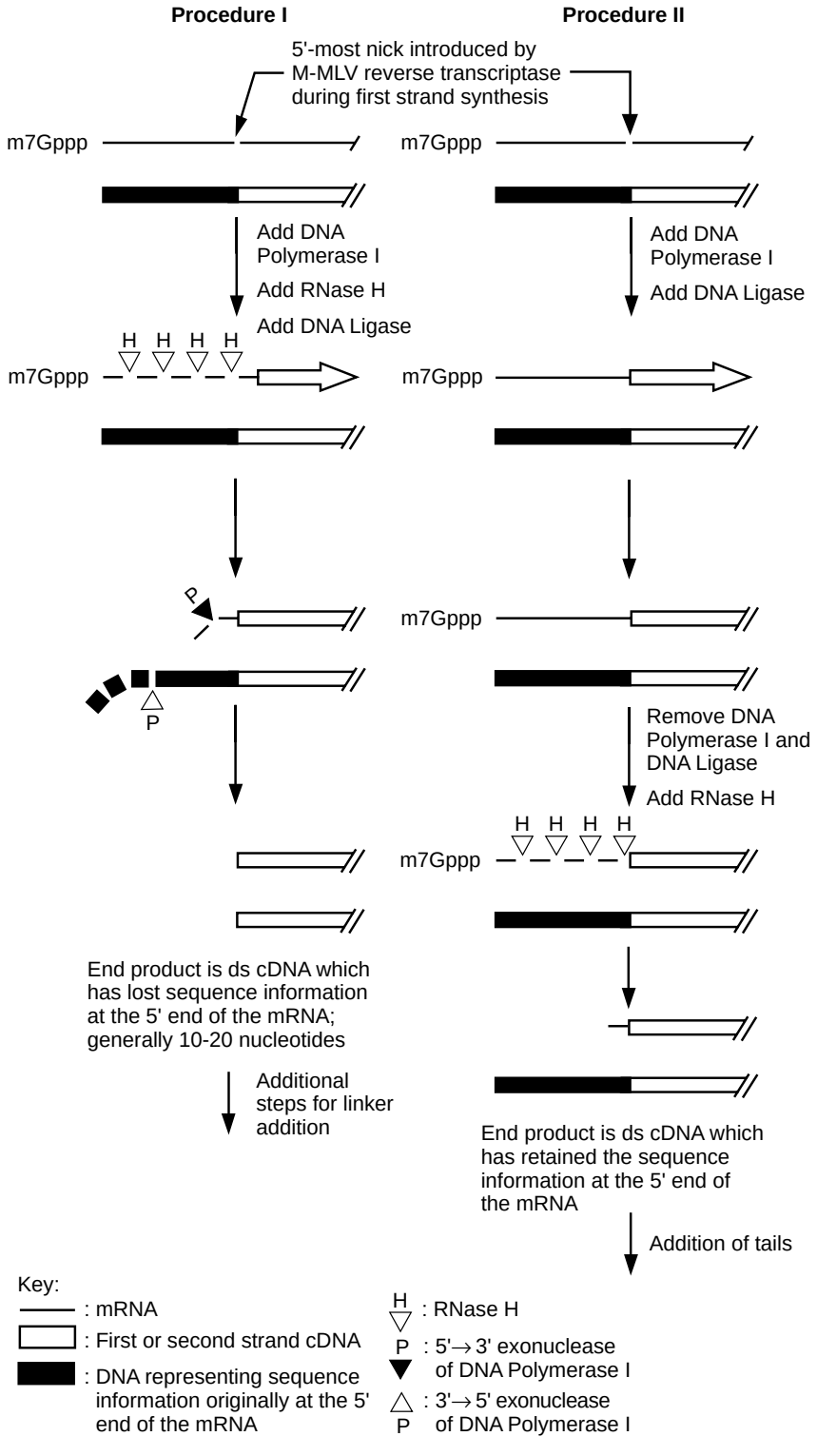

Figure 4. Comparison of second strand procedures using the cDNA synthesis system.

the polymerase I RNase H activity, and the remaining ribonucleotides will be removed. This also leaves the 3′ end of the first strand cDNA single stranded, making it a substrate for the 3′ exonuclease activity of polymerase I. The 3′ exonuclease of the polymerase I degrades this strand to, or very near to, a blunt end.

The result is a population of cDNAs that are blunt ended (or nearly so) and *that have lost some of the sequence information originally encoded in the 5′ end of the mRNA. The amount of sequence lost is random and corresponds to the length of the 5′-most RNA oligonucleotide that primed the second strand reaction*. Therefore, activities of the enzymes used in *Protocol 1* ensure loss of some information at the 5′ end of the RNA. For full-length cDNAs synthesized with M-MLV reverse transcriptase, this will generally be 10 to 20 nucleotides (2).

An alternate method, which retains more of the sequence information at the 5′ end of the RNA, is described in *Protocol 2* (figure 4). In this case, the second strand reaction contains only DNA polymerase I. Thus, the sole priming points will be the nicks introduced by the reverse transcriptase during the first strand reaction. Since M-MLV reverse transcriptase will produce nicks 10 to 20 bases from the 5′ end of the RNA in the hybrid (2), these will provide the 5′-most initiation points for second strand synthesis. If the DNA polymerase I has removed all of the internal RNA primers between the 5′ terminus of the RNA and its poly(A) tail by nick translation, the result will be a duplex that is entirely DNA (except for the surviving capped RNA 5′ oligonucleotide).

Since the loss of sequence information that occurs in *Protocol 1* begins with the hydrolysis of the 5′-most primer RNA by *E. coli* RNase H, when the second strand is synthesized in its absence, the 3′ terminus of the first strand cDNA is protected from the 3′ exonucleolytic activity of the polymerase I. If these molecules are tailed with terminal transferase and cloned into a plasmid, the sequence information at the 5′ end of the RNA will be reclaimed when the single-stranded stretch of the first strand cDNA is repaired *in vivo*. This method gives the same result as the Okayama and Berg protocol, but it does not require specialized DNAs. If *E. coli* RNase H is added *after* removal of the DNA polymerase I, the number of clones containing full-length inserts rises several fold.

For RNAs 1 to 2 kb long, *Protocol 2* and homopolymeric tailing help retain the most sequence information. For larger mRNAs, *Protocol 1* and linker addition are more effective because the sequence nearest the 3′ end of the mRNA is more likely to be deleted by tailing as the length of the mRNA increases (2).

Deletion of the sequence nearest the 3′ end of the mRNA is caused by internal and external tails competing for hybridization to the vector. Discontinuities in the second strand cDNA can provide sites for tailing, and double-stranded cDNA transcribed from a long template may be tailed at one or more internal positions within the second strand in addition to its true 3′ end. Thus, when the tailed double-stranded cDNA is annealed with a vector, the internal tails in the second strand can compete with the tail at the true 3′ terminus for hybridization to the vector. When hybridization to the vector occurs at an internally tailed site, sequence in the double-stranded cDNA between the hybridized tail and the true 3′ terminus of the second strand cDNA is lost during *in vivo* repair and replication of the plasmid after transformation into bacteria. The result is preferential cloning of the ds cDNA sequence derived from the 5′ region of the mRNA.

