evrogen

MINT

cDNA synthesis kit

Cat#SK001

User Manual

This product is intended for research use only.

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I. Intended use

MINT cDNA synthesis kit is designed to synthesize full-length-enriched double stranded (ds) cDNA from total or polyA$^+$ RNA. Synthesized cDNA can be used in various applications including preparation of non-directionally cloned cDNA libraries, Virtual Northern blot (Franz *et al.*, 1999), subtractive hybridization (SSH, Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999), and cDNA normalization using duplex-specific nuclease (Zhulidov *et al.*, 2004; Zhulidov *et al.*, 2005) and Trimmer kit (Evrogen cat #NK001).

II. Method overview

MINT cDNA synthesis kit is based on a novel technology utilizing the specific features of MMLV-based reverse transcriptase (RT). The workflow to prepare cDNA using the MINT cDNA synthesis kit is shown in **Fig. 1**.

First strand cDNA synthesis starts from 3'-primer comprising oligo(dT) sequence to anneal to polyA$^+$ stretch of RNA. When RT reaches the 5' end of the mRNA, it adds several non-template nucleotides, primarily deoxycytidines, to the 3' end of the newly synthesized first-strand cDNA (Schmidt & Mueller, 1999). This oligo(dC) stretch base pairs to complementary oligo(dG) sequence located at the 3' end of a special 30-mer deoxyribooligonucleotide called PlugOligo. RT identifies PlugOligo as an extra part of the RNA-template and continues first strand cDNA synthesis to the end of the oligonucleotide, thus incorporating PlugOligo sequence into the 5' end of cDNA.

The last 3'-dG residue of the PlugOligo is a terminator nucleotide comprising 3'-phosphate group. This blocking group prevents unwanted annealing and extension of the PlugOligo. Under standard conditions RT can hardly use PlugOligo as a template, however our special IP-solution (solution for Incorporation of PlugOligo sequence) dramatically increases the efficiency of this process.

At the third step, ds cDNA synthesis is performed using PCR amplification. cDNA synthesized by the MINT kit is full-length-enriched and comprises the same adapter sequence at both 3'- and 5'- ends.

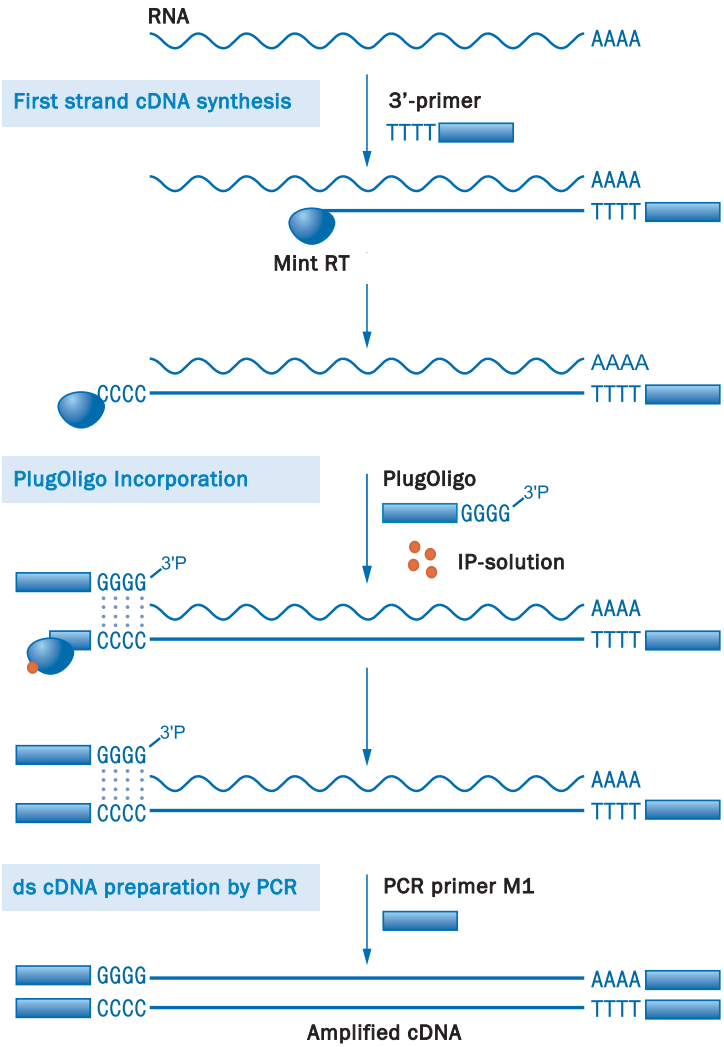

Figure 1. Schematic outline of Mint cDNA synthesis workflow

III. Kit components and storage conditions

A. List of kit components

MINT cDNA Synthesis Kit provides components for 20 reactions of ds cDNA synthesis. Package of the kit includes a free sample of Mint reverse transcriptase for first-strand cDNA synthesis and a free trial-size Encyclo PCR kit (Cat #PK001). For important information about the use of MINT kit, please see the ENDNOTES at the end of this User Manual.

