evrogen

TRIMMER

cDNA Normalization Kit

Cat#NK001

User Manual

This product is intended for research use only.

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I. Intended use

Evrogen TRIMMER kit is specifically designed to normalize amplified cDNA prepared using **SMART** (template switching effect based, Zhu *et al.*, 2001) or **Mint** approaches. Equalized cDNA obtained by TRIMMER normalization can be used to prepare non-directionally cloned cDNA library.

II. Introduction

Eukaryotic cells are known to express from about 10,000 to 50,000 genes, and transcript abundance varies from 200,000 copies to 1 or fewer copies per cell. As a rule, 10-20 abundant genes (several thousands of mRNA copies per cell), several hundreds of genes of medium abundance (several hundreds of mRNA copies per cell), and several thousands of rare genes (from one to several dozens of mRNA copies per cell) are expressed in each cell (Galau *et al.*, 1977). Hence, direct random sequencing of clones from standard cDNA libraries is inefficient for discovering rare transcripts, because cDNAs of medium and high abundance are sequenced repeatedly instead. Normalization performed before cDNA library sequencing decreases the prevalence of clones representing abundant transcripts. Thus, normalization increases the efficiency of random sequencing dramatically, and is essential for rare gene discovery.

The most rational approach to gene discovery through EST sequencing is analysis of cDNA libraries enriched with full-length cDNA. Use of these full-length cDNA libraries allows to obtain the entire sequence information for each transcript in a single cloning step, which is invaluable for high-throughput transcriptome analysis.

Unlike most traditional normalization methods not well suited for normalization of long cDNA, Evrogen TRIMMER is specially developed for normalization of cDNA enriched with full-length sequences. Evrogen TRIMMER allows to obtain normalized cDNA samples with the size of individual cDNAs ranging from 0.5 to 4.5 kb.

TRIMMER is based on a unique DSN normalization technology (Zhulidov *et al.*, 2004). Normalization using TRIMMER is very simple and requires minimum hand-on-time. Several features of this method contribute to its efficiency and reproducibility. Normalization is performed prior to cDNA cloning, and does not include physical separation of single stranded (ss) and double stranded (ds) DNA fractions. Specific suppression PCR-based approach (Shagin *et al.*, 1999) prevents reduction of the average cDNA length during PCR.

Figure 1 presents a brief overview of the procedures required to obtain normalized cDNA library.

Molecular basis of DSN normalization technology

The method involves denaturation-reassociation of cDNA, degradation of ds-fraction formed by abundant transcripts and PCR amplification of the equalized ss-DNA fraction.

The key element of this method is degradation of ds-fraction formed during reassociation of cDNA using Duplex-Specific Nuclease (DSN) enzyme (Shagin *et al.*, 2002). A number of specific features of DSN make it ideal for removing ds-DNA from complex mixtures of nucleic acids. DSN displays a strong preference for cleaving ds DNA in both DNA-DNA and DNA-RNA hybrids, compared to ss-DNA and RNA, irrespective of the sequence length (**Figure 2**).

Moreover, the enzyme remains stable over a wide range of temperatures and displays optimal activity at 55-65°C (**Figure 3**). Consequently, degradation of ds DNA-containing fraction by this enzyme may occur at elevated temperatures, thereby avoiding loss of transcripts due to the formation of secondary structures and non-specific hybridization involving adapter sequences.

Figure 4 details the molecular events that occur during normalization using Evrogen TRIMMER.

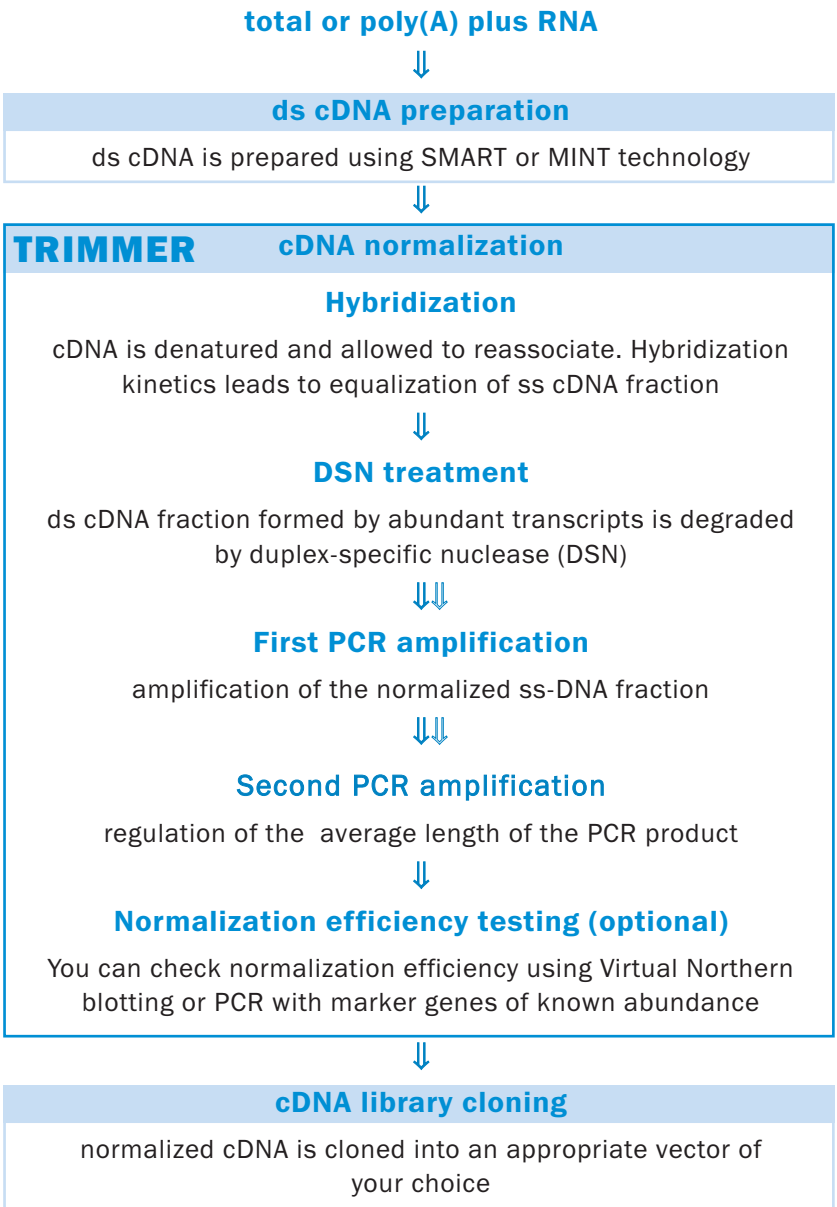


Figure 1. Overview of the normalization procedure using Evrogen TRIMMER.

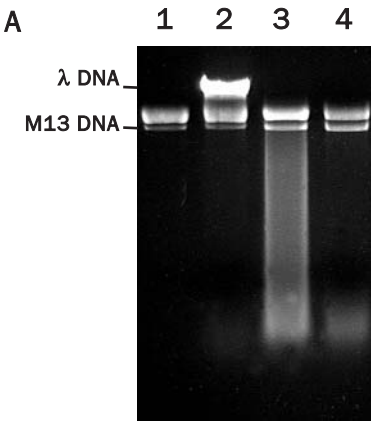
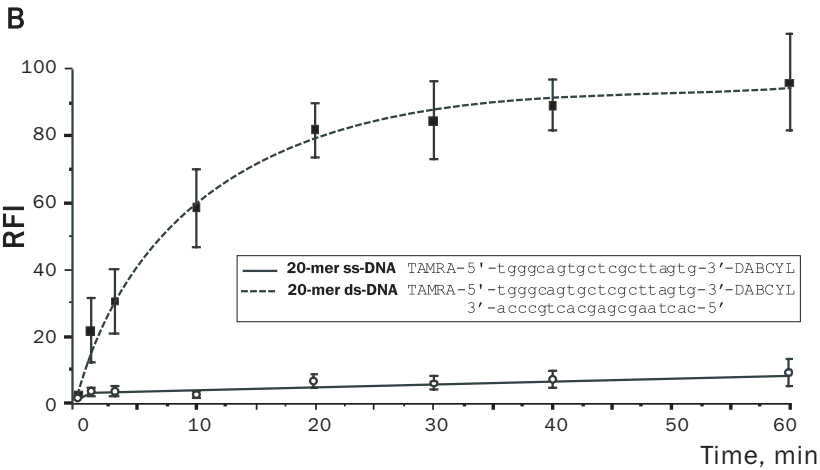


Figure 2. Determination of DSN preference for specific structural features of DNA substrates.

