

## Trimmer-2 cDNA normalization kit, User Manual

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# Trimmer-2

## cDNA normalization kit

Cat # NK003

User manual

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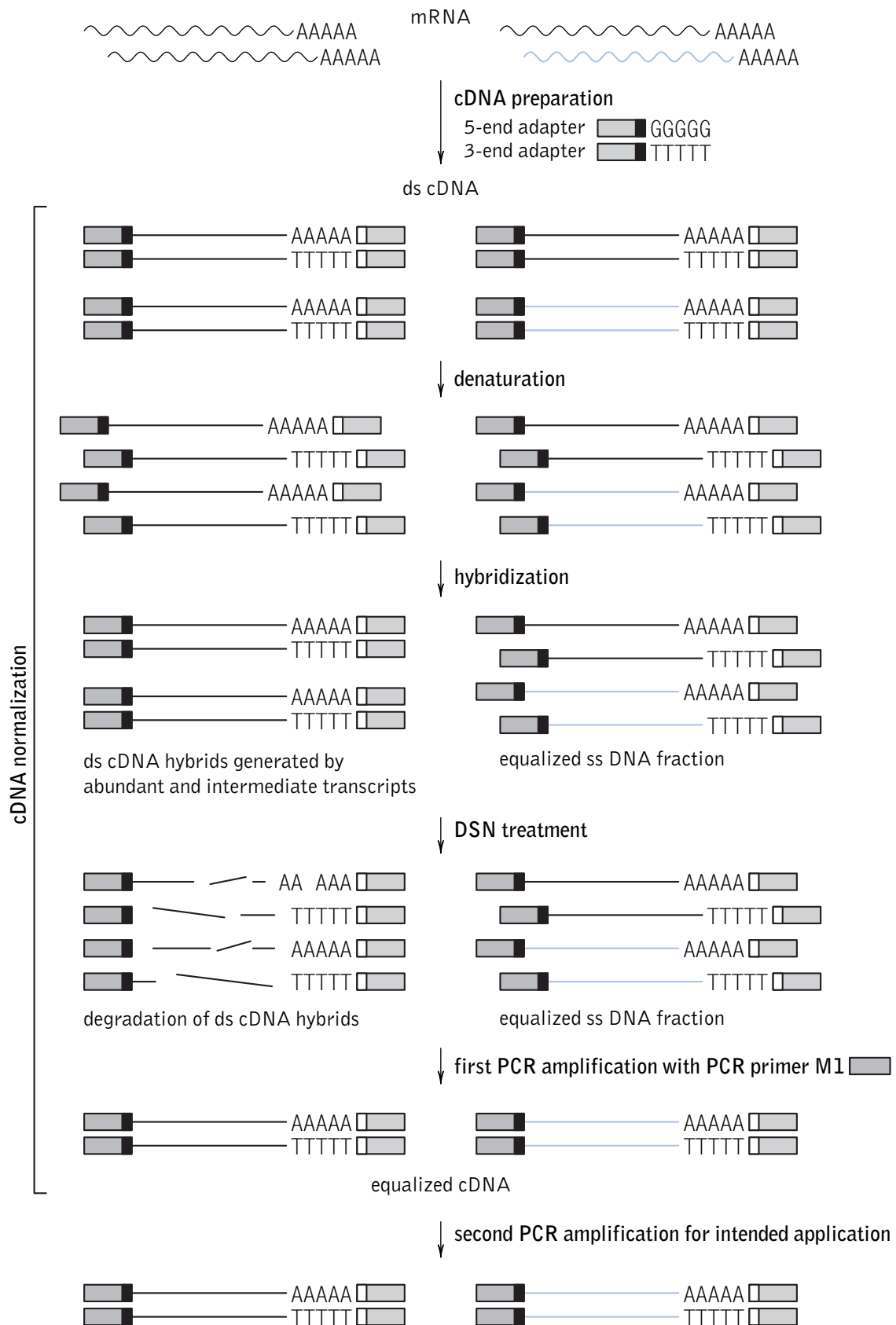
## I Intended use