Component	Amount
5X First-Strand Buffer	80 µl
DTT (20mM)	30 µl
dNTP mix (10mM each)	80 µl
PlugOligo adapter (15 µM)* 5'-AAGCAGTGGTATCAACGCAGAGTACGGGGG-P-3'	25 µl
3'-primer (10 µM)* 5'-AAGCAGTGGTATCAACGCAGAGTAC(T)30VN -3'	25 µl
Mint Reverse Transcriptase	20 µl
IP-solution	130 µl
Control total RNA template (0.5 mg/ml)	15 µl
PCR Primer M1 (10 µM) 5'-AAGCAGTGGTATCAACGCAGAGT-3'	100 µl
50X Encyclo polymerase mix	50 µl
10X Encyclo buffer	300 µl
Sterile RNase free water	1.8 ml

**Rsa* I restriction site is underlined; N = A, C, G or T; V = A, G or C

Shipping & Storage: Encyclo Polymerase mix, Mint Reverse Transcriptase, PlugOligo adapters and control RNA are shipped at -20°C (or below). All other components of the kit can be shipped at ambient temperature. Once arrived, the kit must be kept at -20°C.

B. Materials required but not included:

- Biology grade mineral oil

- RNase Inhibitor (20 u/µl, Ambion) /optional/

- Blue ice

- Sterile 0.5 or 0.2 ml PCR tubes, and sterile microcentrifuge 1.5 ml tubes

- Agarose gel electrophoresis reagents and equipment

- DNA size markers (1-kb DNA ladder)

IV. General considerations

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

1. We recommend that you perform a positive control cDNA synthesis from the total RNA provided in the kit in parallel with your experiment. This control is performed to verify that all components are working properly.

2. After solution is just thawed we strongly recommend that you mix it by gently flicking the tube and spin the tube briefly in a microcentrifuge to deposit contents at the bottom before use.

3. Add enzyme to reaction mixture last and thoroughly mix it by gently pipetting the reaction mixture up and down.

4. Do not increase the amount of enzymes added or concentration of RNA and cDNA in the reactions. The amounts and concentrations have been carefully optimized.

5. Wear gloves to protect RNA and cDNA samples from degradation by nucleases.

6. Use PCR pipette tips containing hydrophobic filters to minimize contamination.

V. RNA requirements

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Note: The sequence complexity and the average length of the MINT cDNA noticeably depend on the quality of starting RNA material.

1. The protocol has been optimized for both total and polyA$^+$ RNA. The minimum amount of starting material for cDNA synthesis is

250 ng of total RNA or 100 ng of polyA$^+$ RNA. However, for better results we recommend that you use at least 1-1.5 µg of total RNA or 0.5 µg of polyA$^+$ RNA to start first strand cDNA synthesis.

Note: Representation of the resulting amplified cDNA depends on the initial amount of RNA used for the first-strand cDNA synthesis. Thus, if possible, use the higher starting amounts of RNA indicated in the following protocol.

2. There are a number of methods suitable for RNA isolation providing stable RNA preparation from a majority of biological objects, for example Trizol method (GIBCO/Life Technologies), Chomczynski & Sacchi method (Chomczynski & Sacchi, 1987), and RNeasy kits (QIAGEN).

3. After RNA isolation, we recommend RNA quality estimation using gel electrophoresis before the first-strand cDNA synthesis. Denaturing formaldehyde/agarose gel electrophoresis should be performed as described (Sambrook *et al*., 1989). Alternatively, standard agarose/ethidium bromide (EtBr) gel electrophoresis can be used to quickly estimate RNA quality (see Appendix A for recommendations to perform a non-denaturing agarose gel electrophoresis of RNA).

The following characteristics indicate successful RNA preparation:

- For mammalian total RNA, two intensive bands at approximately 4.5 and 1.9 kb should be observed against a light smear. These bands represent 28S and 18S rRNA. The ratio of intensities of these bands should be about 1.5-2.5:1. Intact mammalian polyA$^+$ RNA appears as a smear sized from 0.1 to 4-7 (or more) kb with faint 28S and 18S rRNA bands.