(A) Action of DSN on ss phage M13 DNA and ds l DNA. Lanes 1, 2 - negative controls, incubation without nuclease. 1 - phage M13 DNA alone,

2 - mixture containing phage M13 DNA and λ DNA. Lanes 3; 4 - digestion of phage M13 and λ DNA mixture by DSN at 70°C for 1.5 min (lane 3) and 5 min (lane 4).



(B) Action of DSN on synthetic ss and ds 20-mer DNA substrates, labeled by fluorescent donor and quencher pair. The cleavage reaction was performed at 35°C for different periods. Fluorescence intensity was measured at 570 nm (with excitation at 550 nm). The relative fluorescence increase in the oligonucleotide substrate, RFI, was defined as RFI= (Fi-Fo/Fmax-Fo) x 100%, where Fi is the fluorescence intensity of a substrate after incubation with nuclease, Fo is the substrate fluorescence in the absence of enzyme, and Fmax represents fluorescence of 100% cleaved substrate.

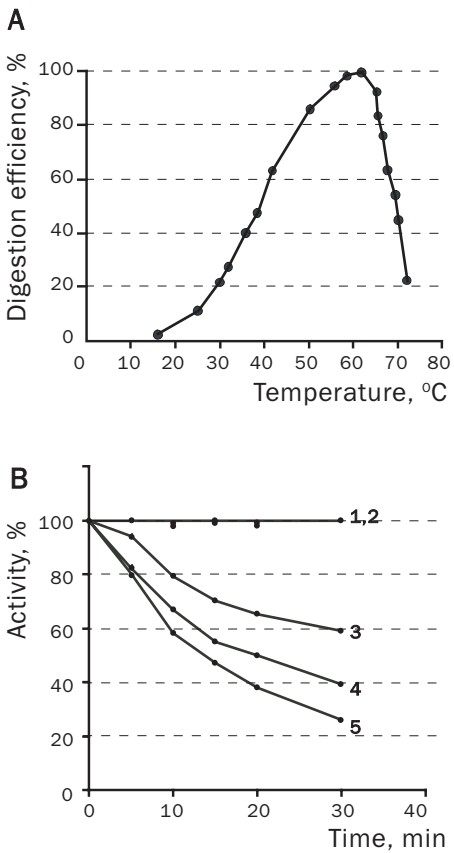


Figure 3. Dependence of the DSN activity and stability upon temperature.

(A) Dependence of the DSN activity upon temperature. Activity of DNAse on ds DNA substrate was measured at different temperatures using Kunitz assay.

(B) Kinetics of thermal denaturation of DSN. DSN was incubated at 50 (1), 60 (2), 70 (3), 80 (4) and 90 (5) °C for 30 min. Activity of DNAse on ds DNA substrate was measured at 65°C using Kunitz assay.

cDNA suitable for DSN-normalization should contain known flanking sequences for subsequent PCR amplification. We adapted TRIMMER-normalization to the amplified cDNA prepared by the SMART (Zhu *et al.*, 2001) or Mint approaches. This cDNA is enriched with full-length sequences and could be obtained both from poly(A)+ and total RNA even if a small amount of starting material is available. Same synthetic adapter sequences are introduced to both 5' and 3' ends of cDNA during cDNA synthesis.

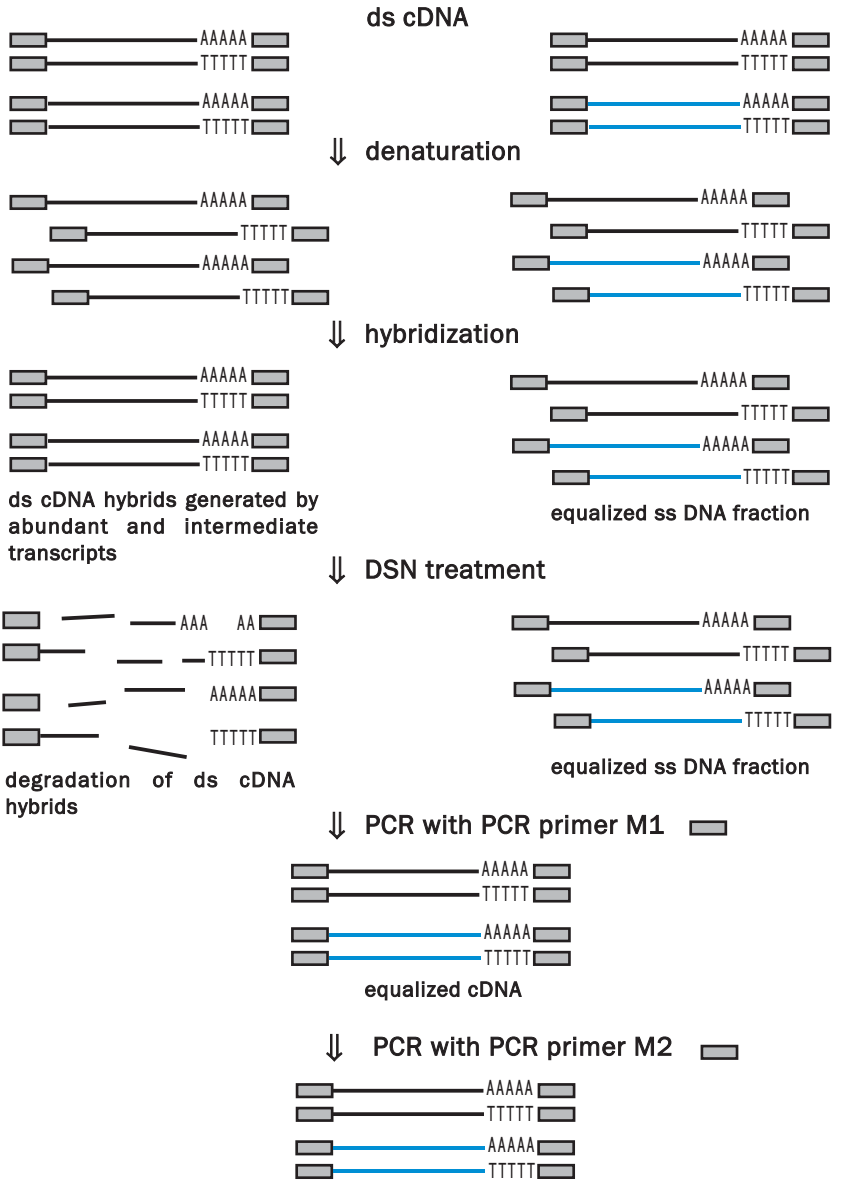


Figure 4. DSN normalization scheme.

Black lines represent abundant transcripts, blue lines - rare transcripts. Rectangle represents adapter sequence and its complement.

PCR primer M1: 5'- aag cag tgg tat caa cgc aga gt - 3';
PCR primer M2: 5'- aag cag tgg tat caa cgc ag- 3'.

During normalization, the cDNA is denatured and subsequently allowed to rehybridize. Due to the second-order hybridization kinetics, abundant transcripts renature quicker than rare sequences, thereby, single-stranded (ss) fraction is equalized to a considerable extent (Gurskaya *et al.*, 1996). Following reassociation, ds DNA fraction (formed by abundant transcripts) is degraded by DSN and the equalized ss-fraction is amplified by PCR.

Additional enrichment with full-length sequences.

PCR has a recognized tendency to amplify shorter fragments more efficiently than longer ones. This may result in the loss of rare long transcripts during PCR and reduction of the average cDNA length. Use of a "long and accurate PCR system" (Barnes, 1994) provides only a partial solution to this problem. To effectively increase the proportion of long fragments in the cDNA sample, we incorporated a previously developed procedure to regulate the average length of complex PCR product (Shagin *et al.*, 1999) in our normalization protocol. Modified PCR primers are specifically designed to ensure that amplification of long cDNA fragments is carried out more effectively than that of shorter ones. Thus, the average cDNA length is maintained during PCR amplification and the adequate size range of normalized cDNA (0.5 to 4.5 kb) is attained.

