

EasyClone cDNA library construction kit

Cat# P01010

User Manual



This product is intended
for research use only.

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I. Intended use

The EasyClone cDNA library construction 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 directionally or 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).

II. Method overview

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

First strand cDNA synthesis starts from the 3'-end adapter 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 final step, ds cDNA is amplified by PCR. Use of EasyClone polymerase and specially designed primers allows synthesis of full-length-enriched cDNA that is flanked by PlugOligo and 3'-end adapter sequences.

2 II. METHOD OVERVIEW ...continued

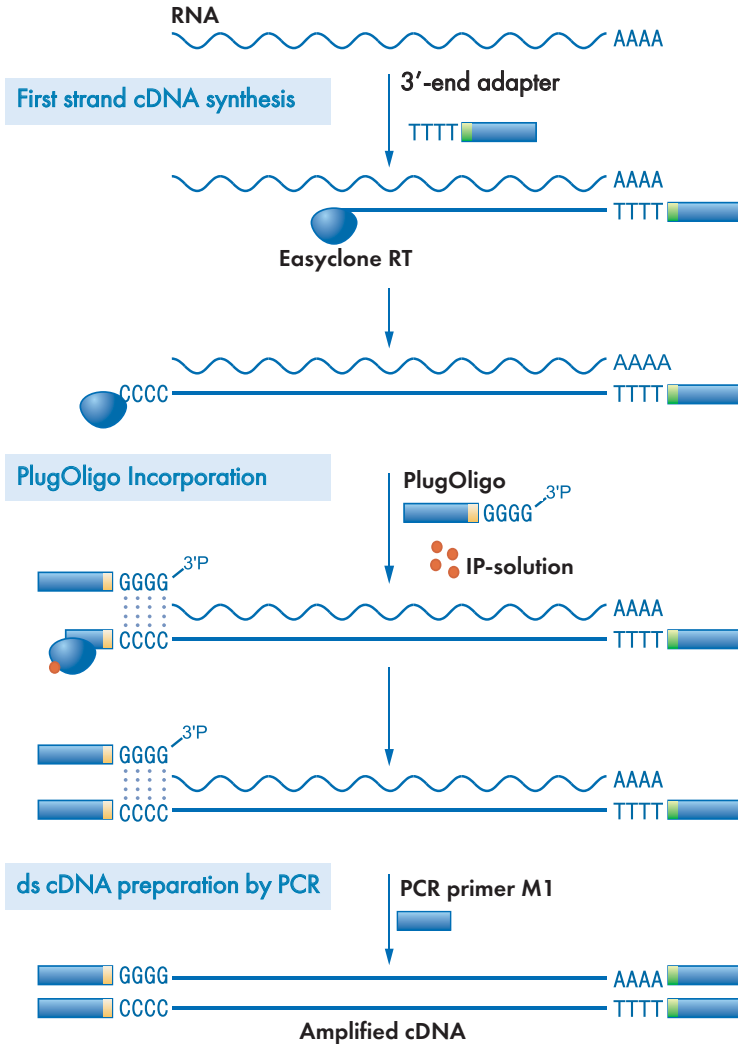


Figure 1. Schematic outline of Easyclone cDNA synthesis workflow.

The EasyClone cDNA library construction kit comprises two pairs of adapters allowing synthesis of cDNA with different flanking sequences.

The first pair is the 3'-end adapter CDS-1 and 5'-end adapter PlugOligo-1. These adapters comprise extensive common sequence and allow synthesis of cDNA to be used for non-directional cloning (see cDNA synthesis protocol-I in Section VI). The cDNA is also well suited for other applications like Virtual Northern blot (Franz et al., 1999) or subtractive hybridization (SSH, Diatchenko et al., 1996; Diatchenko et al., 1999).