- In the case of RNA from other sources (plants, insects, yeast, amphibians), the normal mRNA smear on the non-denaturing agarose gel may not exceed 2-3 kb. Moreover, the overwhelming majority of invertebrates have 28s rRNA with a so-called "hidden break" (Ishikawa, 1977). In some organisms the interaction between the parts of 28s rRNA is rather weak, so the total RNA preparation exhibits a single 18s-like rRNA band even on a non-denaturing gel. In other species the 28s rRNA is more robust, so it is still visible as a second band.

Note: If your experimental RNA is shorter than expected and/or degraded according to electrophoresis data, prepare fresh RNA after checking the quality of RNA purification reagents. If problems persist, you may need to find another source of tissue/cells. In some cases, partially degraded RNA is only available (e.g. tumor samples or hard treated tissues). This RNA can be used for cDNA preparation, however the cDNA sample will contain reduced number of full-length molecules.

4. Commonly, genomic DNA contamination does not exceed the amount seen on the agarose/EtBr gel as a weak band of high molecular weight. Such contamination does not affect cDNA synthesis. DNase treatment to degrade genomic DNA is not recommended. In some cases, excess of genomic DNA can be removed by LiCl precipitation or by phenol:chloroform extraction.

VI. cDNA preparation protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Important Note: To verify that all kit components are working properly, perform a positive control cDNA synthesis with human RNA provided in the kit in parallel with your experimental samples.

A. First-strand cDNA synthesis and PlugOligo incorporation

Note: During the first strand cDNA synthesis, the use of a thermal cycler for incubation steps is recommended. Using the air thermostat may require additional optimization.

1. For each RNA sample, combine the following reagents in a sterile thin 0.2 ml (or 0.5 ml) tube:

x µl	Sterile water
1-3 µl	RNA sample (containing 0.25 - 2 µg of total or 0.1-1.0 µg of polyA+ RNA) For the control reaction use 2 µl of the control RNA
1 µl	3'-primer
1 µl	PlugOligo adapter
5 µl	**Total volume**

Note: Before taking aliquots, heat the RNA samples at 65°C for 1-2 min and mix the content by gently flicking the tubes to prevent RNA aggregation. Spin the tubes briefly in a microcentrifuge.

2. Gently pipette the reaction mixtures and spin the tubes briefly in a microcentrifuge.

3. If you use a thermal cycler that is not equipped with a heated lid, overlay each reaction with a drop of molecular biology grade mineral oil. This will prevent the loss of volume due to evaporation.

4. Close the tubes and place them into a thermal cycler.

5. Incubate the tubes in a thermal cycler at 70°C for 2 min (use heated lid).

6. Decrease the incubation temperature to 42°C. Keep the tubes in the thermal cycler at 42°C for a time required to prepare RT Master mix (from 1 to 3 min).

7. Simultaneously with steps 5-6 prepare a RT Master mix for all reaction tubes by combining the following reagents in the order shown:

per rxn (the recipe must be adjusted for multiple samples)

2 µl	5X First-Strand Buffer
1 µl	DTT (20 mM)
1 µl	dNTP mix (10 mM each)
1 µl	Mint Reverse transcriptase
5 µl	**Total volume**

If required, 0.5 µl of RNase Inhibitor (20 u/µl, Ambion) can be added to the reaction.

8. Gently pipette the RT Master mix and spin the tube briefly in a microcentrifuge.

9. Add 5 µl of the RT Master mix into each reaction tube from Step 6. Gently pipette the reaction mix and if required spin the tubes briefly in a microcentrifuge to deposit contents at the bottom.

Note: Do not remove the reaction tubes from the thermal cycler except for the time necessary to add RT Master mix.

10. Incubate the tubes at 42°C for 30 min, after that proceed immediately to step 11.

11. Add 5 µl of the IP-solution to each reaction tube, mix by gently pipetting, if required spin the tubes briefly in a microcentrifuge and continue incubation of the tubes at 42°C for 1h 30 min.

Note: Do not remove the reaction tubes from the thermal cycler except for the time necessary to add IP-solution.

12. Place the tubes on ice to stop reaction.

Note: A brown sediment may be generated in the reaction(s). It does not affect following procedures.

First strand cDNA prepared can be used immediately for ds cDNA synthesis (Section VI.B) or stored at -20°C up to three months.

B. ds cDNA synthesis by PCR amplification

Important Notes:

1. Use of the optimal number of PCR cycles ensures that the ds cDNA remains in the exponential phase of amplification. This is crucial for many applications like Virtual Northern blot (Franz *et al*., 1999) or selective subtraction hybridization (Diatchenko *et al*., 1996; Diatchenko *et al*., 1999). PCR overcycling yields nonspecific PCR products and is extremely undesirable for these applications. PCR undercycling results in a lower yield of PCR product. The optimal number of PCR cycles must be determined individually for each experimental sample. The protocol provided includes the procedure of evaluative PCR in a small reaction volume to determine the optimal number of PCR cycles (section B1) and subsequent full-size preparation of ds cDNA (section B2).