III. Reagents and materials

A. List of kit components

For 10 normalization reactions

Kit component	Amount	Storage
DSN enzyme, lyophilized	50 Units*	- 20°C
DSN storage buffer 50 mM Tris-HCl pH 8.0	120 µl	- 20°C
4X Hybridization buffer 200 mM Hepes, pH 7.5, 2 M NaCl	70 µl	- 20°C
2X DSN master buffer 100 mM Tris-HCl, pH 8.0 10 mM $MgCl_2$, 2 mM DTT	250 µl	- 20°C
DSN stop solution 5 mM EDTA	500 µl	- 20°C
PCR Primer M1 (10 µM) 5'- aag cag tgg tat caa cgc aga gt - 3'	70 µl	- 20°C
PCR Primer M2 (10 µM) 5'- aag cag tgg tat caa cgc ag- 3'	90 µl	- 20°C
DSN control template, 100ng/µl	20 µl	- 20°C
ACTB plasmid, 100ng/µl	50 µl	- 20°C
UBC plasmid, 100ng/µl	50 µl	- 20°C

*DNAase activity was measured using modified Kunitz assay where unit was defined as: the amount of DSN added to 50 µg/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 $MgCl_2$.

B. Materials required but not included:

- One of the listed kits for cDNA synthesis:

  Mint cDNA synthesis kit (Evrogen Cat. #: SK001),
  Mint-Universal cDNA synthesis kit (Evrogen Cat. #: SK002; protocol 1),
  SMART™ PCR cDNA Synthesis Kit (Clontech Cat. #: 634902),
  Super SMART™ PCR cDNA Synthesis Kit (Clontech Cat. #: 635000);

- Superscript2 reverse transcriptase (Invitrogen) [Required if it is not included in cDNA synthesis kit you use.]

- Sterile nuclease free water;

- Glycerol, 100%;

- Mineral oil;

- Blue ice;

- Sterile 0.5 ml PCR tubes;

- Sterile microcentrifuge 1.5 ml tubes;

- Pipettors (P20, P200) and pipet tips;

- Base and tray/retainer for holding tubes;

- Vortex mixer;

- Microcentrifuge;

- Agarose gel electrophoresis equipment;

- PCR thermal cycler;

- 98% and 80% ethanol;

- 3M NaAc (sodium acetate), pH 4.8;

- PCR kit allowing long-distance PCR like Encyclo PCR Kit (Evrogen Cat.#: PK001) or Advantage™ 2 PCR Kit (Clontech Cat. #: 639206, 639207). Trial-size Encyclo PCR kit is included into Evrogen cDNA synthesis kits listed above.

- PCR product purification kit, such as QIAquick PCR Purification Kit (Cat.# 28104, 28106, QIAGEN Inc., Valencia, CA) or equivalent, suitable for effective removing of primer excess.

IV. General considerations

- This protocol is developed to normalize ds cDNA prepared using one of the following kits:

Mint cDNA synthesis kit (Evrogen Cat. #: SK001),
Mint-Universal cDNA synthesis kit (Evrogen Cat. #: SK002; protocol 1),
SMART™ PCR cDNA Synthesis Kit (Clontech Cat. #: 634902),
Super SMART™ PCR cDNA Synthesis Kit (Clontech Cat. #: 635000);

- The key element of normalization procedure is DSN treatment. Optimal parameters for DSN treatment may vary depending on DSN lot, DSN/cDNA concentration ratio, cDNA sample. Therefore, we strongly recommend to perform the optimization procedure described in this protocol for each cDNA sample.

- Cycling parameters in this protocol have been optimized for MJ Research PTC-200 DNA Thermal Cycler and is provided as an example. Optimal parameters may vary with different thermal cyclers, polymerase mixes, and templates.

- Hot start MUST be used to reduce nonspecific DNA synthesis during the PCR set-up. PCR kits that include automatic hot start are recommended, for example Encyclo PCR Kit (Evrogen Cat.#: PK001) or Advantage™ 2 PCR Kit (Clontech Cat. #: 639206, 639207).

- To resuspend pellets and mix reactions, gently pipet them up and down and centrifuge the tube briefly to deposit contents at the bottom.

- Add enzymes to reaction mixtures last and thoroughly mix the enzyme by gently pipetting the reaction mixture up and down.

- **AVOID** drops of the reaction mixture on the walls of the reaction tubes and inside the mineral oil fraction. Even a small aliquot of non-DSN-treated cDNA will corrupt normalization results.

- Wear gloves to protect cDNA samples from degradation by nucleases.

V. Duplex-specific nuclease (DSN) preparation and testing

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

A. DSN dilution

Note: DSN dilution is performed only once before you begin the first normalization procedure.

Dilute the lyophilized DSN enzyme in DSN storage buffer as follows:

1. Add 25 µl of DSN storage buffer to the lyophilized DSN enzyme.

2. Mix contents by gently flicking the tube. Spin the tube briefly in a microcentrifuge. Avoid foaming of the mixture.

3. Incubate the tube at room temperature for 5 min.

4. Add 25 µl of glycerol to the tube.

5. Mix contents by gently flicking the tube. Spin the tube briefly in a microcentrifuge. Avoid foaming of the mixture.

6. Store the DSN solution at -20°C.

B. DSN activity testing

Note: We strongly recommend to check DSN activity every time before you begin normalization.

1. Combine the following reagents in a sterile 1.5 ml tube:

4 µl	Sterile water
4 µl	DSN control template
10 µl	DSN master buffer
18 µl	**Total volume**

2. Mix contents and spin the tube briefly in a microcentrifuge.

3. Aliquot 9 µl of the reaction mixture into each of the two sterile PCR tubes labeled C (control) and E (experimental).

4. Add 1 µl of DSN storage buffer into C-tube. Mix contents and spin the tube briefly in a microcentrifuge.

5. Add 1 µl of DSN solution into E-tube. Mix contents by gently flicking the tube. Spin the tube briefly in a microcentrifuge.

6. Overlay the reaction mixture in each tube with a drop of mineral oil and spin the tubes briefly in a microcentrifuge.

7. Incubate the tubes in a thermal cycler at 65°C for 10 min.

8. Add 10 µl of DSN stop solution to each tube, mix contents and spin the tubes briefly in a microcentrifuge. Place the tubes at room temperature.

9. Electrophorese 5 µl of each reaction mixture alongside 0.1 µg of 1-kb DNA size markers on a 1.5% agarose/EtBr gel in 1X TAE buffer.

10. Using electrophoresis data, estimate the condition of your DSN enzyme. For comparison, see Figure 5 showing typical gel profile of "DSN control template" digested by DSN with sufficient activity and by partially inactive DSN.

A typical result, indicative of sufficient DSN activity, should have the following characteristics:

1. Two strong DNA bands should be present in the DNA pattern from the C-tube (lane 1, Fig. 5). A strong difference between the patterns of DNA obtained from the C-tube and shown in Figure 5 (lane 1) may indicate that some of the reagents used are contaminated with nuclease (see Troubleshooting Guide, Section A).

2. Low molecular weight DNA should be detected in the E-tube (as in lane 3, Fig. 5). If the pattern of digested DNA from the E-tube looks like smears of various intensities with or without clear bands (see for example lane 2 in Fig. 5), your DSN enzyme is fully or partially inactive and is not appropriate for cDNA normalization.

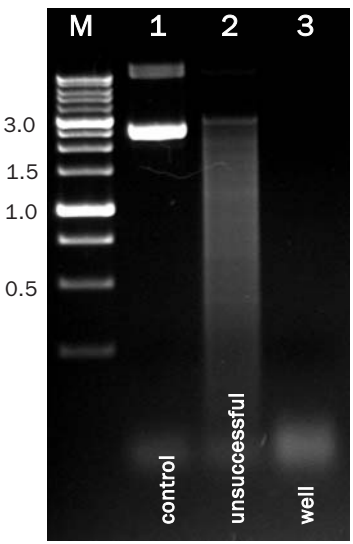


Figure 5. DSN activity testing.