The second adapter pair is the 3'-end adapter CDS-3M and the 5'-end adapter PlugOligo-3M. These adapters comprise asymmetric sites for the *SfiI* restriction enzyme (*SfiI*A & *SfiI*B; Fig. 2). Being incorporated at the 5' and 3' ends of the cDNA, these sites allow directional cloning of the cDNA into DUALhybrid and DUALmembrane library vectors. After digestion with *SfiI* and size fractionation, the cDNA is ready to be ligated into an appropriate *SfiI*-digested library vector. See protocol-II for synthesis of cDNA for directional cloning at the Section VII.

Important note: Adapters used to synthesize cDNA for directional cloning are longer than those used to prepare cDNA for non-directional cloning. Use of the longer adapters leads to a reasonable decrease in the cDNA average length and often to the appearance of a low-molecular-weight fraction in the resulting ds cDNA (which in turn makes it necessary to include a size-separation procedure to remove short cDNA fragments before cloning). Therefore, if directional cloning of cDNA library is not critical for your research we recommend that you use CDS-1 and PlugOligo-1 adapters and cDNA synthesis protocol-I (Section VI).

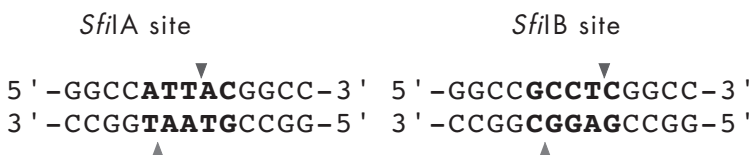


Figure 2. *SfiI* (A & B) recognition sites.

III. Kit components and storage conditions

A. List of kit components

The EasyClone cDNA library construction kit provides components for 20 reactions of ds cDNA synthesis. The kit comprises two adapter pairs, the first pair (CDS-1 and PlugOligo-1) allows synthesis of cDNA suitable for non-directional cloning (see protocol-I, Section VI), while the second (CDS-3M and PlugOligo-3M) is for synthesis of cDNA for directional cloning (see protocol-II, Section VII).

The kit includes a sample of Easyclone reverse transcriptase for first-strand cDNA synthesis and a trial-size EasyClone PCR kit (Cat #P01012).

BOX 1

Component	Amount
5X First-Strand Buffer	80 µl
DTT (20mM)	30 µl
10X dNTP mix (10mM each)	25 µl
PlugOligo-1 adapter (15 µM)* 5'-AAGCAGTGGTATCAACGCAGAGTACGGGGG ^P -3'	25 µl
CDS-1 adapter (10 µM)* 5'-AAGCAGTGGTATCAACGCAGAGTAC(T) ₃₀ VN -3'	25µl
PlugOligo-3M adapter (15 µM)* 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGGGG ^P -3'	25 µl
CDS-3M adapter (10 µM)* 5'-AAGCAGTGGTATCAACGCAGAGTGGCCGAGGCCGGCC(T) ₂₀ VN -3'	25µl
Easyclone Reverse Transcriptase	22 µl
IP-solution	130 µl
Control total RNA template (0.5 µg/µl)	25 µl
Sterile RNase free water	2,00 ml

BOX 2

Component	Amount
50X EasyClone polymerase mix	50 μ l
10X EasyClone buffer	300 μ l
PCR Primer M1 (10 μ M) 5'-AAGCAGTGGTATCAACGCAGAGT-3'	100 μ l
50X dNTP mix (10mM each)	80 μ l
Sterile RNase free water	2,00 ml
Control amplified cDNA sample 1 (for electrophoresis)	25 μ l
Control amplified cDNA sample 2 (for electrophoresis)	25 μ l

**Rsa*I and *Sfi*I restriction sites are underlined; N = A, C, G or T; V = A, G or C

Shipping & Storage: EasyClone Polymerase mix, EasyClone Reverse Transcriptase, PlugOligo adapters and control RNA are shipped at -20°C . 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 μ / μ l, Ambion) /optional/

Blue ice

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

Pipettes (P10, P20, P200) and pipette tips (pipette tips with hydrophobic filters are recommended for all procedures)

Vortex

Microcentrifuge

Agarose gel electrophoresis reagents and equipment

DNA size markers (1-kb DNA ladder)

PCR thermal cycler

Columns for size selection of cDNA, equilibrated in TE buffer (e.g.