2. In parallel with your experimental samples we recommend that you perform a positive control PCR with the first strand cDNA obtained from the control human RNA provided in the kit. This control is used to verify that all components are working properly.

3. Cycling parameters in this protocol have been optimized for a MJ Research PTC-200 DNA. Optimal parameters may vary with different thermal cyclers, polymerase, and templates.

B1. Evaluative PCR

1. For each first strand cDNA sample prepare PCR Master Mix by combining the following reagents in the order shown*:

40 µl	Sterile water
5 µl	10X Encyclo PCR Buffer
1 µl	dNTP mix (10 mM each)
2 µl	PCR Primer M1
1 µl	50X Encyclo Polymerase Mix
1 µl	First-strand cDNA (from Step A.12)**
50 µl	**Total volume**

Notes: * The recipe is for three reactions of 16 ml and must be adjusted for multiple samples or other reaction volumes. In the case of multiple samples, first prepare a PCR Master Mix in a sterile 0.5 ml tube for all samples combining all reagents shown except the first-strand cDNA. Then aliquot 49 µl of the PCR Master Mix into the appropriate number of fresh sterile 0.5 ml tubes and add 1 µl of the first-strand cDNA solutions (from Step A.12).

** If your first-strand cDNA samples were stored at -20°C, pre-heat the first-strand cDNA reactions at 65°C for 1 min and mix contents by gently flicking the tube before taking aliquots. Store the remaining first-strand cDNA in blue ice if you plan to perform full-size cDNA preparation (section B.2) directly after evaluative PCR. If you plan to perform full-size cDNA preparation sometime later, store the remaining first-strand cDNA at -20°C.

2. Mix PCR components by gently flicking the tube. Spin the tube briefly in a microcentrifuge.

3. Aliquot 16 µl of PCR reaction into PCR tubes (three tubes for each first strand cDNA). Label the tubes as <S>1, <S>2, and <S>3, wherein <S> is a sample identifier.

Note: Thin-wall PCR tubes are recommended. These PCR tubes are optimized to ensure more efficient heat transfer and to maximize thermal-cycling performance. We recommend that you use 0.2 ml PCR tubes rather than 0.5 ml ones.

4. Overlay each reaction with a drop of mineral oil (15-20 µl). Close the tubes, and place them into a thermal cycler.

Note: Because of a small reaction volume, we recommend that you perform evaluative PCR under the mineral oil even if you use a thermal cycler equipped with a heated lid.

5. Commence thermal cycling using the following program:

Step	Number of cycles	Temperature
Initial denaturation	1	95°C for 1min
Cycling	X*	95°C for 15 s; 66°C for 20 s; 72°C for 3 min
Final Extension	1	66°C for 15 s; 72°C for 3 min

*X is a number of cycles shown in Table 1 for a given amount of total or polyA$^+$ RNA used in the first-strand synthesis.

Table 1. PCR cycling parameters

Total RNA (µg)	PolyA$^+$ RNA (µg)	Number of PCR cycles for tubes:		
		<S>1	<S>2	<S>3
2.0 or more	0.5-1.0	13-14	16-17	18-20
1.0-2.0	0.25-0.5	14-15	17-18	20-21
0.5-1.0	0.1-0.25	15-16	18-19	21-22
0.25-0.5	0.1 or less	17-18	20-21	23-24

Note: Cycling parameters in this protocol have been optimized for MJ Research PTC-200 DNA thermal cycler and Encyclo polymerase mix. Optimal parameters may vary with different thermal cyclers, polymerases, and templates. If you use another thermal cycler, additional optimization of PCR parameters may be required. See Troubleshooting Guide for details.

6. Analyze 4 µl aliquots of each PCR product alongside 0.1 µg of 1 kb DNA size marker on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare the PCR product you have obtained with that in **Fig. 2** (relative to the 1-kb DNA ladder size markers). Use guidelines below **Fig. 2** to determine samples with optimal number of PCR cycles.

Note: PCR product can be stored at -20°C up to three months. If amplified samples were frozen before electrophoresis, heat them at 72°C for 2 min and mix before loading onto the agarose gel.

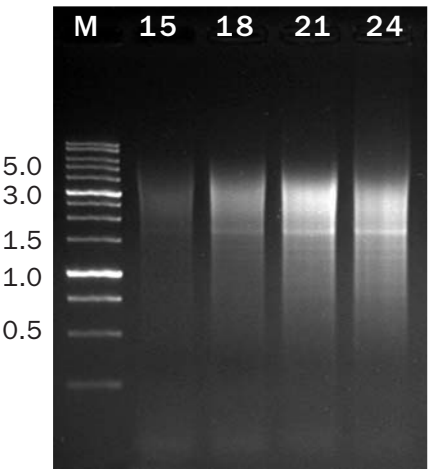

Figure 2. Agarose gel (1.2%) electrophoresis of amplified control cDNA after different number of PCR cycles. The number of PCR cycles performed is indicated at the top . M - 1 kb DNA size marker, SibEnzyme, Russia.

