evrogen

TRIMMER-DIRECT

cDNA Normalization Kit

Cat#NK002

User Manual

This product is intended for research use only.

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I. Intended use

Evrogen TRIMMER-DIRECT kit is specifically designed to normalize amplified cDNA, enriched with full-length sequences. cDNA can be prepared using SMART (Zhu *et al.*, 2001) or Mint approaches. *Sfi*I enzyme restriction sites incorporated into cDNA-flanking adapters allow directional cloning of normalized 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 decreases the prevalence of clones representing abundant transcripts and dramatically increases the efficiency of random sequencing and 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-DIRECT is specially developed to normalize cDNA enriched with full-length sequences (Zhulidov *et al.*, 2004).

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Several features of TRIMMER-normalization 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) equilibrates at least in part reduction of the average cDNA length during PCR.

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

Molecular basis of TRIMMER-normalization

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 occur at elevated temperatures, thereby decreasing loss of transcripts due to the formation of secondary structures and non-specific hybridization involving adapter sequences.

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

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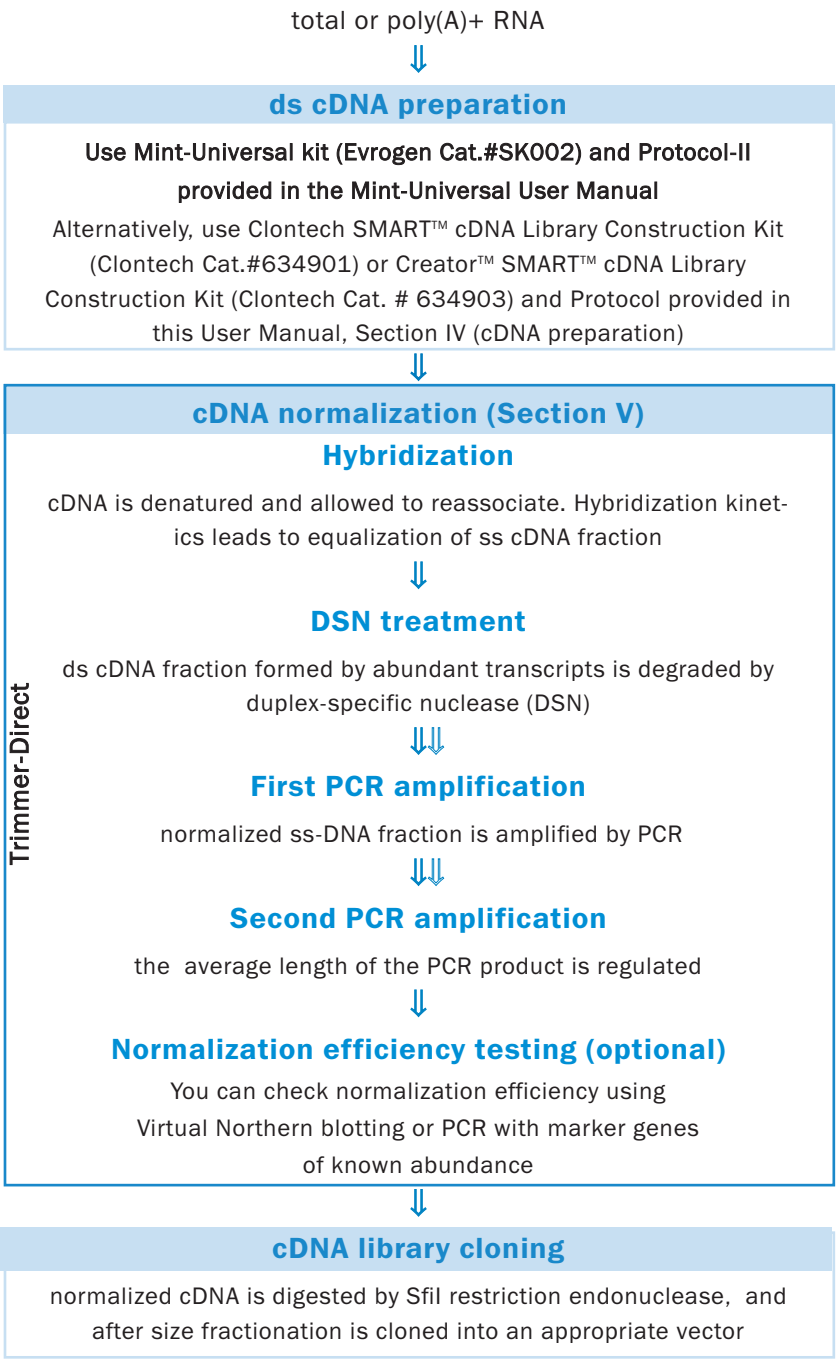

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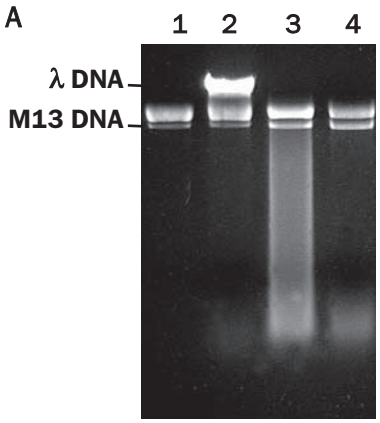
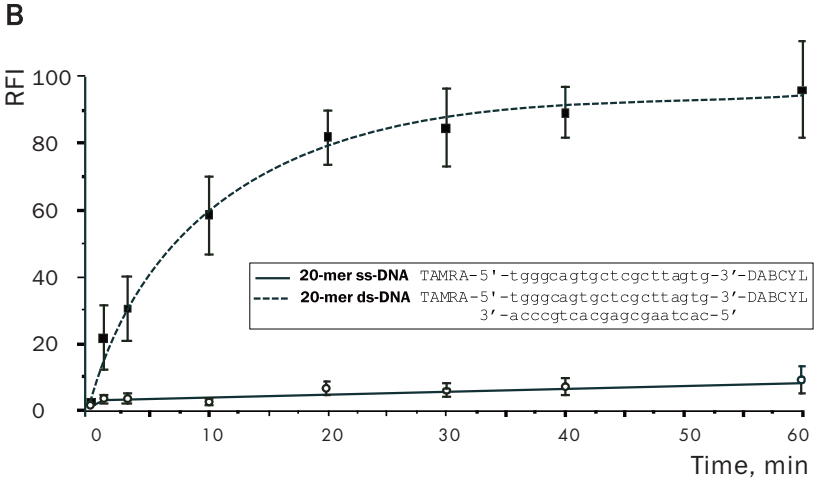