Clontech Δ CHROMA SPINTM-400 or 1000)

IV. General considerations

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

1. Wear gloves to protect RNA and cDNA samples from degradation by nucleases.
2. If possible, perform cDNA synthesis, PCR reaction preparation and post-PCR analysis in separate laboratory areas to avoid cross-contamination of samples.
3. Use PCR pipette tips containing hydrophobic filters to minimize contamination.
4. 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.
5. 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.
6. Add enzyme to reaction mixture last and thoroughly mix it by gently pipetting the reaction mixture up and down.
7. 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.

V. RNA requirements

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Note: The sequence complexity and the average length of the EasyClone 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 the Trizol method (GIBCO/Life Technologies), the Chomczynski & Sacchi method (Chomczynski & Sacchi, 1987), or 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 poly A⁺ RNA appears as a smear sized from 0.1 to 4-7 (or more) kb with faint 28S and 18S rRNA bands.

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- 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-I

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Important Notes: This protocol allows synthesis of cDNA ready to use for non-directional cloning of cDNA libraries, Virtual Northern blot, RACE, and suppression subtractive hybridization. To prepare cDNA for directional cloning please use protocol-II in the Section VII below.

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.

Before you begin the first cDNA synthesis procedure, shake all enzymes solutions and spin the tubes briefly in a microcentrifuge.

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 μ l	Sterile water
1-3 μ l	RNA sample (containing 0.25 - 2 μ g of total or 0.1-1.0 μ g of polyA ⁺ RNA) For the control reaction use 2 μ l of the control RNA
1 μ l	CDS-1 adapter (10 μ M)
1 μ l	PlugOligo-1 adapter (15 μ M)
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.

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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	10X dNTP (10 mM each)
1 µl	Easyclone 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 non-specific 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 μ l	Sterile water
5 μ l	10X EasyClone PCR Buffer
1 μ l	50X dNTP mix (10 mM each)
2 μ l	PCR Primer M1 (10 μ M)
1 μ l	50X EasyClone Polymerase Mix
1 μ l	First-strand cDNA (from Step VI.A.12)**
50 μl	Total volume

Notes: * The recipe is for three reactions of 16 μ l 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 μ l of the PCR

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Master Mix into the appropriate number of fresh sterile 0.5 ml tubes and add 1 μ l of the first-strand cDNA solutions (from Step VI.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 μ l 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 μ l). 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 1 min
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
1.0-2.0	0.5-1.0	13-14	16-17	19-20
0.5-1.0	0.1-0.5	14-15	17-18	20-21
0.1-0.5	0.1 or rather less	16-17	20-21	23-24

Note: Cycling parameters in this protocol have been optimized for a 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 and 4 μg of control cDNA sample 1 on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare the PCR product you have obtained with that in Fig. 3 (relative to the 1-kb DNA ladder size markers). Use guidelines in the step 7 to determine samples with an optimal number of PCR cycles.

Note: The 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.

7. Analysis of PCR results.

When the yield of PCR product 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 3 shows a characteristic gel profile of ds cDNA synthesized using the control human brain total RNA following the Easyclone protocol outlined in Section VI. In the experiment shown, 1 μg 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.

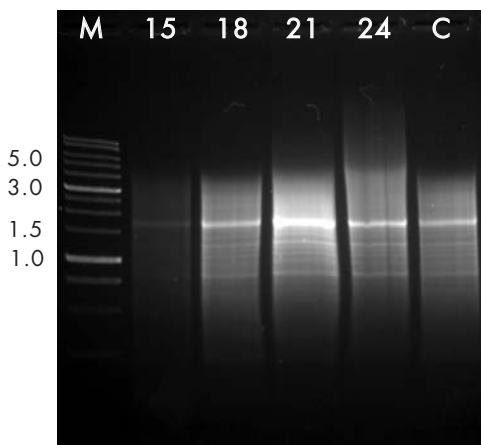


Figure 3. Agarose gel (1.2%) electrophoresis of amplified control cDNA after different number of PCR cycles. The number of PCR cycles performed is indicated at the top. M - 1 kb DNA size marker; C - control cDNA sample 1.