In an eukaryotic cell, the mRNA population constitutes approximately 1% of total RNA with the number of transcripts varying from several thousand to several tens of thousands. Normally, the high abundance transcripts (several thousand mRNA copies per cell) of as few as 5-10 genes account for 20% of the cellular mRNA. The intermediate abundance transcripts (several hundred copies per cell) of 500-2000 genes constitute about 40-60% of the cellular mRNA. The remaining 20-40% of mRNA is represented by rare transcripts (from one to several dozen mRNA copies per cell) [1]. Such an enormous difference in abundance complicates large-scale transcriptome analysis, which results in recurrent sequencing of more abundant cDNAs.

cDNA normalization decreases the prevalence of high abundance transcripts and equalizes transcript concentrations in a cDNA sample, thereby dramatically increasing the efficiency of sequencing and rare gene discovery.

Trimmer-2 kit is designed to normalize amplified full-length-enriched cDNA prepared using Evrogen Mint cDNA synthesis kits. The resulting cDNA contains equalized abundance of different transcripts and can be used for construction of cDNA libraries and for direct sequencing, including high-throughput sequencing on the next generation sequencing platforms (Roche/454, ABI/SOLiD or Illumina/Solexa). The kit also includes special adapters allowing use of Clontech SMART-based kits for construction of cDNA intended for Trimmer-2 normalization.

## I Intended use



**Fig. 1. DSN normalization scheme.** Black lines represent abundant transcripts, blue lines – rare transcripts. Rectangles represent adapter sequences and their complements.

## II Method overview

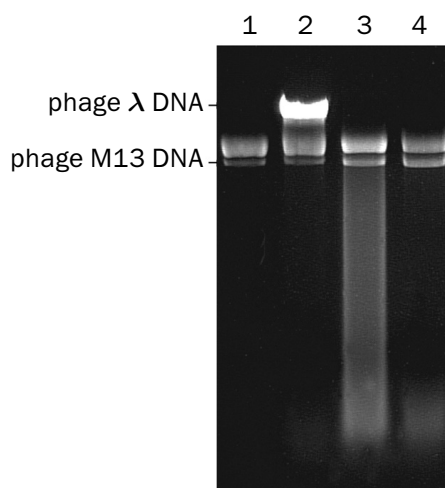
Trimmer-2 kit utilizes a duplex-specific nuclease-based cDNA normalization method [2, 3]. The method is based on nucleic acid hybridization kinetics [4] and unique properties of the duplex-specific nuclease (DSN) specific to the double-stranded (ds) DNA [5].

Normalization procedure is illustrated in Fig. 1. After denaturation of ds cDNA flanked with known adapters, it is subjected to renaturation. During renaturation, abundant transcripts convert to the ds form more effectively than those that are less frequent [4, 6]. Thus, two fractions are formed, specifically, a ds-fraction of abundant cDNA and a normalized single-stranded (ss) cDNA. The ds cDNA fraction is then degraded by DSN.

DSN is an enzyme from Kamchatka crab that displays a strong preference for cleaving ds DNA compared to ss-DNA and RNA, irrespective of the sequence length (Fig. 2). Owing to DSN thermostability (Fig. 3), ds DNA degradation is performed under conditions of cDNA renaturation that prevent the formation of secondary structures and non-specific hybridization involving adapter sequences within the ss cDNA fraction.

The remaining normalized ss DNA is amplified by PCR. Normalized cDNA can then be used for library cloning or sequencing.

cDNA suitable for normalization can be prepared on the basis of total or poly(A)+ RNA and should contain known adapter sequences at both ends for PCR amplification. The quality of the RNA is crucial, especially