In experiments performed at Invitrogen using either procedure with a 6.2-kb RNA as the substrate, approximately 20% of the clones examined were near full length (contained sequences corresponding to within 35 nucleotides of the 5′ terminus of the RNA), yet none were greater than 4 kb long (2). This result did not change significantly when the DNA ligase was omitted, implying that many internal 3′ termini in the second strand cDNA are not substrates for this enzyme. In contrast, addition of molecular linkers to double-stranded cDNA prepared by *Protocol 1* from the 6.2-

kb RNA yielded a large proportion (40%) of clones containing inserts without detectable deletions (2). Thus, when cloning double-stranded cDNA copies of long mRNAs, synthesize the double-stranded cDNA by *Protocol 1* and convert the ends to cohesive termini using molecular linkers.

2.8 Cloning Double-Stranded cDNA

Once the double-stranded cDNA has been synthesized successfully, it must be converted into a form that can be cloned. Further manipulation of the double-stranded cDNA will depend upon the type of cloning vector chosen. Three principal methods are used to prepare the double-stranded cDNA for cloning: tailing, repair of the termini to blunt ends, and addition of molecular linkers. Detailed protocols for cloning into plasmids (1,8,21) or bacteriophage (7,8) should be consulted.

2.8.1 Tailing with Terminal Transferase

One of the most popular methods for insertion of a cDNA into a plasmid is the addition of homopolymer tails to the DNA using terminal deoxynucleotidyl transferase (22,23). This method is fast and relatively simple. When dC tails of 20 to 30 nucleotides are added to the 3′ ends of the double-stranded cDNA, the cDNA can be annealed to a vector that has been linearized and tailed with dGTP. These annealed molecules do not need to be ligated and will transform *E. coli*. If the vector is linearized at a *Pst* I site and tailed with dGTP, the restriction endonuclease site is restored after repair *in vivo*. Thus, the cloned fragments can be excised with *Pst* I.

The cDNA prepared by either of the methods in this manual can be tailed and cloned into a dG-tailed vector, but we recommend *Protocol 2* for preparing cDNA to be cloned by tailing. The cDNA prepared in this manner will retain the greatest amount of sequence information originally encoded at the 5′ end of the message.

2.8.2 Blunt-end Ligation

If the cDNA will be cloned into either bacteriophage or plasmids by blunt-end ligation or the addition of molecular linkers (8), then *Protocol 1* must be followed even though 10 to 20 nucleotides corresponding to the 5′ end of the RNA will be lost. If the RNase H is left out of the second strand reaction (*Protocol 2*), the double-stranded cDNA will contain residual RNA at one end. These molecules are very poor substrates for ligation, even if the ends are blunt, so the RNA must be removed by the combined action of *E. coli* RNase H and DNA polymerase I. The cDNA prepared by *Protocol 1* should be treated with T4 DNA polymerase to render all of the termini perfectly blunt (24). After this treatment, the cDNA can be ligated using T4 DNA ligase into any plasmid that has been digested to produce blunt ends. This approach is simple but relatively inefficient and generally will result in fewer transformants per microgram of cDNA. Molecular linkers are a better alternative.

2.8.3 Addition of Linkers

If it is important to maximize the number of clones obtained from a cDNA sample, the inefficient step of blunt-end ligation should be avoided by the addition of molecular linkers. Linker addition (8) is generally performed when cloning into bacteriophage vectors (7) but is equally applicable to plasmids (1). A disadvantage of linker addition lies in the number of enzymatic steps needed. The cDNA must first be methylated with the methylase specific for the linker that is to be added so that any internal restriction endonuclease sites resist cleavage. After repairing the termini with T4 DNA polymerase, linkers are added with T4 DNA ligase by blunt-end ligation. In this case, the ligation is very efficient because the linkers are present at a very high concentration in the reaction. The DNA is then digested with the appropriate restriction endonuclease to expose the cohesive ends. The digestion products must be purified, usually by column chromatography (7,8), to separate the digested linkers from the cDNA before it can be ligated into a vector. Now that the cDNA has cohesive termini, it is ligated into vectors much more efficiently.

Methods

3

3.1 Components

The components of the cDNA Synthesis System are as follows. Components are provided in sufficient quantities to perform five reactions containing 2 to 10 µg of mRNA per reaction. Store all components at –20°C.

Component	Amount
5X First Strand Buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$]	1 ml
$Oligo(dT)_{12-18}$ (0.5 mg/ml)	50 µl
Control RNA (0.5 mg/ml)	7.5 µg
Cloned M-MLV Reverse Transcriptase (200 U/µl)	10,000 units
0.1 M DTT	250 µl
10X Second Strand Buffer [188 mM Tris-HCl (pH 8.3), 906 mM KCl, 100 mM $(NH_4)_2SO_4$, 46 mM $MgCl_2$, 37.5 mM DTT, 1.5 mM β-NAD^+]	250 µl
E. coli DNA Polymerase I (10 U/µl)	500 units
E. coli RNase H (2 U/µl)	40 units
5X RNase H buffer [100 mM Tris-HCl (pH 7.5), 100 mM KCl, 50 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT]	250 µl
E. coli DNA Ligase	100 units
10 mM dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP)	100 µl
DEPC-treated Water	2 × 1.25 ml

Note: Because the cDNA Synthesis System uses modifications of established procedures, we strongly recommend that all users of this system read Section 2, *Overview*, carefully before proceeding to use the system. This will help you choose the cloning system and method of cDNA synthesis best suited to your needs.

3.2 mRNA Purification

One of the most important steps in the synthesis of double-stranded cDNA is to isolate intact mRNA. One of the most popular and effective methods is based upon the denaturing power of the chaotropic salt, guanidinium isothiocyanate (10). In the presence of high concentrations of 2-mercaptoethanol, this salt effectively denatures ribonucleases and prevents degradation of the RNA. This method can be modified in several ways and is adequately described in *Molecular Cloning: A Laboratory Manual* (8).

After the initial purification of the RNA, it is necessary to select $poly(A)^+$ RNA by chromatography on oligo(dT) cellulose (11). This ensures the most efficient cDNA synthesis and will minimize spurious copying of rRNAs. Additionally, the presence of other RNAs may inhibit the activity of the reverse transcriptase by providing sites of adventitious binding.

An RNase-free environment should be maintained for the RNA at all stages of its purification. The following guidelines should be observed at all times.

1. Never assume that anything is RNase free. Exception: sterile plasticware, preferably individually wrapped, may be used without additional treatment.

2. Dedicate laboratory glassware for use with RNA and clearly mark it. Do not send anything outside of the laboratory to be washed once it has been rendered RNase free. There is no control over what your colleagues send to the dishwasher, and the commonly used RNases (A and T1) are extremely stable to heat and detergents.
3. Autoclaving may not irreversibly inactivate the above RNases. Other RNases, especially if fungal in origin, may resist inactivation by autoclaving. Autoclave stock solutions and maintain sterility to *prevent* microbial growth, *not* to inactivate RNases.
4. Use at least one of the following methods to ensure that your reagents are RNase free. If possible, use two methods for added safety.
   a. Treat all water used for making solutions with diethylpyrocarbonate (DEPC) as follows. From a freshly opened bottle, add DEPC to achieve a final concentration of 0.01% (v/v). Mix well and let the treated water sit overnight at room temperature loosely capped. Autoclave for 30 min to remove traces of DEPC.
   b. Bake glassware at 150°C for at least 4 h.
   c. Rinse glassware with 0.5 N NaOH, followed by DEPC-treated water.
   d. Treat microcentrifuge tubes by immersion in a beaker of water to which DEPC has just been added. Let them sit overnight, remove the water, and then autoclave the entire beaker. Dry the tubes at 120° to 125°C.
   e. Treat solutions with DEPC as described above. This is not appropriate with reagents containing amines such as Tris or nucleotides. Salts commonly used for precipitation (e.g., sodium acetate, NaCl) can be treated in this manner. (Treatment of ammonium acetate with DEPC is not recommended. Salt solutions where some ammonia is present should be made up with DEPC-treated water, as in step 4a., then autoclaved.) A good substitute for Tris is HEPES (pKa = 7.5).
5. Always wear disposable gloves to prevent contamination.
6. Maintain a separate set of automatic pipettes for manipulating RNA. At the very least, maintain a set only for use with RNases.