After 21 cycles, a smear appears in the high-molecular-weight region of the gel, indicating that the reaction is overcycled. Because the plateau is 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. 3, lane 2. If the cDNA smear appears in the high-molecular-weight region of the gel (e.g. as in lane 4), especially if no bright bands are distinguishable, this may indicate overcycled PCR (too many amplification cycles).

If the smear is much fainter (lane 1), this may indicate PCR undercycling (too few cycles).

If the size distribution of cDNA is generally less than expected (for example less than 3 kb for cDNA from mammalian sources), this may indicate that initial RNA is of poor quality or degraded during storage/synthesis.

Note: In general, ds cDNA size distribution should be similar to the corresponding mRNA, which typically appears within the range of 0.5-10 kb on an agarose/EtBr gel. For most mammalian tissues the 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. 4).

2. Several bright bands corresponding to abundant transcripts.

A number of distinct bright bands are usually present in cDNA prepared from many tissue sources. Band visibility depends on gel electrophoresis parameters, RNA source, etc. However, if bright bands become diffuse during PCR cycling, this may indicate PCR overcycling.

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 check the products again.

Note: Representation of the resulting amplified cDNA strongly depends on the initial number of target DNA molecules used for PCR amplification and on the number of PCR cycles required to amplify cDNA to the amount of 5-10 ng/μl (visibility threshold on an agarose/EtBr gel). Please remember that if cDNA requires more than 26 PCR cycles to be amplified it probably will not contain rare transcripts anymore. If no or low yield PCR product is observed after 25 cycles, see the Troubleshooting Guide.

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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 μ l	Sterile water
5 μ l	10X Encyclo PCR Buffer
1 μ l	50X dNTP mix (10 mM each)
2 μ l	PCR Primer M1 (10 μ M)
1 μ l	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 μ l 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 tubes.

4. Add 1 μ l 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 1 min
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 previously (section VI.B.1).

7. Analyze 4 μ l aliquots of each PCR product alongside 0.1 μ g of 1 kb DNA size marker and 4 μ l aliquot of the Control cDNA sample 1 on a 1.2% agarose/EtBr gel in 1X TAE buffer. If required, add 1-2 additional PCR cycles.

You now have obtained amplified ds cDNA.

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

The cDNA can be used for non-directional cloning into TA-cloning vectors. Before cloning, purification of the PCR product is recommended using phenol-chloroform extraction or commercial PCR purification kits.

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

Note: Use unpurified PCR product for polishing.

The cDNA may also be used for Virtual Northern blot (see Appendix C).

VII. cDNA preparation protocol-II

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Important Notes: This protocol allows synthesis of cDNA ready to use for directional cloning of cDNA library. To prepare cDNA for non-directional cloning as well as for other applications (Virtual Northern blot, suppression subtractive hybridization SSH, RACE) please use protocol-I in the Section VI above.

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.

Before you begin the first cDNA synthesis procedure, shake all enzymes solutions and spin the tubes briefly in a microcentrifuge.

A. First-strand cDNA synthesis and PlugOligo-3M 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 μ l	Sterile water
1-3 μ l	RNA sample (containing 0.25 - 2 μ g of total or 0.1-1.0 μ g of polyA ⁺ RNA)
	For the control reaction use 2 μ l of the control RNA
1 μ l	CDS-3M adapter (10 μ M)
1 μ l	PlugOligo-3M adapter (15 μ M each)

5 μ l 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	10X dNTP (10 mM each)
1 µl	EasyClone 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 can be used immediately for ds cDNA synthesis (Section VII.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. PCR overcycling yields nonspecific PCR products and is extremely undesirable for cDNA library cloning. PCR undercycling results in a lower yield of PCR product and also can decrease cloning efficiency. 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 VII.B1) and subsequent full-size preparation of ds cDNA (section VII.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 cycler. 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 μ l	Sterile water
5 μ l	10X EasyClone PCR Buffer
1 μ l	50X dNTP mix (10 mM each)
2 μ l	PCR Primer M1 (10 μ M)
1 μ l	50X EasyClone Polymerase Mix
1 μ l	First-strand cDNA (from Step VII.A.12)**