Figure 2. Determination of DSN preference for specific structural features of DNA substrates.

(A) Action of DSN on ss phage M13 DNA and ds λ 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 (TAMRA) and quencher (DABCYL) 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.

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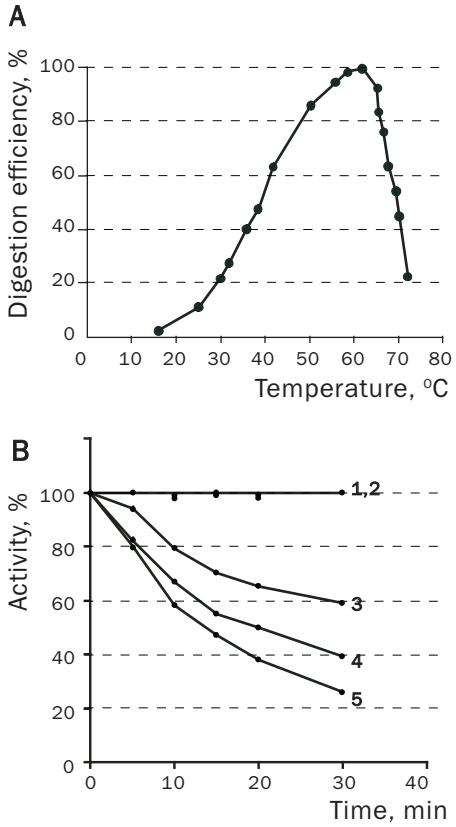

Figure 3. Dependence of the DSN activity and stability upon temperature.

(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 modified Kunitz assay.

Figure 4 details the molecular events that occur during TRIMMER-normalization. cDNA suitable for normalization should contain known flanking sequences for subsequent PCR amplification. We adapted TRIMMER-normalization to the amplified cDNA prepared by SMART (Zhu *et al*., 2001) or Mint (see Mint-Universal, protocol II) methods. Such 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.

Both cDNA synthesis methods utilize the property of MMLV reverse transcriptase to add a few C nucleotide residues at the 3' end of the first strand cDNA. Under specific conditions, a specially designed

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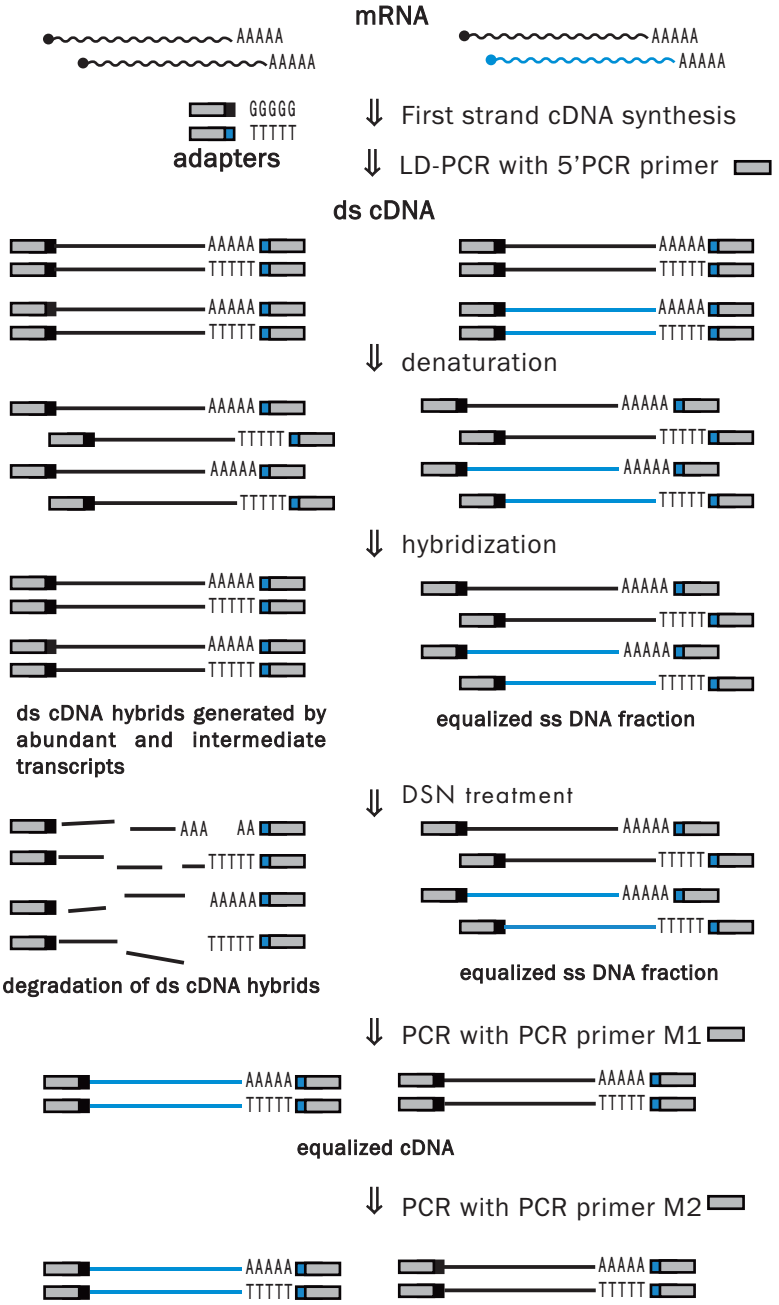