3.3 General Comments

An outline for using the cDNA Synthesis System is presented in figure 5. You may want to refer to it before you begin, or during, the procedure. Certain manipulations required at several points in the procedures are described to streamline the protocols.

"Extract with phenol" means add an equal volume of phenol:$CHCl_3$:isoamyl alcohol [25:24:1 (v/v/v)], vortex thoroughly, and centrifuge for 5 min in a microcentrifuge at room temperature to separate the phases. Carefully remove an amount of the upper, aqueous phase that corresponds to 90% of the original volume (prior to the addition of the phenol:$CHCl_3$:isoamyl alcohol mixture), and transfer it to a fresh microcentrifuge tube.

"Precipitate with ethanol" means add 0.5 volumes of 7.5 M ammonium acetate followed by 3 volumes of absolute ethanol (–20°C). Vortex the mixture thoroughly and centrifuge immediately at room temperature for 15 min in a microcentrifuge. Remove the supernatant carefully and overlay the pellet with 0.5 ml of absolute ethanol (–20°C). Centrifuge for 30 s and remove the ethanol. Dry the pellet at room temperature, or in a 37°C heat block, for 10 min to evaporate the excess ethanol.

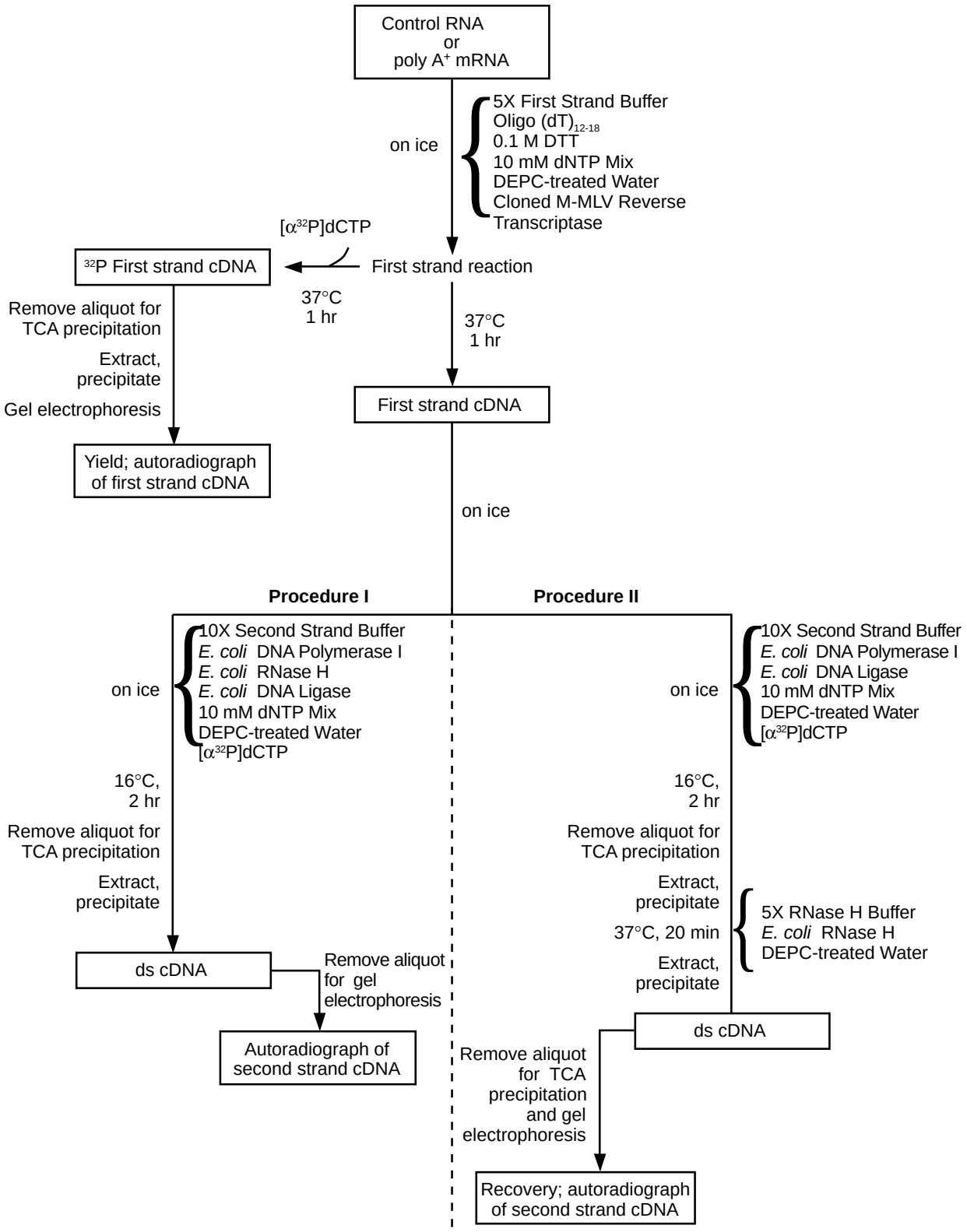

Figure 5. Protocol flow diagram.

3.3.1 First Strand Reaction

This protocol is written for a reaction using 5 μg of the control RNA provided with the kit as a substrate. We highly recommend that you use the control RNA if you are not familiar with cDNA synthesis. When you are ready to proceed with your own material, substitute 2 to 10 μg of poly(A)$^+$ RNA for the control RNA.

A portion of the first strand reaction mix is removed in order to introduce a ^{32}P tracer label for gel analysis and to determine the yield of the cDNA by precipitation with trichloroacetic acid (TCA). We strongly recommend that the first strand reaction be monitored by the inclusion of ^{32}P as described in the procedure. If this is not done, the volumes of the second strand components must be increased by a factor of 1.25 to allow for the increased volume contribution of the first strand reaction. In Section 3.6, *Second Strand Synthesis*, we have provided the volumes to add to the first strand reaction for both situations. Be certain to make the correct additions.

3.3.2 Second Strand Reaction

The second strand reaction can be performed in two ways. *Protocol 1* includes *E. coli* RNase H in the reaction during the second strand synthesis, while *Protocol 2* calls for the use of RNase H in a separate reaction after the *E. coli* DNA polymerase I has been removed. Please refer to Section 3.7, *cDNA Synthesis: Modification of the Gubler and Hoffman Procedure,* to determine which method is appropriate for your needs.

The second strand reaction includes [α^{32}P] dCTP so that the second strand products can be quantitated and examined by gel electrophoresis. If molecular linkers will be added to the cDNA for cloning, often they are labeled with ^{32}P so that the success of the linker ligation and subsequent digestion with the appropriate restriction endonuclease can be monitored (8). Although the cDNA is labeled with ^{32}P in the second strand, labeled linkers still can be used to analyze the linker ligation and restriction endonuclease digestion steps because the polyacrylamide gel used for the analysis resolves the cDNA from the linkers.