Analysis of PCR result

When the yield of PCR products stops increasing with every additional cycle, the reaction has reached its plateau. The optimal number of cycles for your experiment should be one or two cycles less than that needed to reach the plateau. Be conservative: when in doubt, it is better to use fewer cycles than too many.

Figure 2 shows a characteristic gel profile of ds cDNA synthesized using the control human brain total RNA following the Mint protocol outlined in Section VI.

In the experiment showed, 1µg of control RNA was used for cDNA synthesis. PCR products (4 µl per lane) after 15, 18, 21 and 24

cycles were analyzed on a 1.2% agarose/EtBr gel in 1X TAE buffer alongside 0.1 µg of 1 kb DNA size markers.

After 21 cycles, a smear appeared in the high-molecular-weight region of the gel, indicating that the reaction is overcycled. Because the plateau was reached after 20 cycles, the optimal cycle number for this experiment is 18-19.

Typical results, indicative of a successful PCR, should have the following characteristics:

1. A moderately strong cDNA smear of expected size distribution.

For cDNA prepared from most mammalian RNA, the overall signal intensity (relative to the 1-kb DNA ladder size markers, 0.1 µg run on the same gel) should be roughly similar to that shown for the control experiment in **Fig. 2**, lanes 2-3. If the intensity of the cDNA smear is much stronger than that shown for the control (e.g. as in lane 4), especially if no bright bands are distinguishable, this could indicate overcycled PCR (too many amplification cycles).

If the smear is much fainter (lane 1) and the size distribution is generally less than expected (for example less than 3 kb for cDNA from mammalian sources), this could indicate PCR undercycling (too few cycles).

Note: In general, ds cDNA size distribution should be similar to correspondent mRNA, which typically appears within the range of 0.5-10 kb on an agarose/EtBr gel. For most mammalian tissues visible smear of full-length-enriched cDNA should be within the range of 0.5-6 kb, while normal cDNA size for many non-mammalian species is less than 3 kb **(Fig. 3)**.

2. Several bright bands corresponding to abundant transcripts.

Figure 2 shows cDNA prepared from human brain tissues. This cDNA does not display bright bands because of very high complexity of the polyA$^+$ RNA fraction. The same brand pattern is typical also for mammalian cDNA prepared from spleen and thymus. At the same time, a

number of distinct bright bands are usually present in cDNA prepared from many tissue sources (see **Fig. 3**).

A very strong smear of cDNA in the experimental reaction without the characteristic bright bands could indicate PCR overcycling.

If the characteristic bands are present but weak, this could indicate PCR undercycling.

If PCR undercycling is observed in all <S>1-<S>3 samples, subject the samples to two or three additional PCR cycles (plus 1 final extension extra cycle) and recheck the products.

Note: Representation of the resulting amplified cDNA strongly depends on the initial number of target DNA molecules used for PCR amplification and accordingly on the number of PCR cycles required to amplify cDNA to the amount of 5-10 ng/µl (when it becomes visible on agarose/EtBr gel). Please remember that if cDNA requires more than 25 PCR cycles to be amplified it probably doesn't contain rare transcripts. If no or low yield PCR product is observed after 25cycles, see Troubleshooting Guide.

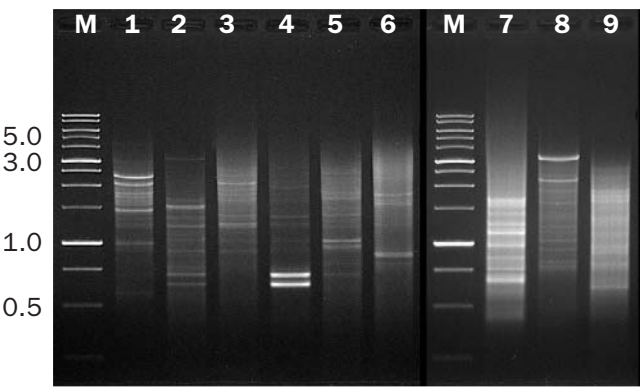

Figure 3. Agarose gel-electrophoresis (1.2%) of Mint-amplified cDNA from different sources: 1 - mouse liver; 2 - mouse skeletal muscle; 3 - mouse brain; 4 - human leucocytes; 5 - human lung; 6 - human skeletal muscle; 7 - mosquito larva; 8 - copepod *Pontella sp.*; 9 - tomato *Lycopersicon esculentum*. M - 1 kb DNA size marker, SibEnzyme, Russia.