Samples containing 100 ng of DSN control template were incubated with or without DSN in 1x DSN master buffer for 10 min at 65°C. Reactions were stopped by DSN stop solution and digestion products were electrophoresed on a 1.5% agarose/EtBr gel in 1X TAE buffer. Lane 1 - control DNA (incubation without DSN). Lane 2 - DNA incubated with ill-conditioned DSN enzyme. Lane 3 - successful digestion of DNA by DSN. Lane M - 1 kb DNA size markers.

VI. cDNA preparation

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

1. For cDNA synthesis, use reagents provided in cDNA synthesis kits listed in the section III.B above.

2. The sequence complexity and the average length of the normalized cDNA library noticeably depend on the quality of experimental RNA starting material. Therefore, we recommend estimating RNA quality using a denaturing formaldehyde/ agarose gel-electrophoresis before the first-strand cDNA synthesis. The adequate RNA length generally depends on the RNA source, however, if your experimental RNA is not larger than 1.5 kb, we suggest you prepare fresh RNA after checking the quality of your RNA purification reagents. If problems persist, you may need to find another source of tissue/cells.

3. To obtain the best results we recommend starting cDNA synthesis with 0.5 - 1.5 µg of poly(A)+ or total RNA. The minimal amount of RNA is 0.1 µg.

4. We strongly recommend performing a positive control cDNA synthesis with control RNA simultaneously with your experimental cDNA synthesis. This control is performed to verify that all components are working properly.

1. Prepare amplified cDNA on the basis of total or poly (A+) RNA using one of the kits listed above (section III.B). Use manufacturer instruction for cDNA synthesis except ds cDNA polishing. If required, perform cDNA polishing after normalization.

Note: Please note, that cDNA samples that require more than 25 PCR cycles to be amplified may be not representative. We do not recommend to use such samples for normalization. Repeat cDNA amplification using larger amounts of first-strand cDNA on start of PCR.

2. Purify the resulting amplified cDNA to remove primer excess, dNTPs and salts using a commercial PCR Purification Kit such as QIAquick PCR Purification Kit (Cat.# 28104, 28106, QIAGEN Inc.) or equivalent.

Note: Be sure that the kit you are using effectively removes primer excess.

3. Aliquot cDNA solution containing about 700-1300 ng of purified cDNA into a separate sterile tube. Store the remaining cDNA solution at -20°C.

Note: Do not use any co-precipitants in the following cDNA precipitation procedure.

4. Add 0.1 volumes of 3M NaAc, pH 4.8, to the reaction tube.

5. Add 2.5 volumes of 98% ethanol to the reaction tube.

6. Vortex the mixture thoroughly.

7. Centrifuge the tube for 15 min at 12,000 - 14,000 rpm at room temperature.

8. Remove the supernatant carefully.

9. Gently overlay the pellets with 100 µl of 80% ethanol.

10. Centrifuge the tubes for 5 min at 12,000 - 14,000 rpm at room temperature.

11. Carefully remove the supernatant.

12. Repeat steps 10-12.

13. Air dry the pellet for 10-15 min at room temperature. Be sure that the pellet has dried completely.

14. Dissolve the pellet in sterile water to the final cDNA concentration of about 100 - 150 ng/µl.

15. To check the cDNA quality and concentration, electrophorese 1 µl of cDNA solution alongside 0.1 µg of 1-kb DNA size markers on a 1.5% agarose/EtBr gel in 1X TAE buffer.

16. Place the tube on ice.

Now, you have obtained ds cDNA ready for normalization.

Note: This amplified cDNA can be stored at -20°C for up to three months and used afterwards for normalization.

VII. Normalization protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Note: Before you begin normalization of your experimental samples, we recommend that you perform normalization of the cDNA, prepared on the basis of the control human RNA provided in the cDNA synthesis kits listed above. After this normalization, you can use the ACTB and UBC plasmids, provided in the TRIMMER kit to check normalization efficiency by Virtual Northern Blotting.

A. Hybridization

Note: Before you start hybridization, make sure that 4X Hybridization buffer has been allowed to stay at room temperature for at least 15-20 min. Be sure that there is no visible pellet or precipitate in the buffer before use. If necessary, warm the buffer at 37°C for about 10 min to dissolve any precipitate.

1. For each sample to be normalized combine the following reagents in a sterile 1.5-ml tube:

4-12 µl	ds cDNA (about 600-1200 ng of dissolved cDNA from stage V.16)
4 µl	4X Hybridization buffer
X µl	Sterile water
16 µl	**Total Volume**

2. Mix contents and spin the tube briefly in a microcentrifuge.

3. Aliquot 4 µl of the reaction mixture into each of the four appropriately labeled (for example, see Table 1) sterile PCR tubes.

4. Overlay the reaction mixture in each tube with a drop of mineral oil and centrifuge the tubes at 14,000 rpm for 2 min.

5. Incubate the tubes in a thermal cycler at 98°C for 2 min.

6. Incubate the tubes at 68°C for 5 hr, then proceed immediately to Section B.

Note: Samples may be hybridized for as little as 4 hr, or as long as 7 hr. Do not allow the incubation to proceed for more than 7 hr. Do not remove the samples from the thermal cycler before DSN treatment.

B. DSN treatment

1. Shortly before the end of the hybridization procedure, prepare the following dilutions of the DSN enzyme in two sterile tubes:

1.1. Add 1 µl of DSN storage buffer and 1 µl of DSN solution (in storage buffer) to the first tube. Mix by gently pipetting the reaction mixture up and down. Label the tube as 1/2 DSN.

1.2. Add 3 µl of DSN storage buffer and 1 µl of DSN solution to the second tube. Mix by gently pipetting the reaction mixture up and down. Label the tube as 1/4 DSN.

1.3. Place the tubes on ice.

2. Preheat the DSN master buffer at 68°C.

3. Add 5 µl of the preheated DSN master buffer to each tube containing hybridized cDNA (from stage A.6), spin the tube briefly in a microcentrifuge and return it to the thermal cycler.

Note: Do not remove the tubes from the thermal cycler except for the time necessary to add preheated DSN master buffer.

4. Incubate the tubes at 68°C for 10 min.

5. Add DSN enzyme as specified in **Table 1.** After DSN adding return the tubes immediately to the thermal cycler.

Note: Do not remove the tubes from the thermal cycler except for the time necessary to add DSN enzyme. When the tube is left at room temperature after DSN adding, non-specific digestion of secondary structures formed by ss-DNA may occur to decrease the normalization efficiency.

6. Incubate the tubes in the thermal cycler at 68°C for 25 min.

7. Add 10 µl of DSN stop solution, mix contents and spin the tubes briefly in a microcentrifuge.

8. Incubate the tubes in the thermal cycler at 68°C for 5 min.

9. Extract the tubes from the thermal cycler and place them on ice.

10. Add 20 µl of sterile water to each tube. Mix contents and spin the tubes briefly in a microcentrifuge. Place the tubes on ice.

Note: The samples obtained can be stored at -20°C for up to two weeks and used afterwards to prepare more normalized cDNA.

Table 1. Setting up DSN treatment

	experimental			control
Component\Tube*	TUBE 1 (S1 DSN1)	TUBE 2 (S1 DSN1/2)	TUBE 3 (S1 DSN1/4)	TUBE 4 (S1 Control)
DSN enzyme in storage buffer	1 µl	-	-	-
1/2 DSN dilution	-	1 µl	-	-
1/4 DSN dilution	-	-	1 µl	-
DSN storage buffer	-	-	-	1 µl

* S <NUMBER>- cDNA sample specification

C. First amplification of normalized cDNA

Note: For PCR amplification, use PCR reagents provided in the Encyclo PCR kit (Evrogen Cat.#: PK001) or Advantage 2 PCR kit (Clontech Cat.#: 639206, 639207) with PCR primers provided in the TRIMMER kit.

1. Preheat a thermal cycler to 95°C

2. Prepare a PCR Master Mix for all reaction tubes. In a sterile 1.5 ml tube, combine the following reagents in the order shown:

per rxn:

40.5 µl	Sterile water
5 µl	10X PCR Buffer
1 µl	50X dNTP mix
1.5 µl	Evrogen PCR primer M1
1 µl	50X Polymerase Mix
49 µl	**Total Volume**

3. Mix well by vortexing and spin the tube briefly in a microcentrifuge.

4. Aliquot 1 µl of each diluted cDNA (from Step B.10) into an appropriately labeled sterile PCR tube.

5. Aliquot 49 µl of the PCR Master Mix into each of the reaction tubes.

6. Mix contents by gently flicking the tubes. Spin tubes briefly in a microcentrifuge.

7. If necessary, overlay the reaction mixture with 2 drops of mineral oil. Close the tubes, and place them into the preheated thermal cycler.