3.4 Advance Preparations

3.4.1 First Strand Reaction

You will need the following items *not* included in this system:

- DEPC-treated 1.5-ml microcentrifuge tubes
- Automatic pipettes capable of dispensing 1 to 20 μl, 20 to 200 μl, and 200 μl to 1 ml
- Individually wrapped, disposable tips for automatic pipettes
- Disposable gloves
- 37°C water bath
- 1 μCi [α^{32}P] dCTP (400 to 3,000 Ci/mmol) for each first strand reaction
- 500 ml 10% (w/v) TCA containing 1% (w/v) sodium pyrophosphate (store at 4°C)
- Glass fiber filters (1 × 2 cm), Whatman GF/C or equivalent
- Buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)]
- 0.25 M Na_2EDTA (pH 7.5)
- 7.5 M ammonium acetate (DEPC-treated and filtered through a sterile 0.45-μm nitrocellulose filter)
- Absolute ethanol (–20° C)

Note: The use of Phenol: Chloroform:Isoamyl Alcohol [25:24:1 (v/v/v)] is recommended. If making your own, saturate the redistilled phenol with TEN buffer, not with distilled water.

Components needed from the cDNA Synthesis System:

Component
5X First Strand Buffer
Oligo(dT)$_{12-18}$
Control RNA
Cloned M-MLV Reverse Transcriptase
0.1 M DTT
10 mM dNTP Mix
DEPC-treated Water

Do not thaw or remove these components until you are ready to begin. Always keep the enzymes at –20°C unless you are making an addition to a reaction. Keep the components on ice while preparing the reactions.

3.4.2 Second Strand Reaction

All of the items listed under the *Advance Preparations* for first strand synthesis will also be needed for second strand synthesis. In addition, you will need the following items *not* included with this system:

16°C water bath
10 µCi [α^{32}P] dCTP (400 to 3,000 Ci/mmol) for each reaction mixture
TE buffer [10 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA]

Components needed from the cDNA Synthesis System:

Component
10X First Strand Buffer
E. coli DNA Polymerase I
E. coli RNase H
5X RNase H Buffer*
E. coli DNA Ligase
10 mM dNTP Mix
DEPC-treated Water

*The 5X RNase H Buffer is needed only if the second strand synthesis is performed using *Protocol 2*.

Do not thaw or remove these components until you are ready to begin. Always keep the enzymes at –20°C unless you are making an addition to a reaction. Keep the components on ice while preparing the reactions.

3.5 First Strand Synthesis

The following reaction is for 5 µg of the control RNA. The reaction conditions have been designed to convert 2 to 10 µg of mRNA into double-stranded cDNA, so it is not necessary to make any volume adjustments when copying your own mRNA.

1. To a sterile, DEPC-treated 1.5-ml microcentrifuge tube on ice, add the following:

Component	**Volume (µl)**
5X First Strand Buffer	10
10 mM dNTP Mix	2.5
Oligo(dT)12-18	5
Control RNA	10
0.1 M DTT	5
DEPC-treated water	15

The final composition of the first strand reaction is:

50 mM Tris-HCl (pH 8.3)
75 mM KCl
3 mM $MgCl_2$
10 mM DTT
500 µM each dATP, dCTP, dGTP and dTTP
50 µg/ml oligo(dT)$_{12-18}$
100 µg/ml poly $(A)^+$ RNA
10,000 units/ml cloned M-MLV reverse transcriptase

2. Prepare a separate tube containing 1 µCi of [α–^{32}P] dCTP tracer.
3. Initiate the reaction by adding 2.5 µl of M-MLV reverse transcriptase to the first strand reaction. Mix and immediately transfer 10 µl to the tube containing the [α-^{32}P] dCTP tracer.
4. Incubate both reactions at 37°C for 1 h, then place the reactions on ice.
5. Terminate the tracer reaction by adding 1 µl of 0.25 M Na_2EDTA (pH 7.5) and keep on ice.
6. Take the 40-µl reaction mixture and proceed with the second strand reaction as described in the next section (see *Second Strand Synthesis, Protocol 1* or *Protocol 2*).
7. While the second strand reaction is incubating, add 89 µl of water to the tracer reaction and spot duplicate aliquots (5 µl) on glass fiber filters.
8. Dry one of the filters under a heat lamp or at room temperature. It will be used to determine the specific activity of the dCTP in the reaction mixture.
9. Wash the other filter in a beaker sequentially with ice-cold TCA (three times) using about 50 ml per wash for 5 min each. Then wash the filter once with 50 ml of 95% ethanol at room temperature for 5 min. Let the filter dry under a heat lamp. This filter will be used to determine the yield of first strand cDNA.
10. Count the filters in standard scintillant to determine the amount of ^{32}P in the reaction mixture as well as the amount of ^{32}P that was incorporated. See Section 3.7, *Analysis of the cDNA Products*, for information needed to convert the data into yield of first strand cDNA.
11. Extract the remaining 90 µl of the tracer reaction with phenol and ethanol precipitate. This material will be used for gel analysis as described in Section 3.7. The pellet may be stored for several days at 4°C.

3.6 Second Strand Synthesis

3.6.1 Protocol 1: cDNA Synthesis for Linker Addition

1. Add the following components, in the order indicated, to the remainder of the first strand reaction (step 7 of the first strand reaction). Keep all components on ice prior to adding them so that the temperature of the reaction mixture does not rise. The volumes of the second strand components that are added depend upon the First Strand Reaction Volume (FSRV):

	Volume (µl)	
Component	**40-µl FSRV**	**50-µl FSRV**
DEPC-treated Water	230.6	288.25
10 mM dNTP Mix	6	7.5
10X Second Strand Buffer	32	40
[α^{32}P] dCTP	1	1.25
E. coli DNA Polymerase I	8	10
E. coli RNase H	1.4	1.75
E. coli DNA Ligase	1	1.25

The final composition of this reaction mixture is

25 mM Tris-HCl (pH 8.3)
100 mM KCl
10 mM $(NH_4)_2SO_4$
5 mM $MgCl_2$
250 µM each dATP, dCTP, dGTP, dTTP
0.15 mM NAD
5 mM DTT
250 U/ml DNA polymerase I
8.5 U/ml RNase H
30 U/ml DNA ligase

Note: In addition to the above components, the M-MLV reverse transcriptase is still present, although its activity is diminished considerably due to its short half-life (25). The oligo$(dT)_{12-18}$ primer is also present at the outset of the reaction, but it is rapidly degraded by the 3´ exonuclease activity of the DNA polymerase I.