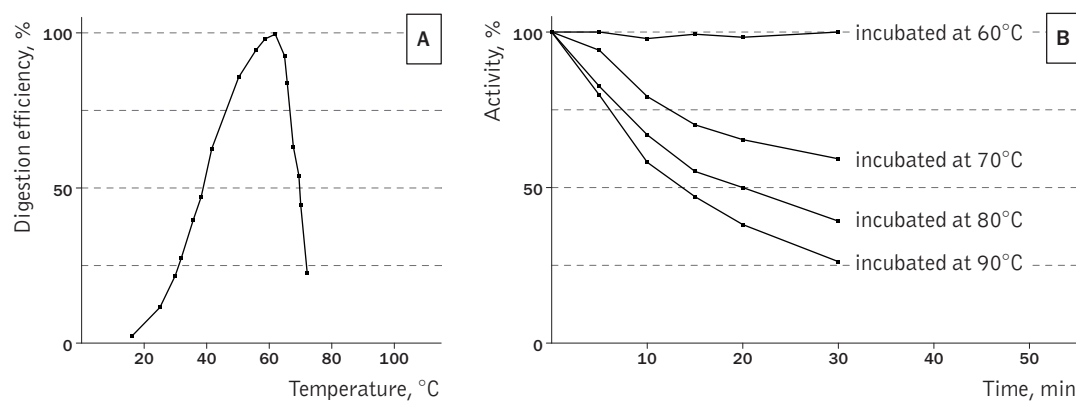


**Fig. 2. Action of DSN on ss DNA of phage M13 and ds DNA of phage λ.**

Lanes 1, 2 - negative controls, incubation without nuclease.

1 - phage M13 DNA alone, 2 - mixture containing phage M13 and lambda DNA. Lanes 3, 4 - digestion of phage M13 and lambda DNA mixture by DSN at 70°C for 1.5 min (3) and 5 min (4).

## II Method overview



**Fig. 3. Dependence of the DSN activity and stability on temperature.** A. Effect of temperature on the DSN activity. Activity of DNase on ds DNA substrate was measured using modified Kunitz assay [7] at different temperatures; B. Effect of temperature on the DSN stability. DSN was incubated at different temperatures for 30 min. Activity of DNase on ds DNA substrate was measured at 65°C using modified Kunitz assay.

when construction of full-length enriched cDNA library is a goal. The flanking sequences can be introduced to the cDNA ends during cDNA synthesis by the template-switching approach (See Section IV «cDNA preparation»).

## III Kit components and storage conditions

### III. A List of kit components

Trimmer-2 cDNA normalization kit provides components for 10 normalization reactions.

Component	Amount
DSN enzyme, lyophilized	50 Units*
DSN storage buffer (50 mM Tris-HCl, pH 8.0)	120 µL
4x Hybridization buffer (200 mM Hepes, pH 7.5, 2M NaCl)	70 µL
2x DSN master buffer (100 mM Tris-HCl, pH 8.0, 10 mM MgCl <sub>2</sub> , 2 mM DTT)	500 µL
DSN stop solution (10 mM EDTA)	1000 µL
DSN control template, 100 ng/µL	20 µL
Control cDNA template, mouse (50-70 ng/µL)	50 µL
GAPD primer mix (10 µM each) Direct primer: 5'-TTAGCACCCCTGGCCAAGG-3' Reverse primer: 5'-CTTACTCCTTGGAGGCCATG-3'	25 µL
<b>Primers</b>	
PCR Primer M1 (10 µM) 5'-AAGCAGTGGTATCAACGCAGAGT-3'	150 µL
454 PCR Primer mix (10 µM each) 5'-CAACGCAGAGTGGCCATTAC-3' 5'-ACGCAGAGTGGCCGAGGCGGCCTTTTGTCTTTTCTTCTGTTTCTTTT-3'	100 µL
<b>3'-end adapters</b>	
CDS-Gsu adapter (10 µM) 5'-AAGCAGTGGTATCAACGCAGAGTACTGGAG(T) <sub>20</sub> VN-3'	25 µL

continued on next page



### III Kit components and storage conditions

Component	Amount
CDS-4M adapter (10 $\mu$ M) 5'-AAGCAGTGGTATCAACGCAGAGT <u>GGCCGAGGCGGCC</u> (T) <sub>4</sub> G(T) <sub>6</sub> C(T) <sub>13</sub> VN-3'	25 $\mu$ L

Sfil and Gsul restriction sites (Fig. 4) are underlined; N=A, C, G or T; V=A, G or C.