8. Commence thermal cycling using the program that you found optimal for cDNA amplification during cDNA synthesis procedure. For example recommended program for MJ Research PTC-200 thermal cycler and Encyclo PCR mix may be as follow:

Step	Number of cycles	Temperature
Initial denaturation	1	95°C for 1 min
Cycling	7	95°C for 15 s; 66°C for 20 s; 72°C for 3 min

Note: Optimal PCR parameters may vary with different polymerase mixes, templates, and thermal cyclers.

Subject all tubes to 7 cycles. Then use the Control tube (**see Table 1**) to determine the optimal number of PCR cycles using the procedure described in Steps 9-10 (below). Store the other tubes on ice.

9. For each Control tube, determine the optimal number of PCR cycles (**Figure 6**):

9.1 Transfer 12 µl from the 7-cycle PCR tube to a clean micro-centrifuge tube (for agarose/EtBr gel analysis)

9.2 Run two additional cycles (for a total of 9) with the remaining 38 µl of the PCR mixture.

9.3 Transfer 12 µl from the 9-cycle PCR tube to a clean micro-centrifuge tube (for agarose/EtBr gel analysis)

9.4 Run two additional cycles (for a total of 11) with the remaining 26 µl of the PCR mixture.

9.5 Transfer 12 µl from the 11-cycle PCR tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis)

9.6 Run two additional cycles (for a total of 13) with the remaining 14 µl of the PCR mixture.

10. Electrophorese 5 µl of each aliquot of each PCR reaction (from Step 9) alongside 0.1 µg of 1-kb DNA size markers on a 1.5% agarose/EtBr gel in 1X TAE buffer. Determine "X", where X = optimal number of cycles required for amplification of each of the control tubes (**see Figure 7**). Store the remaining material on ice.

11. Retrieve the 7-PCR tubes from ice, return them to the thermal cycler, and if necessary, subject them to additional N cycles (where N= X-7), until you reach the optimal number. Then, immediately, subject the tubes to additional 9 cycles.

Note: Altogether, Control tube should be subjected to X PCR cycles, whereas experimental tubes should be subjected to X+9 PCR cycles, where X is the optimal number of PCR cycles determined for the Control tube. In the example shown in Figure 7, the optimal number of PCR cycles determined for control cDNA in the Control tube was 10. Thus, in this example X=10, and N=10-7=3. Hence, in this example, 7-PCR experimental tubes should be subjected to 3+9 additional PCR cycles.

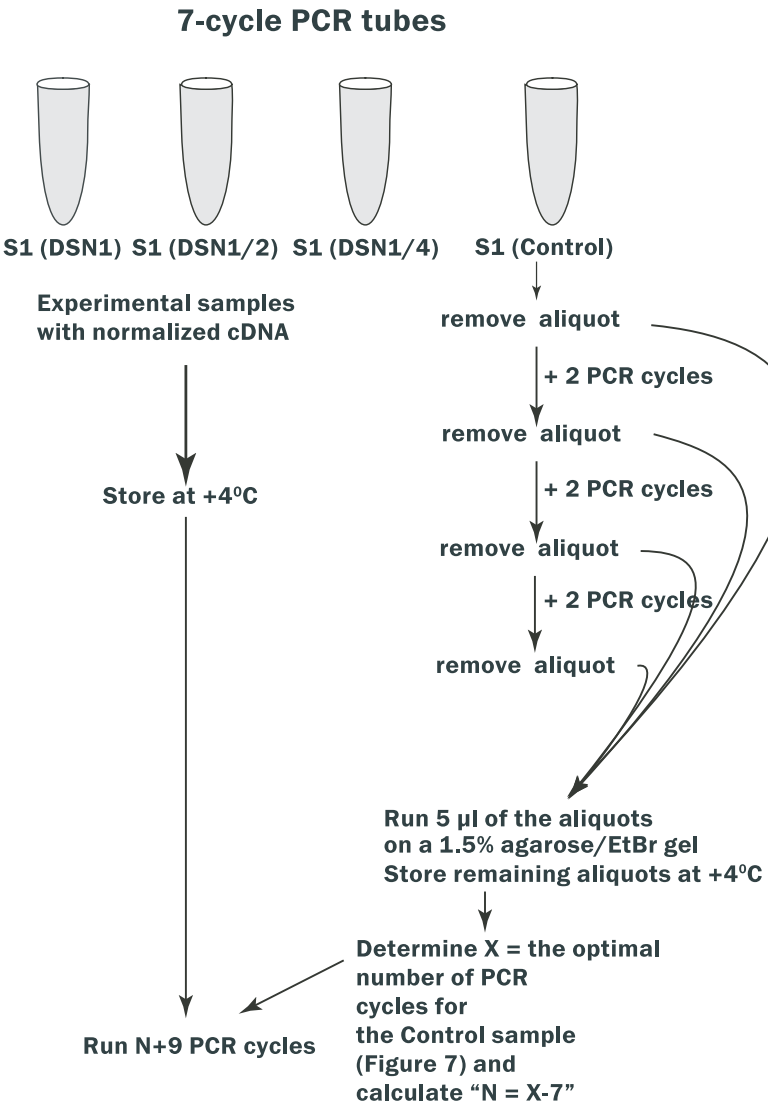


Figure 6. Optimizing PCR parameters for normalized cDNA amplification.

Determination of the Optimal Number of PCR Cycles (Step VII.C.10).

Choosing the optimal number of PCR cycles ensures that the ds cDNA will remain in the exponential phase of amplification. 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.

We have optimized PCR cycling parameters presented in this User Manual using MJ Research PTC-200 DNA Thermal Cycler and the Encyclo PCR Kit. These parameters may vary with different polymerase mixes, templates, and thermal cyclers.

Figure 7 provides an example of how your analysis should proceed. In this experiment, after 13 cycles, a smear appeared in the high-molecular-weight region of the gel, indicating that the reaction was overcycled. Because the plateau was reached after 11 cycles, the optimal number of cycles for this experiment is 10.

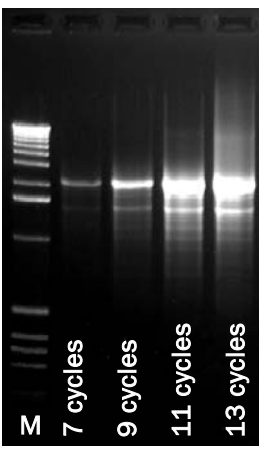


Figure 7. Analysis for optimizing PCR parameters.

5 µl of each aliquot from Control tube (from Step 9) was electrophoresed on a 1.5% agarose/EtBr gel in 1X TAE buffer following the indicated number of PCR cycles. The optimal number of cycles determined in this experiment was 10. Lane M: 1-kb DNA ladder size markers, 0.1 µg loaded.

12. When the cycling is completed, electrophorese 5 µl from each tube alongside 5 µl aliquot from Control PCR tube (with optimal PCR cycle number) and 0.1 µg of 1-kb DNA size markers on a 1.5% agarose/EtBr gel in 1X TAE buffer.

13. Select the tube(s) with efficient normalization. For comparison, Figure 8 shows a characteristic gel profile of TRIMMER normalized human skeletal muscle cDNA.

14. If cDNA from two or more tubes seems well normalized, combine contents of these tubes in one sterile 1.5 ml tube, mix well by vortexing and spin the tube briefly in a microcentrifuge.

Note: This amplified normalized cDNA can be stored at -20°C for up to one month and used afterwards to prepare more normalized cDNA.

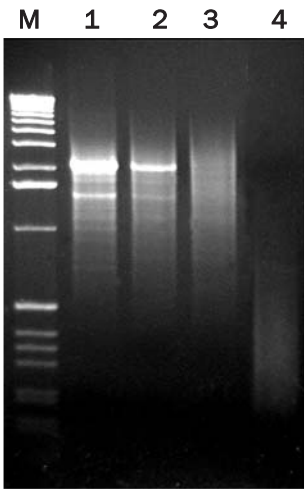


Figure 8. Analysis of cDNA normalization results.