50 μ l Total volume

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

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 1 min
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
1.0-2.0	0.5-0.1	14-15	17-18	20-21
0.5-1.0	0.1-0.5	15-16	18-19	21-22
0.25-0.5	0.1 or rather less	17-18	20-21	23-24

Note: Cycling parameters in this protocol have been optimized for a MJ Research PTC-200 DNA thermal cycler and EasyClone 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 markers and 4 μl of the Control cDNA sample 2 on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare the PCR product you have obtained with that in Fig. 4 (relative to the 1-kb DNA ladder size markers). Use guidelines below (step 7) 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.

7. 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 4 shows a characteristic gel profile of ds cDNA synthesized using the control human brain total RNA following the protocol outlined in Section VII.

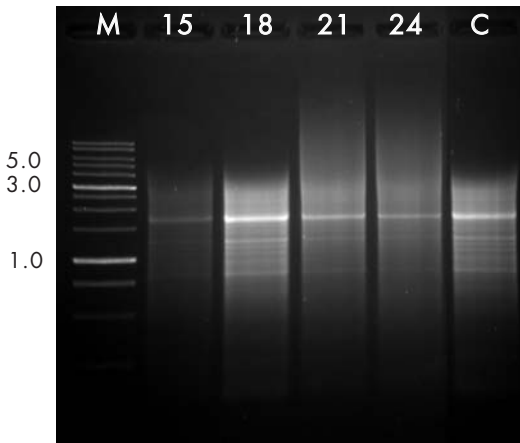


Figure 4. Agarose gel (1.2%) electrophoresis of amplified control cDNA after different number of PCR cycles. The number of PCR cycles performed is indicated at the top. M - 1 kb DNA size marker; C - control cDNA sample 2.

In the experiment shown, 1 μ g of control RNA was used for cDNA synthesis. PCR products (4 μ l 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 μ g of 1 kb DNA size markers.

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

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 μ g run on the same gel) should be roughly similar to that shown for the control experiment in Fig. 5, lane C. If the cDNA smear appears in the high-molecular-weight region of the gel (e.g. as in lane 4, e.g. cDNA after 22 cycles of PCR), especially if no bright bands are distinguishable, this could indicate overcycled PCR (too many amplification cycles).

If the smear is much fainter (lane 1), this may indicate PCR undercycling (too few cycles).

If the size distribution of cDNA is generally less than expected (for example less than 2 kb for cDNA from mammalian sources), this may indicate that initial RNA is of poor quality or degraded during storage/synthesis.

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Note: In general, the ds cDNA size distribution should be similar to corresponding 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.

2. Several bright bands corresponding to abundant transcripts.

A number of distinct bright bands are usually present in cDNA prepared from many tissue sources. Band visibility depends on gel electrophoresis parameters, RNA source, etc. However, if bright bands become diffuse during PCR cycling, this may indicate PCR overcycling.

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 check the products again.

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/ μ l (when it becomes visible on agarose/EtBr gel). Please remember that if cDNA requires more than 25-26 PCR cycles to be amplified it probably does not contain rare transcripts anymore. If no or low yield PCR product is observed after 26 cycles, see Troubleshooting Guide.

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:

80 μ l	Sterile water
10 μ l	10X EasyClone PCR Buffer
2 μ l	50X dNTP mix (10 mM each)
4 μ l	PCR Primer M1 (10 μ M)
2 μ l	50X EasyClone Polymerase Mix
2 μ l	First strand cDNAs (from step A.12)

100 μ l Total volume

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 .

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 tubes.

2. Mix PCR components by gently flicking the tube. Spin the tube briefly in a microcentrifuge. **Note:** PCR amplification in the larger reaction volumes may be less effective than that in the smaller ones. To increase PCR yield, we recommend that you aliquot PCR mix into 3 or 4 tubes and amplify these portions separately.

3. 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.