\*DNase activity was measured using modified Kunitz assay [7] where unit was defined as: the amount of DSN added to 50  $\mu$ g/ml calf thymus DNA that causes an increase of 0.001 absorbance units per min. Activity assay was performed at 25°C, in 50 nM Tris-HCl buffer, pH 7.15, containing 5 mM MgCl<sub>2</sub>.

*Shipping & Storage: All components of the kit can be shipped at ambient temperature. Upon arrival all kit components must be stored at -20°C.*

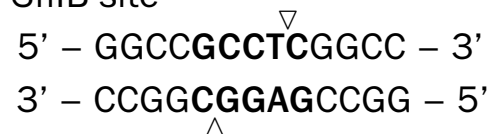
#### III. B Reagents required but not included

- One of the following cDNA synthesis kits:
  - Mint-2 cDNA synthesis kit (Evrogen, Cat.# SK005) – *recommended*
  - SMART<sup>TM</sup> cDNA library construction kit (Clontech, Cat.# 634901)
  - SMARTer<sup>TM</sup> PCR cDNA synthesis kit (Clontech, Cat.# 634925 or 634926)
- Encyclo PCR kit (Cat. # PK001, included in the Mint-2 cDNA synthesis kit) or analogues
- Biology grade mineral oil
- Agarose gel electrophoresis reagents

SfilA site



SfilB site



Gsul site

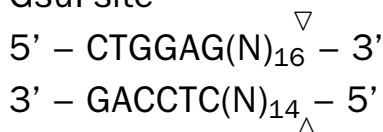


Fig. 4. Sfil and Gsul recognition sites.

- DNA size markers (1-kb DNA ladder)
- 98% and 80% ethanol
- 3M sodium acetate (NaAc), pH 4.8
- QIAquick PCR purification kit (Qiagen Inc., Cat. # 28104 or 28106)
- Sterile molecular biology grade water (sterile RNase-free water)

## IV cDNA preparation

Trimmer-2 protocol is optimized for normalization of ds cDNA flanked by M1-primer sequence at both ends. Such cDNA can be prepared using **Mint-2 cDNA synthesis kit (Cat.# SK005)**.

Alternatively, cDNA compatible with Trimmer-2 protocol can be prepared using Clontech SMART™ cDNA Library Construction Kit (Cat.# 634901) or SMARTer™ PCR cDNA Synthesis Kit (Cat.# 634925; 634926). However, in this case, the original protocol of cDNA synthesis requires special modifications. The modified SMART cDNA synthesis protocol is given in Appendix A of this manual.

Depending on the intended downstream application you should use different 5'- and 3'-end adapters for cDNA synthesis. Importantly, if you plan to synthesize cDNA using Clontech SMART-based kits it might be necessary to substitute the original 3'-end adapter with an alternative adapter included in the Trimmer-2 kit. Please refer to the Table 1 below to find the appropriate adapter pair suitable for your needs.

**Table 1. 5'- and 3'-adapters for cDNA synthesis**

Intended application	Adapter pair	
	5'-end adapter (source kit)	3'-end adapter (source kit)
Non-directional cDNA library cloning and Sanger sequencing	PlugOligo-1 (Mint-2 kit, Evrogen)	CDS-1 (Mint-2 kit, Evrogen)
	SMARTer II A Oligonucleotide (SMARTer™ kit, Clontech)	3'SMART CDS Primer II A (SMARTer™ kit, Clontech)

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## IV cDNA preparation

Intended application	Adapter pair	
	5'-end adapter (source kit)	3'-end adapter (source kit)
Roche/454 sequencing	PlugOligo-3M (Mint-2 kit, Evrogen)	CDS-4M or CDS-Gsu* (Mint-2 kit, Evrogen)
	SMART IV Oligonucleotide (SMART™ cDNA Library Construction Kit, Clontech)	CDS-4M or CDS-Gsu* (Trimmer-2 kit, Evrogen)
SOLiD and Illumina sequencing; directional cDNA library cloning	PlugOligo-3M (Mint-2 kit, Evrogen)	CDS-4M (Mint-2 kit, Evrogen)
	SMART IV Oligonucleotide (SMART™ cDNA Library Construction Kit, Clontech)	CDS-4M (Trimmer-2 kit, Evrogen)

\* The presence of long poly(A:T) tails in cDNA may result in sequencing reads of low quality when using Roche/454 sequencing platform. Two special 3'-end adapters are designed to overcome this problem:

- CDS-4M adapter with a poly(T) part built of thymidines interspersed with other nucleotides.
- CDS-Gsu adapter containing a Gsul recognition site (Fig. 4) just upstream of the poly(T) sequence. Restriction enzyme Gsul cuts cDNA within the poly(A) tail, reducing its length so that all subsequent sequences start with a shorter run of thymidines.