5 µl aliquots of the PCR products were electrophoresed on a 1.5% agarose/EtBr gel. Lane M: 1-kb DNA ladder size markers, 0.1 µg loaded. Lane 1: cDNA from Control tube Lane 2: cDNA from S1_DSN1/4 TUBE. Lane 3: cDNA from S1_DSN1/2 TUBE. Lane 4: cDNA from S1_DSN1 TUBE.

In this experiment efficient normalization was achieved in S1_DSN1/2 TUBE. In S1_DSN1/4 TUBE normalization was not completed, in S1_DSN1 TUBE treatment with DSN enzyme was excessive, resulting in cDNA degradation.

Analysis of the normalization result (Step VII.C.13)

1. Compare the intensity of the banding pattern of your PCR products from experimental tubes with that from the Control tube and with the 1-kb DNA ladder size markers (0.1 µg run on the same gel).

- If the smear from the experimental tubes is much fainter than that shown for the Control, PCR undercycling could be the problem. Subject experimental tubes to two or three additional PCR cycles and repeat electrophoresis. If there is still a strong difference between the overall signal intensity of PCR products from all experimental tubes and from the Control tube, it may indicate that normalization process was superfluous (see Troubleshooting Guide, Section B.3).

- If the overall signal intensity of PCR products from the experimental tubes is much stronger than that shown for the Control, especially if the bright bands are distinguishable, it may indicate that normalization process was not successful (see Troubleshooting Guide, Section B.4).

- If the overall signal intensity of PCR products from the experimental tubes is similar to that in the control tube, select the tube(s) with efficient normalization using the instruction below.

2. A typical result, indicative of efficient normalization, should have the following characteristics:

- The pattern of PCR products from the experimental tube(s) containing efficiently normalized cDNA looks like smears without clear bands, whereas a number of distinct bands are usually present in the pattern of PCR products from the non-normalized Control tube.

- The average length of PCR products from the experimental tube(s) containing efficiently normalized cDNA is congruous with the average length of the PCR products from the non-normalized Control tube.

Note: The upper bound of the cDNA smear normalized using DSN usually does not exceed 4.5 kb.

D. Second amplification of normalized cDNA

To obtain the best results, we recommend that you perform re-amplification of your normalized cDNA before library cloning. If you plan to estimate normalization efficiency before cloning, it is necessary to amplify control non-normalized cDNA simultaneously.

1. Aliquot 2 µl of normalized cDNA (see steps C.13-14) into a sterile 1.5 ml tube; add 20 µl of sterile water to the tube, mix well by vortexing and spin the tubes briefly in a microcentrifuge.

2. Aliquot 2 µl of control cDNA (from aliquot of Step C.10 with optimal PCR cycling) into another sterile 1.5 ml tube; add 20 µl of sterile water to the tube, mix well by vortexing and spin the tubes briefly in a microcentrifuge.

3. Aliquot 2 µl of diluted normalized cDNA from Step 1 into an appropriately labeled sterile PCR tube.

4. Aliquot 2 µl of diluted control cDNA from Step 2 into another appropriately labeled sterile PCR tube.

5. Preheat a thermal cycler to 95°C.

6. Prepare a PCR Master Mix for all reaction tubes. In a sterile 1.5 ml tube, combine the following reagents in the order shown:

per rxn:

80 µl	Sterile water
10 µl	10X PCR Buffer
2 µl	50X dNTP mix
4 µl	Evrogen PCR primer M2
2 µl	50X Polymerase Mix
98 µl	**Total Volume**

7. Aliquot 98 µl of the PCR Master Mix into each of the reaction tubes (from Steps 3 and 4).

8. Mix contents by gently flicking the tubes. Spin the tubes briefly in a microcentrifuge.

9. If necessary, overlay the reaction mixture with 2 drops of mineral oil. Close the tubes, and place them to the preheated thermal cycler.

10. Subject the tubes to 12 PCR cycles using the program that you found optimal for cDNA amplification during cDNA synthesis procedure but decrease annealing temperature to 64°C. For example recommended program for MJ Research PTC-200 thermal cycler and Encyclo PCR mix may be as follow:

Step	Number of cycles	Temperature
Initial denaturation	1	95°C for 1 min
Cycling	12	95°C for 15 s; 64°C for 20 s; 72°C for 3 min
Final Extension	1	64°C for 15 s; 72°C for 3 min

When the cycling is completed, electrophorese 5 µl of the PCR products alongside 0.1 µg of 1-kb DNA size markers on a 1.5% agarose/EtBr gel in 1X TAE buffer to check the PCR quality and concentration. If necessary, subject the tubes to 1-2 additional PCR cycles.

See Troubleshooting Guide if low molecular weight, poor yield, or no PCR products is observed in the samples after the second PCR amplification.

Now, you have normalized ds cDNA ready-to-use for random cloning into a vector of your choice.

This cDNA can be stored at -20°C for up to one month.

To estimate normalization efficiency after cDNA library preparation, sequence 100 randomly picked clones from your library. In a well normalized library, redundancy of the first 100 sequences should not exceed 5%. You can also estimate normalization efficiency before cloning using quantitative PCR or Virtual Northern Blotting with marker genes of known abundance (see Section VIII).

VIII. Analysis of normalization efficiency

cDNA normalization should result in a significant decrease in the concentration of abundant transcripts and in preservation of rare ones. Either quantitative PCR or Virtual Northern blotting (Franz *et al.*, 1999) can be used to estimate the efficiency of normalization. In both cases, it is done by comparing the abundance of known cDNAs before and after normalization.

A. Virtual Northern blot

Using Virtual Northern blots, you can see a relative reduction in the representation level of abundant transcripts in a normalized cDNA sample (in comparison with a non-normalized one).

To perform Virtual Northern blot, electrophorese your normalized and control secondary PCR products (before purification) on a 1.2% agarose/EtBr gel and transfer them onto a nylon membrane (Sambrook *et al.*, 1989). Use [P^{32}]-labeled probes specific to the genes of known abundance in your samples for hybridization with membrane.

To estimate normalization efficiency of human cDNA samples, you can use probes prepared from the ACTB and UBC plasmids, provided in the TRIMMER kit. These plasmids contain fragments of ACTB and UBC housekeeping genes, expressed at high level in most tissues and cell lines. These plasmids can be used directly for [P^{32}]-labeled probe preparation with random primer or gene fragments can be amplified by PCR with standard M13 primers before probe preparation.

A typical result of Virtual Northern blot of non-normalized and normalized cDNA with ACTB and UBC-derived probes is shown in **Figure 9.**

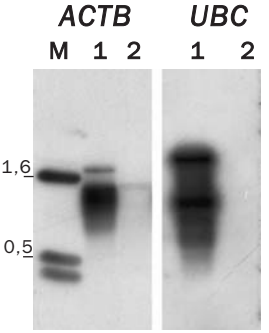


Figure 9. Virtual Northern blot analysis of abundant (ACTB, UBC) transcripts in non-normalized and normalized cDNA.

TRIMMER normalization of human skeletal muscle cDNA was performed. About 0.5 µg of non-normalized (lane 1) and normalized cDNA (lane 2) were resolved on agarose gels and transferred to Hybond-N membranes (Amersham). Gel electrophoresis and subsequent membrane transfer were performed according to standard protocols (Sambrook *et al.*, 1989). PCR amplified fragments of genes with high (ACTB, UBC) abundance in non-normalized cDNA were labeled with [P^{32}]-dATP and hybridized to Virtual Northern blots.

Note: ACTB and UBC genes are expressed at high levels in most human tissues and cell lines, however there could be some exceptions. If in the samples of your particular interest ACTB and UBC transcripts belong to intermediate or low abundance groups, unchanged or slightly increased concentration of these transcripts in normalized cDNA could be observed. In this case, select other marker genes that are abundant in samples of your interest to test normalization efficiency.

IX. Troubleshooting guide

A. DSN activity testing

1. DNA in C-tube is fully or partially degraded.

   a. Electrophorese 1 µl of DSN control template alongside 0.1 µg of 1-kb DNA size markers on a 1.5% agarose/EtBr gel in 1X TAE buffer. If DSN Control template is also fully or partially degraded, use another DNA to test DSN activity. You can use any purified plasmid DNA with concentration of about 100 ng/µl.

   b. If DSN control template is not degraded, while DNA in C-tube is fully or partially degraded, it indicates that your working area, equipment, or solutions are contaminated by nucleases.