2. Vortex the tube gently to mix and incubate the reaction for 2 h at 16°C. Do not allow the temperature to rise above 16°C.
3. Place the reaction tube on ice and add 25 µl of 0.25 M Na_2EDTA (pH 7.5).
4. Remove 10 µl from the second strand reaction mixture, and add it to 90 µl of water. Spot a 5-µl aliquot of this dilution onto a glass fiber filter and let it dry under a heat lamp or at room temperature. This will be used to determine the specific activity of the dCTP in the reaction mixture.
5. Remove a 10-µl aliquot from the second strand reaction mixture and spot it onto another glass fiber filter. Wash this filter in a beaker sequentially with ice-cold TCA (three times) using about 50 ml per wash for 5 min each. Then wash the filter once with 50 ml of 95% ethanol at room temperature for 5 min. Let the filter dry under a heat lamp. This filter will be used to determine the yield of second strand cDNA.
6. Extract the remainder of the reaction (325 µl if a tracer reaction was used in the first strand reaction and 405 µl if it was not used) with phenol and ethanol precipitate.
7. Dissolve the pellet in 200 µl of sterile TE buffer, add 100 µl of 7.5 M ammonium acetate and 500 µl of ethanol to reprecipitate.
8. Dissolve the dry pellet in 20 µl of sterile TE buffer. Reserve 2 µl for gel analysis and use the remainder for the addition of linkers.
9. Count the filters in standard scintillant to determine the amount of ^{32}P in the reaction mixture as well as the amount of ^{32}P that was incorporated. Refer to Section 3.7 for information needed to convert the data into yield of second strand cDNA.

3.6.2 Protocol 2: cDNA Synthesis for Tailing

1. Add the following components, in the order indicated, to the remainder of the first strand reaction (step 7 of the first strand reaction). Keep all components on ice prior to adding them so that the temperature of the reaction mixture does not rise. The volumes of the second strand components that are added depend upon the FSRV:

	Volume (µl)	
Component	**40-µl FSRV**	**50-µl FSRV**
DEPC-treated Water	232	290
10 mM dNTP Mix	6	7.5
10X Second Strand Buffer	32	40
[$\alpha^{32}P$] dCTP	1	1.25
E. coli DNA Polymerase I	8	10
E. coli DNA Ligase	1	1.25

The final composition of this reaction mixture is:

25 mM Tris-HCl (pH 8.3)
100 mM KCl
10 mM $(NH_4)_2SO_4$
5 mM $MgCl_2$
250 µM each dATP, dCTP, dGTP, dTTP
0.15 mM NAD
5 mM DTT
250 U/ml DNA polymerase I
30 U/ml DNA ligase

Note: In addition to the above components, the M-MLV reverse transcriptase is still present, although its activity is diminished considerably due to its short half-life (25). The oligo$(dT)_{12\text{-}18}$ primer is also present at the outset of the reaction, but it is rapidly degraded by the 3′ exonuclease activity of the DNA polymerase I.

2. Vortex the tube gently to mix and incubate the reaction for 2 h at 16°C. Do not let the temperature rise above 16°C.
3. Place the reaction tube on ice and add 25 µl of 0.25 M Na_2EDTA (pH 7.5).
4. Remove 10 µl from the reaction mixture and add it to 90 µl of water. Spot a 5-µl aliquot onto a glass fiber filter and let it dry under a heat lamp or at room temperature. This will be used to determine the amount of ^{32}P in the reaction mixture.
5. Remove a 10-µl aliquot from the second strand reaction mixture and spot it onto another glass fiber filter. Wash this with TCA as described for the first strand reaction. This will be used to determine the incorporation of ^{32}P into the second strand cDNA.
6. Extract the remainder of the reaction (325 µl if a tracer reaction was used in the first strand reaction and 405 µl if it was not used) with phenol and ethanol precipitate.
7. Dissolve the pellet in 200 µl of sterile TE buffer and reprecipitate from ammonium acetate with ethanol.
8. Dissolve the dry pellet in 20 µl of sterile TE buffer. Reserve 2 µl for gel analysis.
9. To the remaining 18 µl, add the following in the order indicated:

Component	**Volume (µl)**
5X RNase H Buffer	20
DEPC-treated Water	61
E. coli RNase H	1

The final composition of the reaction mixture is:

20 mM Tris-HCl (pH 7.5)
10 mM $MgCl_2$
20 mM KCl
0.1 mM Na_2EDTA
0.15 mM NAD
0.1 mM DTT
20 U/ml RNase H

10. Vortex the tube, transfer it to a 37°C water bath, and incubate for 20 min.
11. Place the reaction on ice and add 1 µl of 0.25 M Na_2EDTA (pH 7.5).
12. Extract the reaction with phenol and ethanol precipitate.
13. Dissolve the pellet in 200 µl of sterile TE buffer and reprecipitate from ammonium acetate with ethanol.
14. Dissolve the pellet in 10 µl of sterile TE buffer and count 1 µl by TCA precipitation on a glass fiber filter as described previously to determine the recovery of double-stranded cDNA. The remaining 9 µl will be used for gel analysis and for tailing. For information concerning the tailing reaction, consult section 2.8.
15. Count the filters in standard scintillant to determine the amount of ^{32}P in the reaction mixture, the amount of ^{32}P incorporated into second strand cDNA, and the amount of cDNA recovered after the RNase H digestion. See Section 3.7 for information needed to convert the data into yield and recovery of the double-stranded cDNA.

3.7 Analysis of the cDNA Products

3.7.1 First Strand Yield

The overall yield of the first strand reaction is calculated from the amount of acid-precipitable counts determined as described in First Strand Synthesis. In order to perform the calculation, you must first determine the specific activity (SA) of the radioisotope in the reaction mixture. The specific activity is defined as the counts per minute (cpm) of an aliquot of the reaction mixture divided by the quantity (pmol) of the same nucleotide in that aliquot. The amount of dCTP in the 5-µl aliquot is calculated by multiplying the amount of unlabeled dCTP in the reaction mixture (25,000 pmol) by the actual amount sampled:

$$\text{pmol dCTP/5 µl} = (25{,}000 \text{ pmol dCTP/50 µl}) \times (10 \text{ µl/100 µl}) \times 5 \text{ µl} \quad [1]$$

$$= 250 \text{ pmol dCTP/5 µl}$$

For [α^{32}P] dCTP, the specific activity is given by the relationship:

$$\text{SA (cpm/pmol dCTP)} = \frac{\text{cpm/5 µl}}{\text{250 pmol dCTP/5 µl}} \quad [2]$$

The amount of dCTP contributed by the radiolabeled material is insignificant relative to the unlabeled nucleotide and is ignored in equation 2.

Now that the specific activity is known, the yield of first strand product in the 10-µl reaction can be calculated from the amount of acid-precipitable counts determined from the washed filter:

$$\text{Yield (µg DNA)} = \frac{(\text{cpm}) \times (100 \text{ µl/5 µl}) \times (4 \text{ pmol dNTP/pmol dCTP})}{(\text{cpm/pmol dCTP}) \times (3{,}030 \text{ pmol dNTP/µg DNA})} \quad [3]$$

The correction in the numerator takes into account that, on the average, four nucleotides will be incorporated into the cDNA for every dCTP that is scored by this assay. The factor in the denominator is the amount of nucleotide that corresponds to 1 µg of single-stranded DNA.

Example: The unwashed filter gave 100,000 cpm when it was counted. The specific activity of the dCTP is given by equation 1:

$$\text{SA} = \frac{\text{100,000 cpm/5 µl}}{\text{250 pmol dCTP/5 µl}}$$

$$= 400 \text{ cpm/pmol dCTP}$$

If the TCA-precipitated filter gave 10,000 cpm, then the yield of DNA is calculated using equation 3:

$$\text{Yield} = \frac{(10{,}000 \text{ cpm}) \times (100 \text{ µl/5 µl}) \times (4 \text{ pmol dNTP/pmol dCTP})}{(400 \text{ cpm/pmol dCTP}) \times (3{,}030 \text{ pmol dNTP/µg DNA})}$$

$$= 0.65 \text{ µg DNA (first strand)}$$

This represents the actual yield in the 10-µl reaction. The total yield in the remaining 40 µl would be four times this amount, or 2.6 µg.