Both CDS-4M and CDS-Gsu adapters allow synthesis of cDNA suitable for Roche/454 sequencing. The choice of a particular adapter should be made by the end user. Use of CDS-4M adapter does not require an additional digestion step before cDNA sequencing, however even modified poly(A:T) tails of cDNA may affect the sequence quality on some Roche/454 platforms. cDNA prepared with CDS-Gsu adapter and digested by Gsul enzyme contains shorter poly(A:T) tails that do not harm sequencing. However cDNAs containing intrinsic Gsul recognition sites will be digested as well, potentially resulting in difficulties with contig assembly.

## V General considerations

- AVOID getting drops of the reaction mixture on the walls of the reaction tubes or inside the mineral oil fraction. Even a small aliquot of nonDSN-treated cDNA will corrupt normalization results.
- Wear gloves to protect RNA and cDNA samples from degradation by nucleases.
- Use PCR pipette tips containing hydrophobic filters to minimize contamination.
- After the solution is just thawed, we strongly recommend that you mix it by gently flicking the tube, then spin the tube briefly in a microcentrifuge to deposit contents at the bottom before use.
- Add enzyme to the reaction mixture last and thoroughly mix it by gently pipetting the reaction mixture up and down. Do not increase the amount of enzymes added or concentration of RNA and cDNA in the reactions. The amounts and concentrations have been carefully optimized.
- Thin-wall PCR tubes are recommended. These PCR tubes are optimized to ensure more efficient heat transfer and to maximize thermal-cycling performance. We recommend that you use 0.2 ml PCR tubes rather than 0.5 ml ones.
- PCR cycling parameters in the protocol are optimized for an MJ Research PTC-200 DNA Machine. Please note that the optimal parameters may vary when different thermal cyclers and templates are used.

## VI DSN preparation and activity testing

### VI.A Preparation of DSN stock solution

1. Add 25  $\mu\text{L}$  of DSN storage buffer to the lyophilized DSN enzyme. Mix contents by gently flicking the tube. Spin the tube briefly in a microcentrifuge. Avoid foaming of the mixture.
2. Incubate the tube at room temperature for 5 min.
3. Add 25  $\mu\text{L}$  of glycerol to the tube. Mix contents by gently flicking the tube. Spin the tube briefly in a microcentrifuge. Avoid foaming of the mixture.
4. Store the DSN stock solution at  $-20^{\circ}\text{C}$ .

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*DSN stock solution can be stored at  $-20^{\circ}\text{C}$  up to three months*

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### VI.B DSN activity testing

► **Note:** We strongly recommend that you check DSN activity every time before you begin normalization.

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

4 $\mu\text{L}$	Sterile RNase-free water
4 $\mu\text{L}$	DSN control template
10 $\mu\text{L}$	DSN master buffer
<hr/>	
18 $\mu\text{L}$	Total volume

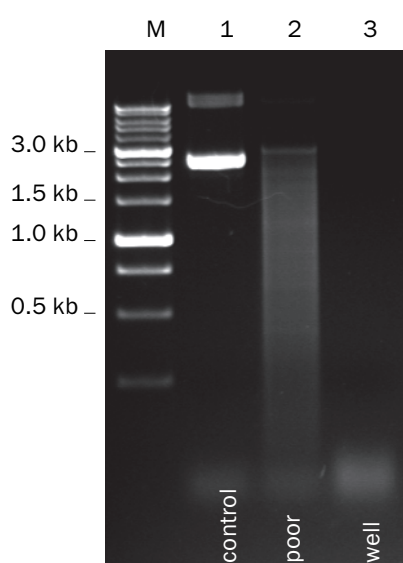
2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Aliquot 9  $\mu\text{L}$  of the reaction mixture into two sterile PCR tubes labeled "C" (control) and "E" (experimental).
4. Add 1  $\mu\text{L}$  of DSN storage buffer into C-tube. Add 1  $\mu\text{L}$  of DSN stock solution into E-tube. Mix contents and spin the tubes briefly in a microcentrifuge.
5. Overlay the reaction mixture in each tube with a drop of mineral oil and spin the tubes briefly in a microcentrifuge.