2. DNA in E-tube is not fully degraded.

   a. Your DSN enzyme is not sufficiently active. Use another DSN enzyme package.

B. Analysis of normalization result

1. Low molecular weight (size distribution < 1.5 kb), poor yield, or no PCR products is observed in the control sample from the Control tube.

   a. TRIMMER is designed for normalization of amplified cDNA prepared using Evrogen Mint kit (Cat.# SK001), Mint-Universal Protocol 1 (Cat.# SK002), or SMART based kits provided by Clontech (SMART™ PCR cDNA Synthesis Kit, Cat.# 634902; or Super SMART™ PCR cDNA Synthesis Kit, Cat.# 635000). Be sure that your cDNA has been prepared using one of these kits and an appropriate protocol.

   b. cDNAs may have degraded during storage and/or hybridization procedure. Poor quality of starting material will reduce chances to obtain representative normalized unfragmented cDNAs. Your working area, equipment, and solutions must be free of contamination by nucleases. Check the quality of starting material on an agarose gel. Repeat ethanol precipitation of cDNA after column purification (see Section VI, Steps 3-16) followed by normalization using a fresh cDNA aliquot.

c. PCR primer M1 may have degraded during storage and/or delivery. To check primer quality, perform control PCR as follow:

1. Dilute 1-2 µl of cDNA from step VI.2 with sterile water to the final cDNA concentration 2 ng/µl.

2. Aliquot 2 µl of Evrogen PCR primer M1 into an appropriately labeled sterile PCR tube.

3. Aliquot 2 µl of 5' PCR Primer provided in the cDNA Synthesis Kit used into another appropriately labeled sterile PCR tube.

4. Preheat a thermal cycler to 95°C.

5. Prepare a PCR Master Mix for all reaction tubes. In a sterile 1.5 ml tube, combine the following reagents in the order shown:

per rxn:	
40 µl	Sterile water
5 µl	10X PCR Buffer
1 µl	50X dNTP mix
1 µl	Diluted cDNA from Step 1
1 µl	50X Polymerase Mix
48 µl	**Total Volume**

6. Aliquot 48 µl of the PCR Master Mix into each of the reaction tubes (from Steps 2 and 3).

7. Mix contents by gently flicking the tubes. Spin the tubes briefly in a microcentrifuge.

8. If necessary, overlay the reaction mixture with drop of mineral oil. Close the tubes, and place them to the preheated thermal cycler.

9. Subject the tubes to 11 PCR cycles using the following program:

95°C for 7 s;

65°C for 20 s;

72°C for 3 min

10. When the cycling is completed, electrophorese 5 µl of the PCR products alongside 0.1 µg of 1-kb DNA size markers on a 1.5% agarose/EtBr gel in 1X TAE buffer to check the PCR quality and concentration:

- If PCR product concentrations are similar in both tubes, it indicates that PCR primer M1 is not degraded;

- If the concentration of the PCR product produced from PCR primer M1 is much less than that of the PCR product produced from another PCR primer (from the cDNA synthesis kit), it may indicate that PCR primer M1 is degraded. Please contact Evrogen Technical Support to replace the PCR primer.

d. The concentration of starting cDNA is low, but the quality is good. Repeat normalization using more cDNA.

e. PCR conditions and parameters might have been suboptimal. The optimal number of PCR cycles may vary with different PCR machines, polymerase mixes, or cDNA samples. Try optimizing PCR cycling parameters by decreasing the annealing and extension temperatures in small increments - each degree lower can dramatically increase the background. First, try reducing annealing temperature from 65°C to 64°C and extension temperature from 72°C to 71°C. After PCR parameter optimization, repeat PCR using fresh aliquots of cDNA after DSN treatment (i.e. from stage VI.C.10).

f. You may have made an error during the procedures, for example used a suboptimal incubation temperature or omitted an essential component. Carefully check the protocol and repeat the procedures using fresh aliquots of cDNA.

2. cDNA from the Control tube is overamplified after 7 PCR cycles:

The concentration of starting cDNA is too high. Repeat normalization using less cDNA.

3. Low molecular weight (size distribution < 1.5 kb), poor yield, or no PCR products is observed in the experimental tubes containing normalized (DSN-treated) cDNA, whereas a high-quality PCR product is generated in the Control tube.

a. DSN treatment was excessive. Make sure that DSN enzyme was entirely diluted in storage buffer. The granules of non-diluted enzyme may dramatically change the DSN concentration in your experimental samples. Repeat normalization using a fresh portion of starting ds cDNA with well-diluted DSN enzyme.

b. If DSN enzyme was diluted successfully, repeat normalization on fresh portion of starting ds cDNA with one modification. Use the following procedure instead of the procedure described in Section VII.B.1:

   In three sterile tubes prepare the following dilutions of DSN enzyme:

   1.1. Add 3 µl of DSN storage buffer and 1 µl of DSN solution (in storage buffer) to the second tube. Mix by gently pipetting the reaction mixture up and down. Mark the tube as 1/4 DSN.

   1.2. Add 5 µl of DSN storage buffer and 1 µl of DSN solution (in storage buffer) to the second tube. Mix by gently pipetting the reaction mixture up and down. Mark the tube as 1/6 DSN.

   1.3. Add 7 µl of the DSN storage buffer and 1 µl of DSN solution (in storage buffer) to the second tube. Mix by gently pipetting the reaction mixture up and down. Mark the tube as 1/8 DSN.

   1.4. Place the tubes on ice.

Use these dilutions in DSN treatment procedure (Step VII.B.6), adding DSN to your experimental tubes as shown in **Table 2**.

Table 1. Setting up DSN treatment

Component\Tube*	TUBE 1 (S1 DSN1/4)	TUBE 2 (S1 DSN1/6)	TUBE 3 (S1 DSN1/8)	TUBE 4 (S1 Control)
1/4 DSN	1 µl	-	-	-
1/6 DSN	-	1 µl	-	-
1/8 DSN	-	-	1 µl	-
DSN storage buffer	-	-	-	1 µl

* S <NUMBER>- cDNA sample specification

4. PCR products in all experimental tubes are overamplified or seem like non-normalized on a gel electrophorese.

a. DSN treatment was insufficient. Make sure that DSN enzyme was entirely diluted in storage buffer. The granules of non-diluted enzyme may dramatically change the DSN concentration in your experimental samples. Repeat normalization using a fresh portion of starting ds cDNA with well-diluted DSN enzyme.

b. If DSN enzyme was diluted sufficiently, test the DSN activity using the procedure described in Section V.B.

c. If DSN works well, the problem may be in microscopic drops of initial cDNA that remained on the experimental tube walls or in the oil layer during hybridization or DSN treatment and were not exposed to DSN treatment. After dilution of the experimental samples, this untreated (non-normalized) cDNA contaminated the experimental samples to generate non-normalized cDNA during the following PCR reaction. Repeat normalization more carefully.

d. DSN enzyme has lower concentration than necessary. Repeat normalization using 2 µl of DSN solution for DSN treatment instead of 1/4 DSN dilution and 1.5 µl of DSN solution instead of 1/2 DSN dilution.

Use these dilutions in DSN treatment procedure (Step VII.B.6) as shown in Table 3.

Table 3. Setting up DSN treatment

Component\Tube*	TUBE 1 (S1 DSN1)	TUBE 2 (S1 DSN1.5)	TUBE 3 (S1 DSN2)	TUBE 4 (S1 Control)
DSN	1 µl	1,5 µl	2 µl	-
DSN storage buffer	-	-	-	1,5 µl

* **S <NUMBER>**- cDNA sample specification

C. Second PCR amplification

1. Low molecular weight (size distribution < 1.5 kb), poor yield, or no PCR products is observed in the samples after second PCR amplification.

a. cDNAs may degrade during storage. Your working area, equipment, and solutions must be free of contamination by nucleases. Check the quality of starting material on agarose gel. Repeat PCR amplification using a fresh cDNA aliquots.

b. PCR primer M2 may have degraded during storage and/or delivery. To check primer quality, perform control PCR as follow:

1. Dilute 1-2 µl of cDNA from Step VI.2 with sterile water to the final cDNA concentration 2 ng/µl.

2. Aliquot 2 µl of Evrogen PCR primer M2 into an appropriately labeled sterile PCR tube.

3. Aliquot 2 µl of 5' PCR Primer provided in the cDNA Synthesis Kits used (the appropriate kites are listed in Section III.B) into another appropriately labeled sterile PCR tube.