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5'-end adapter, capable of formation base pairs with the added C residues, serves as a prolonged template for reverse transcription. Consequently, cDNA prepared using these methods is flanked with asymmetric adapter sequences comprising SfiIA and SfiIB restriction sites and can be used for directionally cloned cDNA library preparation. During normalization, cDNA is denatured and subsequently allowed to rehybridize. Due to the second-order hybridization kinetics, abundant transcripts renature quicker than rare sequences, thereby, 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 in 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

Figure 4. DSN normalization scheme.

Black lines represent abundant transcripts, blue lines - rare transcripts. Rectangles represent adapter sequences and their complements.

GGGGG - 5' adapter (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3');

TTTTT - CDS-3M adapter
(5'-AAGCAGTGGTATCAACGCAGAGTGGCCGAGGCGGCCd(T)20VN*-3');

5' PCR primer: 5'- AAGCAGTGGTATCAACGCAGAGT-3'

PCR primer M1: 5'- AAGCAGTGGTATCAACGCAGAGT- 3';

PCR primer M2: 5'- AAGCAGTGGTATCAACGCAG- 3';

*N = A, C, G or T; V = A, G or C

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previously developed procedure to regulate the average length of complex PCR product (Shagin *et al*., 1999) in our normalization protocol.

Briefly, this procedure is based on the fact that under certain conditions amplification involving a single primer ("single-primer PCR") proceeds less efficiently for shorter molecules than for longer ones (Lukyanov *et al*., 1995). This can be explained as follows: a DNA fragment being amplified by a single primer contains inverted terminal repeats (ITRs) corresponding to the primer sequence. Thus in a ss-form of this molecule its ends are represented by complementary sequences, which tend to anneal to each other - the process competing with primer annealing. Evidently, the shorter the molecule, the higher the probability for its ends to meet and anneal, and therefore the stronger the competition. Suppression degree of short molecule amplification depends on a primer/ITR length ratio, primer nucleotide composition and primer concentration in PCR.

To apply the regulation procedure to cDNA prepared using SMART™ cDNA Library Construction Kits (Clontech Cat.# 634901, 634903), we modified SMART adapters so that they could form ITRs. We developed a modified 3'-CDS adapter (CDS-3M) that comprises an oligo(dT) part to anneal to a poly(A) RNA tail, *Sfi*IB site for cloning and an outer part identical to that of SMART IV™ Oligonucleotide (Clontech). CDS-3M adapter should be used instead of SMART CDS III Primer in cDNA synthesis procedure when you use one of the SMART™ cDNA Library Construction Kits.

Additionally, PCR primers were 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.

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cDNA library cloning

Asymmetric *Sfi*I restriction enzyme sites (A & B; Figure 5) incorporated at the 5' and 3' ends of normalized cDNA allow directional cloning of cDNA library. After digestion with SfiI and size fractionation, normalized cDNA is ready for ligation into an appropriate *Sfi*I-digested vector, like lTriplEx2 vector provided in SMART™ cDNA Library Construction Kit (Clontech Cat.#634901) or pDNR-LIB vector, provided in Creator™ SMART™ cDNA Library Construction Kit (Clontech Cat.# 634903).

*Sfi*IA site	*Sfi*IB site
5'-GGCC**ATTAC**GGCC-3'	5'-GGCC**GCCTC**GGCC-3'
3'-CCGG**TAATG**CCGG-5'	3'-CCGG**CGGAG**CCGG-5'

Figure 5. *Sfi*I (A & B) recognition sites.

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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
CDS-3M adapter (10 µM) 5'-AAGCAGTGGTATCAACGCAGAGTGGCCGAGGCGGCC(T)20VN*-3'	30 µl	- 20°C
PCR Primer M1 (10 µM) 5'- AAGCAGTGGTATCAACGCAGAGT- 3'	70 µl	- 20°C
PCR Primer M2 (10 µM)* 5'- AAGCAGTGGTATCAACGCAG- 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

*N = A, C, G or T; V = A, G or 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 $MgCl_2$.

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B. Materials required but not included:

- One of the following cDNA synthesis kits:

  Mint-Universal cDNA synthesis kit (Evrogen Cat.# SK002);

  SMART™ cDNA Library Construction Kit (Clontech Cat.# 634901);

  Creator™ SMART™ cDNA Library Construction Kit (Clontech Cat.# 634903);

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

- PCR kit suitable for 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 Mint-Universal kit;

- 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 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 Mint or SMART-prepared ds cDNA. For cDNA preparation we strongly recommend that you use

(1) Mint-Universal cDNA synthesis kit (Evrogen Cat.# SK002, protocol-II) or

(2) one of the SMART™ cDNA Library Construction Kits (Clontech Cat# 634901; 634903) together with CDS-3M adapter provided in the Evrogen TRIMMER-DIRECT kit.

- 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 that you perform the optimization procedure described in this protocol for each cDNA sample.

- Cycling parameters in the normalization protocol have been optimized for MJ Research PTC-200 DNA Thermal Cycler and Encyclo PCR kit (Evrogen). 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.

- 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.

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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).

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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 6 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. 6). 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. 6). 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. 6), your DSN enzyme is fully or partially inactive and cannot be used for cDNA normalization.