B2. Full-size preparation of ds cDNA

1. For each first strand cDNA sample prepare a PCR Master Mix by combining the following reagents in the order shown:

per rxn (the recipe must be adjusted for multiple samples or other reaction volumes):

40 µl	Sterile water
5 µl	10X Encyclo PCR Buffer
1 µl	dNTP mix (10 mM each)
2 µl	PCR Primer M1
1 µl	50X Encyclo Polymerase Mix
49 µl	**Total volume**

2. Mix PCR components by gently flicking the tube. Spin the tube briefly in a microcentrifuge.

3. Aliquot 49 µl of PCR Master Mix into the appropriate number of PCR tubes.

Note: Thin-wall PCR tubes are recommended. These PCR tubes are optimized to ensure more efficient heat transfer and to maximize thermal-cycling performance. We recommend that you use 0.2 ml PCR tubes rather than 0.5 ml ones.

4. Add 1 µl aliquot of the first strand cDNAs (from step A.12) into the tubes.

Note: If your first-strand cDNA samples were stored at -20°C, pre-heat the first-strand cDNA reactions at 65°C for 1 min and mix by gently flicking the tubes before taking aliquots. Store the remaining first-strand cDNA at -20°C.

5. If you use a thermal cycler that is not equipped with a heated cover, overlay each reaction with a drop of mineral oil. Close the tubes, and place them into a thermal cycler.

6. Commence thermal cycling using the following program:

Step	Number of cycles	Temperature
Initial denaturation	1	95°C for 1min
Cycling	N*	95°C for 15 s; 66°C for 20 s; 72°C for 3 min
Final Extension	1	66°C for 15 s; 72°C for 3 min

*N is the optimal number of cycles determined as described in the section B.1.

7. Analyze 4 µl aliquots of each PCR product alongside 0.1 µg of 1 kb DNA size marker on a 1.2% agarose/EtBr gel in 1X TAE buffer.

Now, you have obtained amplified ds cDNA.

This cDNA can be stored at -20°C up to six months.

It can be used for non-directional cloning of cDNA library into TA-cloning vectors. Before cloning, purification of a PCR product is recommend using phenol-chloroform extraction or commercial PCR purification kits.

After polishing procedure (see Appendix B) the cDNA can be non-directionally cloned using blunt ends into any other vector of choice.

Note: Use unpurified PCR product for polishing.

This cDNA can be also used for Virtual Northern blot (see Appendix C), SSH (see Clontech SMART™ PCR cDNA Synthesis Kit User Manual, Cat #PT3041-1, Section VIII. Protocol for PCR-Select™ cDNA Subtraction), and cDNA normalization using Trimmer kit (Evrogen cat #NK001).

VII. Troubleshooting guide

A. Low molecular weight (size distribution < 1,5 kb), poor yield, or no PCR product observed for the control brain total RNA.

1. RNA may have degraded during storage and/or first-strand cDNA synthesis. Your working area, equipment, and solutions must be free of contamination by RNases. Check the quality of starting RNA on denaturing formaldehyde/agarose gel electrophoresis.

2. You may have made an error during the procedure, such as using a suboptimal incubation temperature or omitting an essential component. Carefully check the protocol and repeat the first-strand synthesis and PCR using 1 µl of the control RNA on a start. One of the typical mistakes is that RNA samples were not well mixed after defrosting.

3. PCR conditions and parameters might have been suboptimal. The optimal number of PCR cycles may vary with different PCR machines, shelf life of enzymes, or RNA samples. If your PCR reaches its plateau after 25 cycles or more, the conditions of your PCR may have not been optimal. Perform optimization of PCR parameters and repeat the PCR using a fresh aliquot of the first-strand cDNA product.

Optimization of PCR parameters:

a. Annealing temperature is too high: decrease the annealing temperature in increments of 2-4°C

b. Denaturation temperature is too high or low: optimize denaturation temperature by decreasing or increasing it in 1°C increments

c. Extension time too short: increase the extension time in 1-min increments.

4. If RNA degradation during cDNA synthesis is suspected, add 0.5 µl RNase Inhibitor (20 u/µl, Ambion) into the first-strand synthesis reaction as described in the section VI.A.7.

5. If the positive control does not work anyway, contact Evrogen technical support: customer-support@evrogen.com

B. Poor yield or no PCR product is generated from your experimental RNA. The PCR product has size distribution less than expected. At the same time, a high-quality PCR product is generated from the control RNA.