4. Preheat a thermal cycler to 95°C.

5. Prepare a PCR Master Mix for all reaction tubes. In a sterile 1.5 ml tube, combine the following reagents in the order shown:

per rxn:

40 µl	Sterile water
5 µl	10X PCR Buffer
1 µl	50X dNTP mix
1 µl	Diluted cDNA from Step 1
1 µl	50X Polymerase Mix
48 µl	**Total Volume**

6. Aliquot 48 µl of the PCR Master Mix into each of the reaction tubes (from Steps 2 and 3).

7. Mix contents by gently flicking the tubes. Spin the tubes briefly in a microcentrifuge.

8. If necessary, overlay the reaction mixture with drop of mineral oil. Close the tubes, and place them to the preheated thermal cycler.

9. Subject the tubes to 11 PCR cycles using the following program:

95°C for 7 s;

64°C for 20 s;

72°C for 3 min

10. When the cycling is completed, electrophorese 5 µl of the PCR products alongside 0.1 µg of 1-kb DNA size markers on a 1.5% agarose/EtBr gel in 1X TAE buffer to check the PCR quality and concentration:

- If PCR product concentrations are similar in both tubes, it indicates that PCR primer M2 is not degraded;

- If the concentration of the PCR product produced from PCR primer M2 is much less than that of the PCR product from another PCR primer, it may indicate that PCR primer M2 is degraded. Please contact Evrogen Technical Support to replace the PCR primer.

c. The concentration of starting cDNA is low, but the quality is good. Repeat PCR amplification using more cDNA.

d. PCR conditions and parameters might have been suboptimal. The optimal number of PCR cycles may vary with different PCR machines, polymerase mixes, or cDNA samples. Try optimizing PCR cycling parameters as recommended by polymerase manufacturer. After PCR parameter optimization, repeat PCR using fresh aliquots of cDNA.

e. You may have made an error during the procedures, for example omitted an essential component. Carefully check the protocol and repeat amplification using fresh aliquots of cDNA.

D. Analysis of normalization efficiency

1. Abundance of the transcripts tested remains unchanged after normalization procedure, whereas cDNA sample seems efficiently normalized (see Step VII.C.13: Analysis of normalization result).

a. Concentrations of non-normalized and normalized cDNA used for comparison are not equal. Equalize the concentrations of these cDNAs and repeat the test.

b. Transcripts selected for testing are not abundant in non-normalized samples of your interest. For abundant transcripts, in non-normalized cDNA, you should see PCR products after 18-23 cycles. Intermediate and rare transcripts may not change the representation levels during normalization procedure. In some cases, a slight increase in the representation level of such transcripts may occur.

c. Normalization process was unsuccessful. The problem may be in microscopic drops of initial cDNA that remained on the experimental tube walls or in the oil layer during hybridization or DSN treatment and were not exposed to DSN treatment. After dilution of the experimental samples, this untreated (non-normalized) cDNA contaminated the experimental samples to generate non-normalized cDNA during the following PCR reaction. Repeat normalization more carefully.

X. References

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XI. Related products and services

A. TRIMMER-DIRECT kit (for preparation of directionally cloned cDNA library)

Product	Cat.#	Amount
TRIMMER-DIRECT	NK002	for 10 reactions

TRIMMER-DIRECT is designed to normalize SMART cDNA prepared using Evrogen Mint-Universal (Cat.# SK002, protocol II). Clontech SMART™ cDNA Library Construction Kit (Cat.# 634901) or Creator™ SMART™ cDNA Library Construction Kit (Cat.# 634903) can be also used for cDNA preparation with a specially designed Evrogen CDS-3M adapter.

Normalized cDNA contains asymmetric adapter sequences and can be used to generate directionally cloned normalized cDNA library.

B. Duplex-specific nuclease, lyophilized

Product	Cat.#	Amount	Storage
Duplex-Specific Nuclease	EA001	50 Units*	- 20°C
Duplex-Specific Nuclease	EA002	100 Units*	- 20°C

*DNAase activity was measured using modified Kunitz assay where unit definition was defined as: the amount of DSN added to 50 μg/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 $MgCl_2$.

Duplex-Specific Nuclease (DSN) enzyme 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.

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 a pH of greater than 6, and temperatures below 75°C.

DSN exhibited strong cleavage preference for ds DNA substrates and little activity against ss DNA. No significant cleavage activity on ss RNA substrates is observed. However, 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 can be used for:

- Degradation of double-stranded DNA;

- Discrimination between perfectly matched short DNA-DNA duplexes (8-12 bp) and duplexes of the same length with at least one mismatch.

C. Mint cDNA synthesis kits

Product	Cat.#	Amount
Mint kit	SK001	for 20 reactions
Mint-Universal kit	SK002	for 20 reactions

Mint cDNA synthesis kits are designed to synthesize full-length-enriched double stranded (ds) cDNA from total or poly(A+) RNA.

D. Encyclo PCR kit

Product	Cat.#	Amount
Encyclo kit	PK001	for 100 reactions (50 µl each)

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

E. cDNA normalization service

Cat.# CS010-CS011

EVROGEN offers Custom cDNA Normalization Service using TRIMMER-normalization. Depending on your particular needs, we offer

- cDNA normalization for further non-directional (random) cloning or
- cDNA normalization for directional cloning.

cDNA normalization procedure is set up and monitored by the inventors of the TRIMMER-normalization technology.

You provide:

0.5-2.0 µg of total or poly(A)+ RNA

We perform (Levels 1 to 3):

Level 1

1. Full-length cDNA preparation using SMART-method
2. cDNA normalization

Note: Depending on your particular needs we can prepare normalized cDNA flanked by adapter sequences suitable for directional cloning using SfiIA - SfiIB, SalI - NotI, SdaI - NotI, or blunt end - NotI restriction sites.

Level 2 (also includes all services provided in level 1)

3. Ligation of normalized cDNA into an appropriate vector
4. Transformation of ligate from step 4 into *E. coli*.

Note: Normalized cDNA is cloned into a vector from our collection using blunt ends (non-directional cloning) or using the following restriction sites (directional cloning): *Sda*I - *Not*I, or *Srf*I - *Not*I. Cloning using other sites can be performed by agreement.

Level 3 (also includes services provided in levels 1 and 2)

5. Plating of the resulting library on one 96-well plate
6. Purification of plasmid DNA from 90 clones
7. Single run sequence of all 90 clones

Turnaround time

Level 1: 4 to 6 weeks

Level 2: 5 to 7 weeks

Level 3: 7 to 9 weeks

You will receive:

Level 1

- Any leftover starting material (on request)
- normalized cDNA (at least 1 µg)
- non-normalized cDNA (at least 1 µg)
- PCR primers for cDNA amplification
- cDNA normalization report

Additionally for Level 2

- Amplified and characterized *E.coli* library

Additionally for Level 3

- Purified plasmid DNA from 90 clones with sequencing data of the inserts

Normalization efficiency analysis (optional, on request):

Normalization efficiency analysis using PCR or Virtual Northern blotting with two marker genes.

For samples from human and mouse:

- Virtual Northern blot with ACTB and UBC gene-derived probes (high abundance in most tissues);

For samples from another sources:

- PCR analysis, if sequences of two abundant transcripts are provided by a customer, OR
- Virtual Northern blotting, if two cloned fragments corresponding to abundant transcripts are provided by a customer.

Support/Ordering Information

Technical support

If you encounter a problem using TRIMMER kit or have a question, contact our Technical Support staff using e-mail: customer-support@evrogen.com

Service ordering

For any questions related to our services please contact service@evrogen.com

Product ordering

For any questions related to ordering please contact order@evrogen.com

Notice to Purchaser:

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B. use the Product to provide a service, information, or data;
C. use the Product in manufacturing, including use of the Product in quality control or quality-assurance procedures;
D. use the Product for diagnostic or therapeutic purposes.

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