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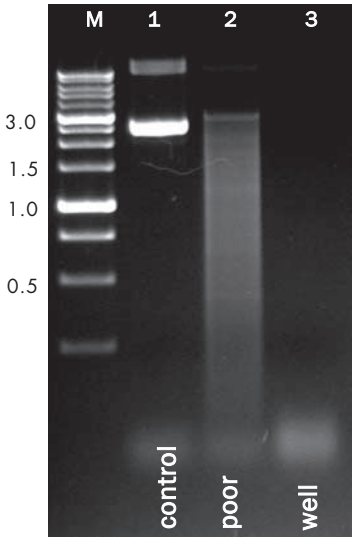

Figure 6. DSN activity testing.

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

Important notes:

1. The sequence complexity and the average length of the normalized cDNA library noticeably depend on the quality of experimental RNA starting material. Therefore, estimate 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.

2. 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.

3. We strongly recommend that you perform a positive control cDNA synthesis with control RNA provided in the cDNA synthesis kit, that you use, parallel with your experimental cDNA synthesis. This control is performed to verify that all components are working properly.

TRIMMER-DIRECT is specially developed to normalize cDNA prepared using following cDNA synthesis kits:

- Mint-Universal, protocol II (Evrogen Cat.#SK002) - Section VI.A;
- SMART™ cDNA Library Construction Kit (Clontech Cat.#634901) - Section VI.B;
- Creator™ SMART™ cDNA Library Construction Kit (Clontech Cat.#634903) - Section VI.B.

Note: Clontech kits must be used with a specially designed Evrogen CDS-3M adapter and the protocol in the Section VI.B.

A. cDNA synthesis and amplification using Mint-Universal kit (Evrogen Cat.#SK002)

1. Prepare amplified ds cDNA as described in the protocol-II provided in the Mint-Universal User Manual. Use Encyclo PCR kit included into Mint-Universal for cDNA amplification.

2. Proceed to the Section VI.C “cDNA purification”.

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B. cDNA synthesis and amplification using SMART-based kit (Clontech) and CDS-3M adapter

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Note: This protocol is optimized for Advantage™ 2 PCR kit (Clontech Cat.#639206, 639207) and SuperScript reverse transcriptase (Invitrogene) .

B1. First-strand cDNA synthesis

Note: For the first-strand synthesis and PCR, all components and reaction vessels should be pre-chilled on ice.

1. For each RNA sample, combine the following reagents in a sterile 0,5-ml tube:

1-3 µl	RNA sample (0.5 - 2 µg of RNA; for the control reaction, use 1 µl of the control RNA)
1 µl	CDS-3M adapter
1 µl	SMART IV Oligonucleotide
x µl	Sterile water
5 µl	**Total volume**

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

3. Incubate the tube at 72°C for 2 min.

4. Incubate the tube on ice for 2 min.

5. Spin the tube briefly in a microcentrifuge to collect its contents at the bottom.

6. Add the following to each reaction tube:

2 µl	5X First-Strand Buffer
1 µl	DTT (20 mM)
1 µl	50X dNTP (10 mM)
1 µl	SuperScript Reverse Transcriptase

7. Mix contents by gently pipetting and spin the tube briefly in a microcentrifuge.

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8. Incubate the tube at 42°C for 1.5 hr in an air incubator.

Note: If you use a water bath or thermal cycler for incubation, cover the reaction mixture with one drop of mineral oil before you close the tube. This will prevent the loss of volume due to evaporation.

9. Place the tube on ice to terminate first-strand synthesis.

10. If you plan to proceed directly to the PCR step (Section VI.B2), transfer a 2-µl aliquot from the first-strand synthesis reaction to a clean, prechilled, 0.5-ml reaction tube. Place the tube on ice. If you used mineral oil in your first-strand reaction tube, be careful to take the aliquot from the bottom of the tube to avoid the oil.

Note: First-strand reaction mixture that is not used right away should be placed at -20°C. First-strand cDNA can be stored at -20°C up to one month.

B2. cDNA amplification

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:

80 µl	Sterile water
10 µl	10X Advantage 2 PCR Buffer
2 µl	50X dNTP mix
4 µl	5' PCR Primer (SMART™ technology-based kit)
2 µl	50X Advantage 2 Polymerase Mix
98 µl	**Total volume**

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

4. Aliquot 98 µl of the PCR Master Mix into each reaction tube from step B1.10.

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

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6. If necessary, overlay the reaction mixture with 2 drops of mineral oil. Close the tube, and place it into the preheated thermal cycler.

7. Commence thermal cycling using the following program:

95°C for 7 s; 66°C for 20 s; 72°C for 4 min

Note: Cycling parameters are optimized for MJ Research PTC-200 and Advantage™ 2 Polymerase mix. They may vary depending on thermal cycler, polymerase mix and cDNA template.

8. Subject the tube to PCR cycling. To determine the optimal number of PCR cycles for a given amount of total or poly(A)+ RNA used for the first-strand cDNA synthesis, see Table 1.

9. When the cycling is completed, analyze 5 µl of each PCR product alongside 0.1 µg of 1 kb DNA size marker on a 1.5% agarose/EtBr gel in 1X TAE buffer. For comparison, Figure 7 shows a characteristic gel profile of ds cDNA synthesized from the control human placental poly(A)+ RNA. In the case of PCR undercycling, subject the PCR reaction to two more cycles and recheck the product.

Table 1. PCR cycling parameters

Total RNA (µg)	poly(A)+ RNA (µg)	Number of PCR cycles
1.0-1.5	0.5-1.0	13-15
0.5-1.0	0.25-0.5	15-18
0.25-0.5	0.25-0.1	18-21

Note: Table 1 was developed using placenta and skeletal muscle total poly(A)+ RNA and MJ Research PTC-200 Thermal Cycler. Optimal parameters may vary with different thermal cyclers, polymerase mixes, and templates. Use the minimal possible number of cycles since overcycling may yield a nonspecific PCR product. If necessary, undercycling can be easily rectified by placing the reaction tube back into the thermal cycler for a few more cycles (see Troubleshooting Guide, Section B).