1. Your experimental RNA can be too diluted or degraded. If you have not already done so, analyze your RNA samples using formaldehyde/ agarose/EtBr gel electrophoresis to estimate its concentration and quality.

2. Experimental RNA can be partially degraded (e.g. due to RNase contamination) before or during the first-strand synthesis. Check the stability of your experimental RNA by incubating a small aliquot in water for 1 hr at 42°C. Then, analyze it on a formaldehyde/agarose/EtBr gel alongside an unincubated aliquot. If the RNA is degraded during the incubation, it will not yield good results in the first strand cDNA synthesis. In this case, re-isolate RNA. Perform several additional rounds of phenol:chloroform extraction because they can considerably increase RNA stability. Repeat the experiment using a fresh lot or preparation of RNA.

3. If RNA degradation during cDNA synthesis is suspected, add 0.5 µl RNase Inhibitor (20 u/µl, Ambion) into the first-strand synthesis reaction as described in the section VI.A.7.

4. Your experimental RNA sample can contain impurities that inhibit cDNA synthesis. In some cases, ethanol or LiCl precipitation of RNA can remove impurities. If this does not help, re-isolate RNA using a different technique.

C. The concentration of the PCR product generated from the experimental RNA samples is low, but the quality is good.

1. PCR undercycling resulting in a low yield of PCR product may be a problem. Subject the samples to two or three additional PCR cycles (plus 1 final extension extra cycle) and recheck the products. If the increase in the cycle number of does not improve the yield of PCR product, repeat PCR using a fresh aliquot of the first-strand cDNA. If you still obtain a low yield of PCR product, it could indicate a low yield of first-strand cDNA. Repeat the experiment using more RNA.

Note: We do not recommend that you use cDNA samples obtained after more than 25 PCR cycles because these samples may be not representative.

D. No expected bright bands are distinguishable in the PCR product visualized by agarose gel-electrophoresis.

1. For most cDNA samples, there should be several intensive bands distinguishable against the background smear when the PCR product is visualized on agarose gel. If these bands are expected but not visible, and the background smear is very intense, it could indicate PCR overcycling. Repeat PCR amplification with a fresh first-strand cDNA sample, using 2-3 fewer cycles.

Note: cDNA prepared from some mammalian tissues (e.g., human brain, spleen, and thymus) may not display bright bands due to a very high complexity of the starting RNA.

2. Gel running parameters can alter band visibility. Be sure to use the following conditions for optimal quality of your electrophoresis picture: a 1X TAE buffer instead of 1X TBE, a gel concentration of 1.1%-1.5% agarose, and running voltage up to 10 V/cm.

VIII. Appendixes

Appendix A. Recommendations to perform non-denaturing agarose gel electrophoresis of RNA

1. The following gel electrophoresis conditions are recommended:

- use 1X TAE buffer instead of 1X TBE
- use agarose gel in the concentration of 1.1%-1.2%
- add ethidium bromide (EtBr) to the gel and electrophoresis buffer to avoid the additional (potentially RNAse-prone) step of gel staining
- always use fresh gel and buffer as well as clean electrophoresis equipment for RNA analysis. Wear gloves to protect RNA samples from degradation by nucleases and avoid a hand contact with EtBr
- use running voltage up to 10 V/cm (10V per each cm of space between the electrodes in electrophoretic chamber). Do not use high voltage to avoid RNA degradation during electrophoresis.

2. Heat an aliquot of the RNA solution at 70°C for 1 min and place it on ice before loading on a gel.

3. Load a known amount of DNA or RNA ladder alongside your RNA sample as a standard for determining the RNA concentration. RNA concentration can be roughly estimated assuming that the efficiency of EtBr incorporation in rRNA is the same as for DNA (the ribosomal RNA may be considered a double-stranded molecule due to its extensive secondary structure).

4. The first sign of RNA degradation on the non-denaturing gel is a slight smear starting from the rRNA bands and extending to the area of shorter fragments. RNA showing this extent of degradation is still good for further procedures. However, if the downward smearing is so pronounced that the rRNA bands do not have a discernible lower edge, this RNA should be discarded.

Appendix B. ds cDNA Polishing

A. Materials required for cDNA polishing

- T4 DNA Polymerase
- 96% ethanol
- 3 M potassium acetate (pH5,2)
- 80% ethanol
- Tris-HCl-saturated phenol
- chlorophorm-isoamyl alcohol mix (24:1)
- 10mM Tris-HCl, pH 7,5 - 8,5

B. ds cDNA polishing protocol

1. Combine the following reagents in a sterile 0,5-ml tube:

50 µl of non-purified amplified dsDNA (after step B2.6)

1.0 µl dNTP mix (10 mM)

3.0 µl (15 units) of T4 DNA polymerase

Mix components by gently flicking the tube. Spin the tube briefly in a microcentrifuge.