6. Incubate the tubes in a thermal cycler at 65°C for 10 min.
7. Add 5 µL of DSN stop solution to each tube, mix contents and spin the tubes briefly in a microcentrifuge. Place the tubes at room temperature.
8. Analyze 5 µL aliquots of each reaction mixture alongside 0.1 µg of 1-kb DNA ladder on a 1.5% agarose/EtBr gel in 1X TAE buffer.
9. Using electrophoresis data, estimate the activity of the utilized DSN. For comparison, Fig. 5 shows the typical gel profile of "DSN control template" digested with acceptably active and partially inactive DSN.

A typical result should have the following characteristics:

- (a) Two strong DNA bands should be present in the DNA pattern from the C-tube (as in lane 1, Fig. 5).
- (b) Low molecular weight DNA should be detected in the E-tube (as in lane 3, Fig. 5).

► **Note:** *If a strong difference between the patterns of DNA from the C-tube and that shown in Fig. 5 (lane 1) occurs or the pattern of DNA from the E-tube looks like smears of various intensities with or without clear bands (as in lane 2, Fig. 5), see «Troubleshooting», subsection XI. A.*



**Fig. 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 analyzed on a 1.5% agarose/EtBr gel in 1X TAE buffer. Lane 1 – control DNA (incubation without DSN). Lane 2 – DNA incubated with partially inactive DSN. Lane 3 – successful digestion of DNA by DSN. Lane M – 1 kb DNA size markers.

## VII Normalization protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

### VII.A cDNA precipitation

- ▶ **Note:** Do not use any co-precipitants in the following cDNA precipitation procedure.
  1. Aliquot ds cDNA solution containing about 0.7-1.3 µg of purified ds cDNA to a fresh sterile tube.
    - ▶ **Note:** If the purified ds cDNA samples were stored at -20°C, pre-heat them at 65°C for 1 min and mix by gently flicking the tubes before taking aliquots. Store the remaining purified ds cDNA at -20°C.
  2. Add 0.1 volume of 3M sodium acetate, pH 4.8, and 2.5 volumes of 98% (v/v) ethanol. Vortex the mixture thoroughly.
  3. Centrifuge the tube for 15 min at maximum speed in a microcentrifuge at room temperature.
  4. Carefully remove and discard the supernatant.
  5. Gently overlay the pellets with 100 µL of 80% ethanol.
  6. Centrifuge the tubes for 5 min at maximum speed in a microcentrifuge at room temperature.
  7. Carefully remove and discard the supernatant.
  8. Repeat steps 5-7.
  9. Air-dry the pellet for 10-15 min at room temperature. Be sure that pellet is completely dry before moving to the next step.
  10. Resuspend the cDNA pellet in sterile RNase-free water to a final cDNA concentration of about 50-150 ng/µL.
  11. To check the ds cDNA quality and concentration, analyze 1 µL of cDNA solution alongside 0.1 µg of 1-kb DNA ladder on a 1.5% (w/v) agarose/EtBr gel in 1X TAE buffer.

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*ds cDNA from step 10 can be stored at -20°C up to three months and used for normalization afterwards.*

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## VII. B cDNA denaturation and 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.