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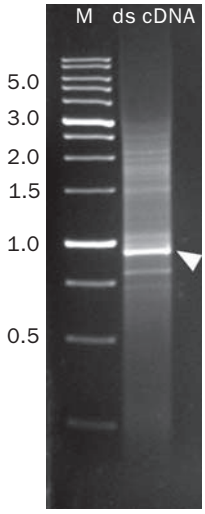
Figure 7. ds cDNA synthesized from poly(A)+ placenta RNA using SMART protocol.

1 µg of poly(A)+ RNA was used as starting material in a first-strand cDNA synthesis. 2 µl of the first-strand cDNA was then used as template for SMART cDNA amplification in 100 µl reaction volume. 16 PCR cycles were performed. 5 µl of the PCR product was electrophoresed on a 1.5% agarose/EtBr gel.

Lane M: 1-kb DNA size marker (0.1 µg loaded). The arrow indicates a strong band at 900 bp typically seen for human placenta cDNA.



Typical results for ds cDNA synthesis using LD PCR and SMART protocol should have the following characteristics:

1. A moderately strong smear of cDNA ranging from 0.1 to 4 kb (or more).

   Compare the intensity of the banding pattern of your PCR product with the 1-kb DNA size marker (0.1 µg run on the same gel). For cDNA from mammalian RNA sources, the overall signal intensity (relative to the DNA size marker) should be roughly similar to that shown in the control experiment in Figure 7. If the smear is much fainter and the size distribution is less than 4 kb, it could indicate that too few thermal cycles (i.e., PCR undercycling) have been used. Subject the PCR reaction to two more cycles and recheck the product. If the intensity of the cDNA smear is much stronger than that shown in the control (relative to 0.1 µg of size markers), especially if no bright bands are distinguishable, it could indicate that too many thermal cycles have been used, i.e., PCR overcycling has occurred.

Notes: 1. Normal mRNA size distribution ranges from 0.1 to 4 kb (or more) for mRNA from a mammalian source. For other sources, such as insect species, the normal mRNA size distribution may be less than 2-3 kb.

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2. Please note, that cDNA with low molecular weight does not represent full-length transcripts. Such cDNA will not become full-length during the normalization procedure and is not suitable for full-length library preparation. However, such cDNA is suitable for DSN normalization and preparation of cDNA library comprising non-full-length cDNA fragments.

2. Several bright bands corresponding to abundant transcripts

The pattern of bright bands shown in Figure 7 is characteristic of the PCR product obtained using the control human placenta poly(A)+ RNA. A very strong smear of cDNA in the control reaction without the characteristic bright bands may be indicative of PCR overcycling. If the characteristic bands are present but weak, this may be indicative of PCR undercycling.

Note: The number and position of the bands you obtain with your experimental RNA may differ from those shown for the control reaction in Figure 7. Furthermore, cDNA prepared from some mammalian tissue sources (e.g., human brain, spleen, and thymus) may not display bright bands due to a very high complexity of poly(A)+ RNA.

3. Some low-molecular-weight material

Most raw PCR reaction products will contain some small cDNA fragments (<0.4 kb) and some very small (i.e., <0.1 kb) non-cDNA contaminants. The non-cDNA contaminants include unincorporated primers, oligonucleotides, and primer dimers. A preponderance of material in the lower part of the gel (i.e., <0.1 kb) may indicate that PCR overcycling has occurred.

See also Troubleshooting Guide, Section B.

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C. cDNA purification

1. Purify the resulting amplified cDNA (from the step A.1 or B.9) to remove primer excess, dNTPs and salts using a commercial PCR Purification Kit.

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

2. 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.

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

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

5. Vortex the mixture thoroughly and centrifuge the tube for 15 min at 12,000 - 14,000 rpm at room temperature.

6. Remove the supernatant carefully.

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

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

9. Carefully remove the supernatant.

10. Repeat steps 7-9.

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

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

13. 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.

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14. Place the tube on ice.

Now, you have obtained ds cDNA ready for normalization.

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

VII. Normalization protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

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 0.5-ml tube:

4-12 µl	ds cDNA (about 600-1200 ng of dissolved cDNA from stage VI.C.14)
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 2) 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. Do not remove the samples from the thermal cycler before DSN treatment.

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.

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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 VII.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.

Table 2. Setting up DSN treatment

Component\Tube*		experimental		control
	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

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5. Add DSN enzyme as specified in the Table 2. 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. Then, place the tubes on ice.

9. 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 up to two weeks and used afterwards to prepare more normalized cDNA.

C. First amplification of normalized cDNA

Note: For PCR amplification, please use PCR primers provided in the TRIMMER-DIRECT 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.

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4. Aliquot 1 µl of each diluted cDNA (from Step VII.B.9) 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 the 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 2) to determine the optimal number of PCR cycles using the procedure described in Step 9-10 (below). Store the other tubes on ice.

9. For each Control tube, determine the optimal number of PCR cycles (Figure 8):

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

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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 microcentrifuge 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.