2. Incubate the tube at room temperature for 5-10 min.

3. Purify blunt-ended cDNA using either phenol-chloroform extraction followed by ethanol precipitation, or commercial PCR purification kits.

4. Dissolve cDNA in 30-50 ml of 5mM Tris-HCl buffer (pH7.5-8.5) to the final DNA concentration of about 30-50 ng/µl.

This cDNA can be ligated to any adapter you choose. Consult your protocol for cDNA library construction.

Appendix C. Virtual Northern blot

To perform Virtual Northern blot, perform gel-electrophoresis of your unpurified PCR products on a 1.2% agarose/EtBr gel and transfer them onto a nylon membrane (Sambrook *et al*., 1989). Load 150-200 ng of ds cDNA onto a gel slot (about 8-12 µl of the PCR reaction). Use [P^{32}]-labeled probes specific to the genes of interest for hybridization with the membrane. For example, TurboBlotter equipment and protocol from Schleicher & Schuell should be used to perform Virtual Northern blot.

IX. References

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X. Related products

A. Encyclo PCR kit

Encyclo PCR kit is suitable for most PCR applications. It is especially recommended for cDNA amplification due to optimal combination of high fidelity and processivity provided by Encyclo polymerase mix.

Evrogen Encyclo polymerase mix produces high yields of PCR products from a wide variety of templates and is suitable for difficult templates, long PCR (up to 15 kb), and cloning.

Product	Cat.#	Amount
Encyclo PCR kit	PK001	100 PCR rxn (50 ml each)

B. TRIMMER kit (for nondirectionally cloned cDNA library preparation)

TRIMMER kit is designed to normalize full-length-enriched cDNA prepared using Evrogen MINT cDNA synthesis kit (Cat. #SK001) or Clontech SMART™ technology-based kits:

SMART™ PCR cDNA Synthesis Kit (Cat. #634902);

Super SMART™ PCR cDNA Synthesis Kit (Cat. #635000).

cDNA generated using these kits contains symmetric adapter sequences, 5'-aagcagtggtatcaacgcagagt-3', at both ends and can be used after normalization with TRIMMER kit to generate nondirectionally (randomly) cloned normalized cDNA library.

Product	Cat.#	Amount
TRIMMER	NK001	for 10 rxn

C. Duplex-specific nuclease, lyophilized

Duplex-Specific Nuclease (DSN) enzyme exhibited strong cleavage preference for ds DNA substrates and little activity against ss DNA. No significant cleavage activity on ss RNA substrates is observed. In addition, the nuclease effectively cleaves DNA molecules in DNA-RNA hybrid duplexes.

Analysis of DSN action on synthetic oligonucleotide substrates revealed that the enzyme discriminates between perfectly matched short DNA-DNA duplexes (8-12 bp) and duplexes of the same length with at least one mismatch.

DSN acquires its enzymatic activity in the presence of Mg^{2+} ions and is inhibited by EDTA. The pH and temperature optima for activity are 7-8 and 55-65°C, respectively. The nuclease is stable at temperatures below 70°C.

DSN is purified from Kamchatka crab hepatopancreas using acetone precipitation and following column chromatography on DEAE-MacroPrep, Phenyl-Agarose, Hydroxyapatite, Heparin-Sepharose, and Sephadex G-75 columns.

Product	Cat.#	Amount	Storage
Duplex-Specific Nuclease	EA001	50 Units*	+4°C
Duplex-Specific Nuclease	EA002	100 Units*	+4°C
Duplex-Specific Nuclease	EA003	10 Units*	+4°C

*DNAase activity was measured using modified Kunitz assay where unit definition was defined as: the amount of DSN added to 50 mg/ml calf thymus DNA that causes an increase of 0.001 absorbance units per minute. Activity assay was performed at 25°C, in 50 mM Tris-HCl buffer, pH 7.15, containing 5 mM $MgCl_2$.

Endnotes

This product is intended to be used for research purposes only. It is not to be used for drug or diagnostic purposes nor is it intended for human use. Evrogen products may not be resold, modified for resale, or used to manufacture commercial products without written approval of Evrogen.

PCR is the subject of patents issued in certain countries. The purchase of this product does not include a license to perform PCR. However, many researchers may not be required to obtain a license. Other investigators may already have a license to perform PCR through use of a thermal cycler with the appropriate label license.

Material safety data sheet information

EVROGEN JSC (Moscow, Russia) hereby confirms that to the best of our knowledge this product does not require a Material Safety Data Sheet. However, all of the properties of this product (and, if applicable, each of its components) have not been thoroughly investigated. Therefore, we recommend that you use gloves and eye protection and wear a laboratory coat when working with this product.

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