3.7.2 Second Strand Yield

The yield of the second strand reaction is determined in an identical manner, except that the composition of the second strand reaction dictates the specific activity of the isotope. The amount of dCTP in the aliquot spotted for specific activity determination is given by equation 4:

$$\text{pmol dCTP/5 µl} = (80{,}000 \text{ pmol dCTP/345 µl}) \times (10 \text{ µl/100 µl}) \times 5 \text{ µl} \quad [4]$$

$$= 116 \text{ pmol dCTP/5 µl}$$

For [α^{32}P] dCTP, the specific activity is given by the relationship:

$$\text{SA (cpm/pmol dCTP)} = \frac{\text{cpm/5 µl}}{\text{116 pmol dCTP/5 µl}} \quad [5]$$

Again, the amount of dCTP contributed by the radiolabeled material is insignificant relative to the unlabeled nucleotide and is ignored in equation 5.

Now that the specific activity is known, the yield of second strand product can be calculated from the amount of acid-precipitable counts determined from the washed filter.

$$\text{Yield (µg DNA)} = \frac{(\text{cpm}) \times (345\ \mu\text{l}/10\ \mu\text{l}) \times (4\ \text{pmol dNTP/pmol dCTP})}{(\text{cpm/pmol dCTP}) \times (3{,}030\ \text{pmol dNTP/µg DNA})} \quad [6]$$

Example: The unwashed filter gave 29,000 cpm when counted. The specific activity of the dCTP is given by equation 5:

$$\text{SA} = \frac{29{,}000\ \text{cpm}/5\ \mu\text{l}}{116\ \text{pmol}/5\ \mu\text{l}} = 250\ \text{cpm/pmol dCTP}$$

If the TCA-precipitated filter gave 12,000 cpm, then the yield of DNA is calculated using equation 6:

$$\text{Yield} = \frac{(12{,}000\ \text{cpm}) \times (345\ \mu\text{l}/10\ \mu\text{l}) \times (4\ \text{pmol dNTP/pmol dCTP})}{(250\ \text{cpm/pmol dCTP}) \times (3{,}030\ \text{pmol dNTP/µg DNA})} = 2.2\ \mu\text{g DNA (second strand)}$$

Note: This corresponds to 4.4 µg of double-stranded cDNA.

3.7.3 Amount Recovered After RNase H Digestion

The amount of double-stranded cDNA recovered following RNase H digestion *(Protocol 2)* is determined from the number of cpm in the 1-µl aliquot counted after TCA precipitation (step 14 of *Protocol 2*):

$$\text{µg ds cDNA} = \frac{(\text{cpm}) \times (9\ \mu\text{l}/1\ \mu\text{l}) \times (4\ \text{pmol dNTP/pmol dCTP})}{(\text{cpm/pmol dCTP}) \times (1{,}515\ \text{pmol dNTP/µg ds DNA})} \quad [7]$$

The factor in the denominator is the amount of nucleotide which corresponds to 1 µg of double-stranded DNA, and the SA of the dCTP is the same as calculated by equation 5. Generally, you can expect to recover 50% to 75% of the amount of double-stranded cDNA calculated from the second strand yields.

Example: The TCA-precipitated filter gave 34,000 cpm when counted. The amount of double-stranded cDNA recovered is given by equation 7:

$$\text{µg ds cDNA} = \frac{(34{,}000) \times (9\ \mu\text{l}/1\ \mu\text{l}) \times (4\ \text{pmol dNTP/pmol dCTP})}{(250\ \text{cpm/pmol dCTP}) \times (1{,}515\ \text{pmol dNTP/µg ds DNA})} = 3.2\ \mu\text{g}$$

In this case, the recovery is 3.2 µg/4.4 µg, or 72%.

3.7.4 Gel Analysis

The products of the first and second strand reactions can be analyzed by alkaline agarose gel electrophoresis (26) if they are labeled with ^{32}P. This system denatures the hybrids formed in the first and second strand reactions and fractionates the DNA by length. With ^{32}P-labeled molecular weight markers, the sizes of the cDNA products can be estimated and the success of the synthesis evaluated.

3.7.5 Advance Preparations

You will need the following items *not* included in the cDNA Synthesis System:

Buffers	Composition
10X gel buffer	0.3 M NaCl 20 mM Na_2EDTA (pH 7.5)
10X electrophoresis buffer	0.3 M NaOH 20 mM EDTA
2X sample buffer	20 mM NaOH 20% glycerol 0.02% bromophenol blue

Other items needed:

7.5 M ammonium acetate

Ethanol

DNA fragments labeled with ^{32}P using T4 polynucleotide kinase or the large fragment of DNA polymerase I (Klenow fragment) for molecular weight markers. The 1 Kb DNA Ladder is useful for this purpose.

3.7.6 Procedure

1. Assemble a vertical gel mold with 1.5-mm spacers; a 15 × 17-cm format is sufficient for most applications. A horizontal gel apparatus may also be used.
2. Prepare a 1.4% agarose gel in 1X gel buffer. Microwave for 3 min to dissolve the agarose.
3. Swirl the dissolved agarose solution gently and pour it into the mold.
4. Insert a comb (5- to 10-mm wide teeth) and let the gel solidify 30 min. Remove the comb and place the gel in the apparatus.
5. Fill the apparatus with 1X electrophoresis buffer. If a horizontal apparatus is used, submerge the gel for 1 h prior to electrophoresis so that the alkaline buffer can diffuse into the gel.
6. Prepare the samples by addition of appropriate amounts of 2X sample buffer and water to achieve a final concentration of about 2,000 cpm/µl. Load approximately 10,000 counts in each lane so that the autoradiographic images will be uniform. Prepare the ^{32}P DNA fragments for electrophoresis in the same manner as the cDNA.
7. Load the samples and attach to a power supply. Perform electrophoresis at 75 mA (regulated) if a vertical apparatus is used and at 7.5 V/cm (regulated) if a horizontal apparatus is used. Continue electrophoresis until the dye migrates approximately 10 cm.
8. Remove the gel, and dry it in a gel dryer for several hours with heat and vacuum. Place onto x-ray film overnight at room temperature for a trial exposure. Repeat the exposure, if necessary, for an appropriate amount of time.

This gel resolves fragments in the range of 500 to 10,000 nucleotides. For the control RNA, full-length material (2,300 nucleotides) should be evident in both the first and second strand reactions. Lower molecular weight material will be evident on both lanes. If the distribution of radioactivity is determined from the gel, usually 30% to 40% of the first strand reaction will be full-length material. The second strand reaction should look similar to the first strand reaction. There may be a small amount of a fragment at approximately 2,800 nucleotides in both lanes. This is double-stranded cDNA that was made by hairpin-primed synthesis and should not be a cause for concern as it usually comprises <10% of the double-stranded cDNA products.

When a heterogeneous messenger RNA population is examined, a smear of labeled fragments in the range of 500 to several thousand nucleotides will be seen in both lanes. Ideally, one would like to compare the distribution of the starting mRNA population with the products of the first strand reaction on the same gel, but this often is impractical because the amount of RNA is limiting. Additionally, the alkaline gel described above could not be used because the RNA would be hydrolyzed by the alkali, so an alternative system would be needed (*e.g.*, glyoxal; reference 27).