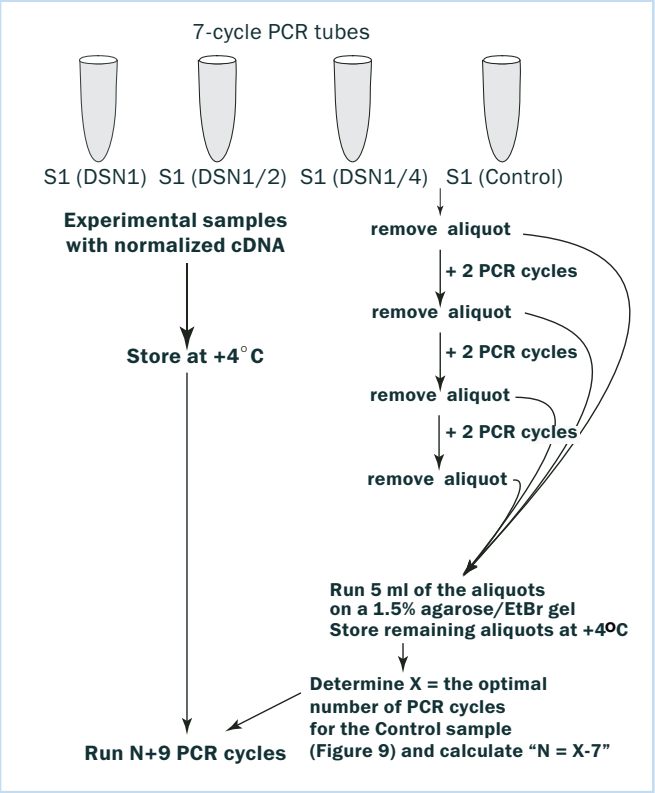

Figure 8. Optimizing PCR parameters for normalized cDNA amplification.

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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 9). Store the remaining material on ice.

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.

Figure 9 provides an example of how your analysis should proceed. In this experiment, after 11 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 9.

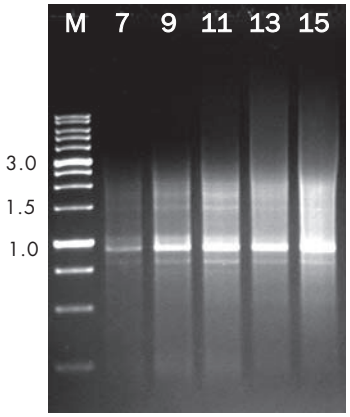

Figure 9. Analysis for optimizing PCR parameters.

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 9. Lane M: 1-kb DNA ladder size markers, 0.1 µg loaded.

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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 9, the optimal number of PCR cycles determined for control cDNA in the Control tube was 9. Thus, in this example X=9, and N=9-7=2. Hence, in this example, 7-PCR experimental tubes should be subjected to 2+9 additional PCR cycles.

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 1.5% agarose/EtBr gel in 1X TAE buffer.

13. Select the tube(s) with efficient normalization. For comparison, Figure 10 shows a characteristic gel profile of human placenta cDNA normalized using TRIMMER-DIRECT kit.

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 could indicate that normalization process was superfluous.
- 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 could indicate that normalization process was not successful.

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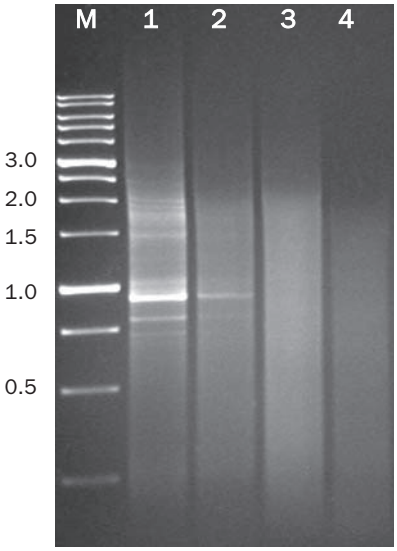

Figure 10. Analysis of cDNA normalization results.

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

In this experiment efficient normalization was achieved in the S1_DSN1/2 tube (lane 3). In the S1_DSN1/4 tube (lane 1) normalization was not completed, in the S1_DSN1 tube (lane 4) DSN treatment was excessive, resulting in partial cDNA degradation.

- 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.

See also Troubleshooting Guide, Section C.

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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 up to one month and used afterwards to prepare more normalized cDNA.

D. Second amplification of normalized cDNA

To obtain the best results, we recommend to perform reamplification 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 diluted normalized cDNA from step 1 into an appropriately labeled sterile PCR tube.

4. Aliquot 2 µl 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**

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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. Commence thermal cycling 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

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

Subject the tubes to 12 cycles. When the cycling is completed, electrophorese 5 µl of the PCR products alongside 0.1 µg of 1-kb DNA size markers on 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.

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

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Now, you have normalized ds cDNA flanked by asymmetric adapters containing *Sfi*I restriction enzyme sites.

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

Normalized cDNA can be used to prepare directionally cloned normalized cDNA library. **Before cloning, cDNA purification using phenol-chloroform extraction or commercial PCR purification kits and size fractionation is strongly recommended.** Size fractionation can be performed using CHROMA SPIN™-400 or 1000 columns (Clontech) or equivalent.

Appropriate vectors for library cloning using *Sfi*I sites include, for example, lTripIEx2 and pDNR-LIB vectors provided in Clontech SMART™ cDNA Library Construction Kit (Cat.# 634901) and Creator™ SMART™ cDNA Library Construction Kit (Cat.# 634903), respectively. Please use correspondent manufacturer protocols (start from "SMART cDNA Synthesis by LD PCR. Proteinase K digestion" section) for library cloning.

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.

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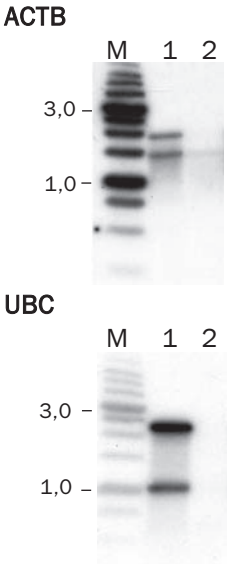

Figure 11. Virtual Northern blot analysis of abundant (ACTB, UBC) transcripts in non-normalized and normalized cDNA.

TRIMMER-normalization of human placenta SMART-cDNA was performed. About 0.5 mg 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.