4. Commence thermal cycling using the following program:

Step	Number of cycles	Temperature
Initial denaturation	1	95°C for 1 min
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 in the section B.1.

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7. Analyze 4 μ l aliquots of each PCR product alongside 0.1 μ g of 1 kb DNA size marker and 4 μ l aliquot of the Control cDNA sample 2 on a 1.2% agarose/EtBr gel in 1X TAE buffer. If required add 1-2 additional PCR cycles for the experimental samples.

You now have obtained amplified ds cDNA.

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

The cDNA may now be used for directional cloning into DUALmembrane or DUALhybrid library vectors restricted with asymmetric *Sfi*-I sites. Before cloning, cDNA purification using phenol-chloroform extraction or commercial PCR purification kits and size separation using Δ CHROMA SPIN™-400/1000 columns (Clontech) is strongly recommend.

The following library vectors are compatible with cDNA generated with the EasyClone kit:

DUALmembrane library vectors pPR3-N (P03230) and pPR3-C (P03231); DUALhybrid library vector pGAD-HA (P03103); mammalian expression vectors pHA-MEX (P03401) and pMEX-HA (P03402).

VIII. 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 μ l of the control RNA on a start. One of the typical mistakes is that RNA samples were not well mixed after defrosting. In some cases heating of RNA samples before aliquoting (65°C for 2-3 min) may help.

3. PCR conditions and parameters might have been suboptimal. The optimal number of PCR cycles may vary with different PCR machines, RNA samples, etc. 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 μ l RNase Inhibitor (20 μ g/ μ l, Ambion) into the first-strand synthesis reaction as described in the sections VI.A.7 (protocol I) or VII.A.7 (protocol II).

5. If the positive control does not work, contact our technical support.

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 sections VI.A.7 (protocol I) or VII.A.7 (protocol II).

4. Your experimental RNA sample may 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 re-check the products. If the increase in cycle number does not improve the yield of PCR product, repeat the PCR using a fresh aliquot of the first-strand cDNA. If you still obtain a low yield of PCR product, this may 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 by more than 25 PCR cycles because these samples may be not representative.

2. Your experimental RNA may be too diluted. When total RNA concentration is determined on a spectrophotometer, high amounts of tRNA may lead to incorrect estimation of mRNA concentration. If you have not already done so, analyze your RNA samples using formaldehyde/agarose/EtBr gel electrophoresis to estimate RNA concentration and quality. If high amounts of tRNA are a problem, reduce the low-molecular-weight RNA fraction using RNA purification columns.

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 an agarose gel. If these bands are not visible and the background smear is very intense, this may indicate PCR overcycling. Repeat the 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: 1X TAE buffer instead of 1X TBE, a gel concentration of 1.1%-1.5% agarose, and running voltages lower than 10 V/cm.

IX. Appendices

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 1.1%-1.2% agarose gels
 - 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 contact with EtBr.
 - use running voltages up to 10 V/cm. Do not use high voltages to avoid RNA degradation during electrophoresis.
2. Heat the RNA at 70°C for 1 min and place it on ice before loading on the 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 (New England Biolabs Cat. No. M0203S)
- 96% ethanol
- 3 M potassium acetate (pH5,2)
- 80% ethanol
- TE-saturated phenol
- chloroform-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 μ l of non-purified amplified dsDNA (after step B2.6)

1.0 μ l dNTP mix (10 mM each)

3.0 μ l (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 μ l of 5mM Tris-HCl buffer (pH7.5-8.5) to the final DNA concentration of about 30-50 ng/ μ l.

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 μ l 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.

X. References

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XI. Related products

A. EasyClone PCR kit

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

The EasyClone 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
EasyClone PCR kit	P01012	100 PCR rxn (50 μ l each)

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. Dualsystems products may not be resold, modified for resale, or used to manufacture commercial products without written approval of Dualsystems

The EasyClone cDNA library construction kit is produced by EVROGEN, Moscow, Russia, and contains Evrogen proprietary technologies.

For further information please contact evrogen@evrogen.com.

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

Dualsystems Biotech 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.