4 Troubleshooting

4.1 mRNA Purification

The quantity and quality of the isolated mRNA influences the quantity and quality of the double-stranded cDNA product. The need for all of the precautions outlined in Section 3, *Methods*, of this manual cannot be stressed enough. Unfortunately, it is not possible to evaluate the quality of a heterogeneous population of RNA without resorting to methods such as Northern blots (28), which require homologous probes, or translation *in vitro* if the product can be identified immunologically by Western blots or by immunoprecipitation. If these techniques are not applicable, it is possible to evaluate the efficacy of an isolation procedure by adding a known RNA to an initial lysate as a tracer, and then assaying for the integrity of the tracer RNA by probing Northern blots after the RNA has been purified. The most convenient source of RNA is material that has been synthesized *in vitro* with SP6 or T7 RNA polymerases. Probes can be prepared easily by nick translation of the plasmid or by synthesis of the RNA complement of the tracer RNA.

We recommend the guanidinium isothiocyanate procedure (10) if another protocol has not been used before. Since all starting materials do not require the denaturing power of this technique, other, less rigorous procedures may be adequate in some cases.

The oligo(dT) cellulose column is extremely important in determining the purity of the mRNA preparation. Certain lots of oligo(dT) cellulose may function better than others, resulting in a greater enrichment of polyadenylated molecules. Inclusion of dimethyl sulfoxide at 9% (v/v) in the chromatography buffers may decrease the nonspecific binding of ribosomal RNA to the oligo(dT) cellulose.

4.2 First Strand Reaction

Before attempting to generate double-stranded cDNA for cloning, use the control RNA to verify the performance of the cDNA Synthesis System *in your hands.* This will allow you to become familiar with the protocols before using a precious RNA sample.

The yield of the first strand cDNA will generally be between 30% and 40% with the control RNA, and upon gel electrophoresis, a prominent band at 2,300 nucleotides will be evident. If the yield appears to be good, but the gel does not contain full-length material, then it is likely that RNase contamination has been introduced. The contamination may be in the RNA itself or in one of the components. The integrity of the RNA may be checked by running 1 µg on a glyoxal gel (26) or on a formaldehyde gel (28) followed by staining with ethidium bromide. If the RNA appears to be intact, then one of the reagents may be contaminated. Since determining this is a tedious procedure, most laboratories will throw out suspect materials rather than try to identify a contaminated reagent. In our experience, contamination is a rare event if the recommended precautions are followed.

If the yield of first strand is low, again, there may be a contamination problem, but for it to affect the yield of acid-precipitable material, it would have to be extremely severe. If low yield is a problem and you have determined that the RNA is intact, be certain that the directions for the composition of the first strand reaction are being followed explicitly. The following points should also be noted.

(a) Unlike the AMV reverse transcriptase, incubation of reactions with the M-MLV reverse transcriptase at temperatures >37°C will have a deleterious effect upon the yield because of the thermal lability of the enzyme. Be certain that the water bath you are using does not fluctuate greatly in temperature.

(b) It is not necessary to include exogenous RNase inhibitors when using the control RNA. This also will be true for any mRNA population being used as a template if it is RNase free.

(c) Do not add sodium pyrophosphate to the reaction mixtures. Although reported to increase the yield of full-length cDNA and prevent hairpin synthesis with the avian enzyme (29), it is a potent inhibitor of M-MLV reverse transcriptase.

(d) Do not pulse the reaction mixture by adding aliquots of M-MLV reverse transcriptase periodically during the incubation. The primary result will be increased hairpin synthesis due to the elevated RNase H activity.

(e) M-MLV reverse transcriptase has a half-life of approximately 15 min in a first strand reaction, so increasing the incubation time beyond 1 h will not increase appreciably the yield of cDNA. Since the enzyme reverse transcribes RNA at about 200 nucleotides/minute, 1 h is more than enough time to copy most mRNAs.

If low yields are obtained with an mRNA population and nuclease does not appear to be a problem, there may be an inhibitor present (*e.g.*, sodium dodecyl sulfate, EDTA, or guanidinium salts) that has carried over from the purification of the RNA. This possibility can be checked by adding a small amount of the control RNA to some of the RNA preparation in question and making first strand from this mixture. The yield should be similar to that obtained with the control RNA alone, and gel analysis should reveal the 2,300-nucleotide fragment.

Occasionally, a batch of radioisotope will not perform as expected. Although this is rare when used at the low specific activities recommended, it is worth checking, especially if the ^{32}P isotope is over 2 weeks old.

Please note that ^{3}H-labeled nucleoside triphosphates can be used in place of ^{32}P to determine yields, but corrections for the quenching of ^{3}H must be applied to the data. In general, ^{3}H will count with about 25% to 50% of the efficiency of ^{32}P. The counting efficiency should be determined empirically.

4.3 Second Strand Reaction

The second strand reaction is not as sensitive to RNase contamination as the first strand reaction because the RNA moiety is now part of an RNA:DNA hybrid. However, it is still good practice to adhere to the strict guidelines that have been discussed previously.

The second strand reaction should be carried out on the first strand synthesized from the control RNA to verify that the system is working and that you are familiar with the reaction. The second strand reaction is generally very efficient, and yields approaching 100% are common. Thus, if the amount of first strand synthesized is 1 µg, then the yield of the second strand should be approximately 1 µg. The actual yield will deviate from 100%, mostly due to differences in the base composition of the first and second strands and errors in radioactivity measurement. Yields of >75% to 80% are satisfactory. The second strand reaction will be more successful if the following guidelines are observed:

(a) The second strand reaction is performed by diluting the first strand reaction 8-fold. This dilution factor is critical for efficient second strand synthesis.

(b) The temperature of the second strand reaction should remain at 16°C. If the temperature is allowed to rise, the DNA polymerase can synthesize unusual structures that result from the enzyme priming from a hairpin formed on the strand just synthesized. This is a common problem with polymerase I in nick translation reactions if the temperature rises above 20°C.

(c) The concentration of the polymerase I is set at 250 units/ml to ensure maximal

Troubleshooting Guide

continuous second strand synthesis with large templates (2).

4.4 General Guidelines

We recommend the following books for protocols on the generation of cDNA libraries in plasmid and phage vectors:

1. *DNA Cloning: A Practical Approach (Vol. 1)* (D.M. Glover, ed.) IRL Press Limited, Oxford, England.
2. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual, 2nd edition*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
3. *Current Protocols in Molecular Biology* (1989) (F.M. Ausubel et al. eds.) Greene Publishing Associates and Wiley-Interscience, New York, New York.