A. Virtual Northern blot

To perform Virtual Northern blot, electrophorese your normalized and control secondary PCR products (from step VII.D.10) on 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-DIRECT kit. These plasmids contain fragments of ACTB and UBC housekeeping genes, expressed at high level in most tissues and cell lines. The 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 11.

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

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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. cDNA preparation

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

a. Template switching in SMART method requires the use of an MMLV RNase H- point mutant reverse transcriptase such as SuperScript Reverse Transcriptase.

b. RNA may degrade during storage and/or hybridization procedure. Poor quality of starting material will reduce the chances to obtain representative unfragmented cDNAs. Your working area, equipment, and solutions must be free of RNase contamination. Check the quality of starting RNA on denaturing formaldehyde/agarose gel electrophoresis.

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c. CDS-3M adapter may degrade during storage and/or delivery. To check adapter quality, perform control cDNA preparation using the reagents and protocol provided in SMARTTM technology-based kits.

- If cDNA synthesis using Clontech reagents is successful, it indicates that CDS-3M adapter is degraded. Please contact Evrogen Technical Support to replace the adapter.

- If cDNA synthesis using Clontech reagents is not successful, it indicates that CDS-3M adapter is not degraded and other reagents used for cDNA preparation must be checked.

d. PCR conditions and parameters might have been suboptimal. The optimal number of PCR cycles may vary with different PCR machines, polymerase mixes, or RNA samples. If your PCR reaches its plateau after 24 cycles or more, the conditions of your PCR may have not been optimal. Check the protocol and repeat the PCR using a fresh aliquot of the first-strand cDNA product.

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

2. Poor yield or truncated PCR product is generated from your experimental RNA, whereas a high-quality PCR product is generated from the control RNA.

a. Your experimental RNA sample may be too diluted or degraded. If you have not already done so, electrophorese your RNA sample on a formaldehyde/ agarose/EtBr gel to estimate its concentration and analyze its quality.

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b. If your RNA sample was prepared from a non-mammalian species, the seemingly truncated PCR product may actually have the size distribution normal for that species. For example, for insects, the normal RNA size distribution may be <2-3 kb.

c. Experimental RNA has been partially degraded (by contaminating RNases) before or during the first-strand synthesis. Repeat the experiment using a fresh lot or preparation of RNA. Check the stability of your experimental RNA by incubating a small aliquot in water for 2 hr at 42°C. Then, electrophorese 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.

d. Your experimental RNA sample contains impurities that inhibit cDNA synthesis. In some cases, ethanol precipitation of your RNA, followed by washing twice with 80% EtOH, may remove impurities. If this does not help, reisolate RNA using a different technique.

3. The concentration of your PCR product is low, but the quality is good.

a. Too few thermal cycles were used in the PCR step resulting in a low yield of PCR product. If you suspect that undercycling is the problem, subject the PCR mixture to two or three more cycles and recheck the product. If the increase of the number of cycles does not improve the yield of PCR product, repeat PCR using a fresh aliquot of the first-strand product.

Note: Representation of the resulting amplified cDNA library strongly depends on the initial number of target DNA molecules used for PCR amplification. There is a direct relationship between the initial number of target DNA molecules and the number of PCR cycles

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required to amplify cDNA in the amount of about 5-10 ng/µl (visible on agarose/EtBr gel). If you require 25 or fewer cycles to amplify cDNA, your sample is representative. In practice, we prefer to achieve at least one order of magnitude higher representation (i.e., get a robust cDNA product in 16-21 cycles) to ensure that even the rarest messages were included.

b. The concentration of your experimental RNA is low. Repeat the experiment using more RNA.

4. No bright bands are distinguishable in the PCR product visualized on agarose gel-electrophoresis.

a. For most mammalian RNA sources, there should be several bright bands distinguishable against the background smear when the PCR product is run on a gel. If bright bands are expected but are not visible, and the background smear is very intense, it could indicate PCR overcycling. Repeat the PCR step (VI.B.8) with a fresh first-strand cDNA sample, using 2-3 fewer cycles.

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

5. Low-molecular-weight (<0.1 kb) material is observed in the ds cDNA product.

a. The raw cDNA (e.g., before size fractionation) is expected to contain some low-molecular-weight DNA contaminants, including unincorporated primers, adapters and very short PCR products. However, these small fragments are generally removed from the ds cDNA preparation in the size fractionation step (after normalization). Nevertheless a preponderance of low-molecular-weight (<0.1 kb) material in the raw PCR product could indicate PCR overcycling. If you suspect overcycling, repeat the PCR step with a fresh sample of first-strand cDNA, using 2-3 fewer cycles.

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C. 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. Be sure that your cDNA has suitable adapters at the 5'and 3'-ends. TRIMMER-DIRECT is designed for normalization of amplified cDNA prepared using following kits and protocols:

- Mint-Universal (Evrogen Cat.# SK002), **protocol-II** ;

- Clontech SMART™ technology-based kits (SMART™ cDNA Library Construction Kit, Cat.# 634901, or Creator™ SMART™ cDNA Library Construction Kit, Cat.# 634903) with the replacement of the CDS III/ 3' PCR Primer by Evrogen CDS-3M adapter.

b. cDNAs may degrade 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 cDNA on agarose gel electrophoresis. Repeat ethanol precipitation of cDNA after column purification (see Section VI.C) and normalization using a fresh cDNA aliquot.

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

1. Dilute 1-2 µl of ds cDNA (prepared as described in the Section VI.A or VI.B) with sterile water to the final cDNA concentration of 2 ng/µl.

2. Aliquot 2 µl of PCR primer M1 from Trimmer-Direct kit into an appropriately labeled sterile PCR tube.

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

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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 a drop of mineral oil. Close the tubes, and place them to the preheated thermal cycler.

9. 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: 95°C for 15 s; 66°C for 20 s; 72°C for 3 min. Subject the tubes to 11 cycles.

10. When the cycling is completed, electrophorese 5 µl of the PCR products alongside 0.1 µg of 1-kb DNA size markers on 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 concentration of the PCR product produced from PCR primer M1 is much less than that for the PCR pro-

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duct produced from PCR primer taken from cDNA synthesis kit, it could 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. After PCR parameter optimization, repeat PCR using fresh aliquots of cDNA after DSN treatment (i.e. from stage VII.B.10).

f. You may have made an error during the procedures, such as using a suboptimal incubation temperature or omitting 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.

a. 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.

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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.5-6), adding DSN to your experimental tubes as shown in Table 3.

Table 3. Setting up DSN treatment

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

* S <NUMBER>- cDNA sample specification

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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. 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.5-6) as shown in Table 4.

Table 4. Setting up DSN treatment

Component\Tube*	TUBE 1 (S1 DSN1)	experimental TUBE 2 (S1 DSN1.5)	TUBE 3 (S1 DSN2)	control TUBE 4 (S1 Control)
1/4 DSN dilution	1 µl	1.5 µl	2 µl	-
DSN storage buffer	-	-	-	1 µl

* S <NUMBER>- cDNA sample specification

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D. 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 have degraded during storage. Your working area, equipment, and solutions must be free of nuclease contamination. Check the quality of starting material on an agarose gel. Repeat PCR amplification using a fresh cDNA aliquot.

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

1. Dilute 1-2 µl of ds cDNA (prepared as described in the Section VI.A or VI.B) with sterile water to the final cDNA concentration of 2 ng/µl.

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

3. Aliquot 2 µl of 5' PCR Primer M1 into another appropriately labeled sterile PCR tube.

4. 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.

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8. If necessary, overlay the reaction mixture with a 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 15 s; 64°C for 20 s; 72°C for 3 min.

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

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 PCR primer M1, it could indicate that PCR primer M2 is degraded. Please contact Evrogen Technical Support to replace the PCR primer or use PCR primer M1 for second amplification as described in the following section.

c. The concentration of starting cDNA is low, but the quality is good. In some cases, PCR amplification from PCR primer M2 is inefficient. In these cases please use PCR primer M1 for second amplification using the following protocol:

1. Aliquot 2 µl of normalized cDNA (see steps VII.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 VII.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.

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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:

81 µl	Sterile water
10 µl	10X PCR Buffer
2 µl	50X dNTP mix
3 µl	PCR primer M1
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, for example

Step	Number of cycles	Temperature
Initial denaturation	1	95°C for 1 min
Cycling	12	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

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

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11. When the cycling is completed, electrophorese 5 µl of the PCR products alongside 0.1 µg of 1-kb DNA size markers on 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.

E. 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.

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XI. Related products and services

A. Mint-Universal cDNA synthesis kit

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

Use

MINT-Universal cDNA synthesis kit is designed to synthesize full-length-enriched double stranded (ds) cDNA from total or poly(A)+ RNA. Synthesized cDNA can be used in various applications including preparation of directionally or non-directionally cloned cDNA libraries, Virtual Northern blot, subtractive hybridization (SSH), and cDNA normalization using duplex-specific nuclease.

Protocol-I provides instruction for cDNA synthesis for non-directional cloning of cDNA library;

Protocol-II provides instruction for cDNA synthesis for directional cloning of cDNA library.

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

Product	Cat.#	Amount
TRIMMER	NK001	for 10 reactions

Use

TRIMMER kit is optimized for ds cDNA prepared using following cDNA synthesis kits:

Mint (Evrogen Cat.# SK001);
Mint-Universal, Protocol-I (Evrogen Cat.# SK002);
SMART™ PCR cDNA Synthesis Kit (Clontech Cat.# 634902);
Super SMART™ PCR cDNA Synthesis Kit (Clontech Cat.# 635000).

cDNA generated using these kits contains symmetric adapter sequences, 5'-aagcagtggtatcaacgcagagt-3', at both ends and can

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be used after normalization with TRIMMER kit to generate nondirectionally (randomly) cloned normalized cDNA library.

C. Duplex-specific nuclease, lyophilized

Product	Cat.#	Amount	Storage
Duplex-Specific Nuclease	EA001	50 Units*	- 20°C
Duplex-Specific Nuclease	EA002	100 Units*	- 20°C
Duplex-Specific Nuclease	EA003	10 Units*	- 20°C

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

Purity

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 use

- Degrade double-stranded DNA

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

DSN features

DSN acquires its enzymatic activity in the presence of Mg^{2+} ions (7 mM required for optimal activity) 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 RNA substrates is observed.

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D. cDNA normalization service

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

- cDNA normalization for further nondirectional cloning (Cat.#CS013)

- cDNA normalization for directional cloning (Cat.#CS014).

Processing of cDNA normalization order is set up and monitored by the inventors of the TRIMMER-normalization technology. Various options are available, including cDNA preparation and normalization for 454 sequencing.

E. Encyclo PCR kit

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

Encyclo PCR kit provides reagents for 100 standard PCR reactions (50 µl each). The kit is especially recommended for cDNA amplification due to optimal combination of high fidelity and processivity provided by Encyclo polymerase mix.

Encyclo polymerase mix features:

5'>3' DNA polymerase activity with high processivity;
Proofreading 3'>5' exonuclease activity;
Automatic hot start;
TA cloning compatibility.

Encyclo buffer has been developed to facilitate the amplification of specific PCR products and provide successful amplification of long DNA templates. Encyclo PCR kit includes a mix of high-purity deoxyribonucleotides as well as sterile PCR water. Control DNA template and primer mix enclosed can be used for positive control PCR reaction.

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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

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ver. NK002-03111

Evrogen JSC

Miklukho-Maklaya str, 16/10
117997, Moscow, Russia

Tel: +7(495) 988 4084
Fax: +7(495) 988 4085
www.evrogen.com