References

5

1. Williams, J.G. (1981) in *Genetic Engineering: 1* (R. Williamson, ed.) Academic Press, London, pp. 1-59.
2. D'Alessio, J.M. and Gerard, G.F. (1988) *Nucleic Acids Res. 16,* 1999.
3. Young, R.A. and Davis, R.W. (1983) *Science, 222,* 778.
4. Helfman, D.M., Feramisco, J.R., Fiddes, J.C., Thomas, G.P., and Hughes, S.H. (1983) *Proc. Nat. Acad. Sci. 80,* 31.
5. Grunstein, M. and Hogness, D. (1975) *Proc. Nat. Acad. Sci., 72,* 3961.
6. Benton, W.D. and Davis, R.W. (1977) *Science, 196,*180.
7. Huynh, T.V., Young, R.A., and Davis, R.W. (1985) in *DNA Cloning: A Practical Approach (Vol. 1)* (D.M. Glover, ed.) IRL Press Limited, Oxford, England, pp. 49–78.
8. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
9. Kirby, K.S. (1968) *Methods Enzymol., Vol. XII* (L. Grossman and K. Moldave, eds.) Academic Press, New York, pp. 87-98.
10. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) *Biochemistry 18,* 5294.
11. Aviv, H. and Leder, P. (1972) *Proc. Nat. Acad. Sci. 69,* 1408.
12. Verma, I.M. (1981) in *The Enzymes: Nucleic Acids (Part A) XIV* (P.D. Boyer, ed.) Academic Press, New York, pp. 87–103.
13. Kotewicz, M.L., D'Alessio, J.M., Driftmier, K.M., Blodgett, K.P., and Gerard, G.F. (1985) *Gene 35,* 249.
14. Efstradiatis, A., Kafatos, F.C., Maxam, A.M., and Maniatis, T. (1976) *Cell 7,* 279.
15. Okayama, H. and Berg, P. (1982) *Molecular and Cell Biology 2,* 161.
16. Gubler, U. and Hoffman, B.J. (1983) *Gene 25,* 263.
17. Gerard, G.F. (1985) *Focus® 7:1,* 1.
18. Gerard, G.F. (1981) *Biochemistry 20,* 256.
19. Lehman, I.R. (1981) in *The Enzymes: Nucleic Acids (Part A) XIV,* (P.D. Boyer, ed.) Academic Press, New York, pp. 15–37.
20. Crouch, R.J. (1981) in *Gene Amplification and Analysis (Vol. 2)* (J.G. Chirikjian and T.S. Papas, eds.) Elsevier/North Holland, New York, pp. 218–228.
21. Watson, C.J. and Jackson, J.F. (1985) in *DNA Cloning: A Practical Approach (Vol. 1)* (D.M. Glover, ed.) IRL Press Limited, Oxford, England, pp. 79–88.
22. Wu, R. and Deng, G. (1983) *Methods Enzymol., Vol. 100,* (R. Wu, L. Grossman, and K. Moldave, eds.) Academic Press, New York, pp. 96–116.
23. Michaelson, A.M. and Orkin, S.K. (1982) *J. Biol. Chem. 257,* 14773.
24. Challberg, M.D. and Englund, P.T. (1980) *Methods Enzymol. Vol. 65* (L. Grossman and K. Moldave, eds.) Academic Press, New York, pp. 39–43.
25. McDonnell, M.W., Simon, M.N., and Studier, F.W. (1977) *J. Mol. Biol. 110,* 119.
26. McMaster, G.K. and Carmichael, G.G. (1977) *Proc. Nat. Acad. Sci. 74,* 4835.
27. Alwine, J.C., Kemp, D.J., and Stark, G.R. (1977) *Proc. Nat. Acad. Sci. 74,* 5350.
28. Lehrach, H., Diamond, D., Wozney, J.M., and Boedtker, H. (1977) *Biochemistry 16,* 4743.
29. Kacian, D.L. and Meyers, J.C. (1976) *Proc. Nat. Acad. Sci. 73,* 2191.

6

Related Products

Product	Size	Cat. No
Combination Systems		
GeneTrapper™ cDNA Positive Selection System and ElectroMAX™ DH10B™ Competent Cells	one set	10356-038
GeneTrapper cDNA Positive Selection System and MagnaSep Magnetic Particle Separator	one set	10356-046
GeneTrapper cDNA Positive Selection System and ElectroMAX DH10B Competent Cells plus MagnaSep Magnetic Particle Separator	one set	10356-053
GeneTrapper cDNA Positive Selection System and UltraMAX™ DH5α-FT™ Competent Cells	one set	10356-145
GeneTrapper cDNA Positive Selection System and UltraMAX DH5α-FT Competent Cells plus MagnaSep Magnetic Particle Separator	one set	10356-137
Products for Purification		
MessageMaker® mRNA Isolation System	10 reactions	10551-018
MessageMaker Reagent System	10 reactions	10298-016
TRIzol® Reagent	100 ml	15596-026
	200 ml	15596-018
TRIzol LS Reagent	100 ml	10296-010
	200 ml	10296-028
Guanidine Hydrochloride	500 g	15502-016
Guanidine Isothiocyanate	500 g	15535-016
Oligo(dT) Cellulose	5 g	15940-026
DEPC-Treated Water	4 × 0.25 ml	10813-012
Phenol	500 g	15509-037
Phenol:Chloroform:Isoamyl Alcohol, (25:24:1, v/v/v)	100 ml	15593-031
Ribonuclease Inhibitor, Cloned	1,000 units	15518-012
RNase *AWAY* Reagent	250 ml	10328-011
Products for Transformation		
UltraMAX DH5α-FT Competent Cells		
MAX Efficiency DH5α Competent Cells	1 ml	18258-012
MAX Efficiency DH10B Competent Cells	1 ml	18297-010
IPTG	1 g	15529-019
S.O.C. Medium	10 × 10 ml	15544-018
X-gal	100 mg	15520-034
Products for Electroporation		
ElectroMAX DH10B Cells	5 × 0.1 ml	18290-015
ElectroMAX DH12S Cells	5× 0.1 ml	18312-017
Other Related Products		
SuperScript™ II RNase H- Reverse Transcriptase	10,000 units	18064-014
GeneTrapper cDNA Positive Selection System	5 reactions	10356-020
SuperScript Plasmid System for cDNA Synthesis and Cloning	3 reactions	18248-013
SuperScript Choice System for cDNA Synthesis and Cloning	3 reactions	18090-019
SuperScript Lambda System for cDNA Synthesis and Cloning	3 reactions	18248-013

Related Products

Product	Size	Cat. No
Second Strand Buffer	500 µl	10812-014
Oligo(dT)$_{12-18}$ Primer	25 µg	18418-012
Glycogen	100 µl	10814-010
Ampicillin, Sodium salt	20 ml	11593-019
cDNA Size Fractionation Columns	3 columns	18092-015
1 Kb DNA Ladder	250 µg	15615-016
	1 mg	15615-024
0.24-9.5 Kb RNA Ladder	75 µg	15620-016
Acrylamide	500 g	15512-023
Agarose	100 g	15510-019
	500 g	15510-027
10 mg/ml Ethidium Bromide	10 ml	15585-011
10X TAE Buffer	1 L	15558-042
	4 L	15558-026
	10 L	15558-034
10X TBE Buffer	1 L	15581-044
	10 L	15581-028
1 M Tris-HCl (pH 7.5)	1 L	15567-027

Notes

Notes

Notes

United States Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, California 92008
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 603 7229
Email: tech_service@invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287
Email: eurotech@invitrogen.com

International Offices:
Argentina 5411 4556 0844
Australia 1 800 331 627
Austria 0800 20 1087
Belgium 0800 14894
Brazil 0800 11 0575
Canada 800 263 6236
China 10 6849 2578
Denmark 80 30 17 40

France 0800 23 20 79
Germany 0800 083 0902
Hong Kong 2407 8450
India 11 577 3282
Italy 02 98 22 201
Japan 03 3663 7974
The Netherlands 0800 099 3310
New Zealand 0800 600 200
Norway 00800 5456 5456

Spain & Portugal 900 181 461
Sweden 020 26 34 52
Switzerland 0800 848 800
Taiwan 2 2651 6156
UK 0800 838 380
For other countries see our website

www.invitrogen.com

Part No. 18267A
Date: 012502