12. For each cDNA sample to be normalized combine the following reagents in a sterile 0.5 mL tube:

4-12 $\mu$ L	ds cDNA (from step 10, about 0.6-1.2 $\mu$ g of cDNA)
4 $\mu$ L	4X Hybridization buffer
X $\mu$ L	Sterile RNase-free water
<hr/>	
16 $\mu$ L	Total volume

13. Mix contents and spin the tube briefly in a microcentrifuge.
14. Aliquot 4  $\mu$ L of the reaction mixture into each of the four appropriately labeled (see Table 2) sterile PCR tubes.
15. Overlay the reaction mixture in each tube with a drop of mineral oil and centrifuge the tubes for 2 min at maximum speed in a microcentrifuge.

**Table 2. Setting up DSN treatment**

Component	Experimental tubes*			Control tube
	TUBE1 (S1 DSN1)	TUBE2 (S1 DSN1/2)	TUBE3 (S1 DSN1/4)	TUBE4 (S1 Control)
DSN solution**	1 $\mu$ L	–	–	–
1/2 DSN dilution	–	1 $\mu$ L	–	–
1/4 DSN dilution	–	–	1 $\mu$ L	–
DSN storage buffer	–	–	–	1 $\mu$ L

\* S<NUMBER> specifies the cDNA sample.

\*\* DSN solution is from step 4 of section VI. A "Preparation of DSN stock solution" (page 10).



## VII Normalization protocol

16. Incubate the tubes in a thermal cycler at 98°C for 2 min.
17. Incubate the tubes at 68°C for 5 h, then proceed immediately to step 18. Do not remove the samples from the thermal cycler before DSN treatment.
  - ▶ **Note:** *Samples may be hybridized for as little as 4 h, or as long as 7 h. Do not allow the incubation to proceed for more than 7 h.*

### VII. C DSN treatment

18. Shortly before the end of the hybridization procedure, prepare two DSN dilutions in two sterile tubes to final DSN concentrations of 0.5 U/μL and 0.25 U/μL, as follows:
  - (a) Combine 1 μL of DSN storage buffer and 1 μL of DSN stock solution (from step 4 of VI. A section) to the first tube. Mix by gently pipetting the reaction mixture up and down. Label the tube "1/2 DSN".
  - (b) Combine 3 μL of DSN storage buffer and 1 μL of DSN stock solution to the second tube. Mix by gently pipetting the reaction mixture up and down. Label the tube "1/4 DSN".
  - (c) Place the tubes on ice.
19. Preheat the DSN master buffer at 68°C for 3-5 min.
20. Add 5 μL of the hot DSN master buffer to each tube containing hybridized cDNA (step 17), spin the tube briefly in a microcentrifuge and return it quickly 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.*
21. Incubate the tubes at 68°C for 10 min.
22. Add DSN enzyme as specified in Table 2. After the addition of DSN, return the tube immediately to the thermal cycler.
  - ▶ **Note:** *Do not remove the tubes from the thermal cycler except for the time needed for addition of the DSN enzyme. If the tube is left at room temperature after the addition of DSN, non-specific digestion*

*of secondary structures formed by ss DNA may occur, decreasing the efficiency of the normalization.*

23. Incubate the tubes in the thermal cycler at 68°C for 25 min.
24. Add 5 µL of DSN stop solution, mix contents and spin the tubes briefly in a microcentrifuge.
25. Incubate the tubes in the thermal cycler at 68°C for 5 min. Then, place the tubes on ice.
26. Add 25 µL of sterile RNase-free water to each tube. Mix contents and spin the tubes briefly in a microcentrifuge. Place the tubes on ice.

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

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#### **VII. D Amplification of normalized cDNA**

27. Prepare a PCR master mix by combining the following reagents in the order shown:

162 µL	Sterile RNase-free water
20 µL	10X Encyclo buffer
4 µL	50X dNTP mix (10 mM each)
6 µL	PCR primer M1 (10 µM)
4 µL	50X Encyclo polymerase mix

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196 µL Total volume

- **Note:** *If you normalize several cDNA samples, increase the volume of PCR master mix accordingly.*
28. Mix the contents by gently flicking the tubes. Spin the tubes briefly in a microcentrifuge.
29. Aliquot 1 µL of each sample from step 26 into an appropriately labeled sterile 0.2 mL PCR tubes.
30. Add 49 µL of the PCR master mix into the tubes.

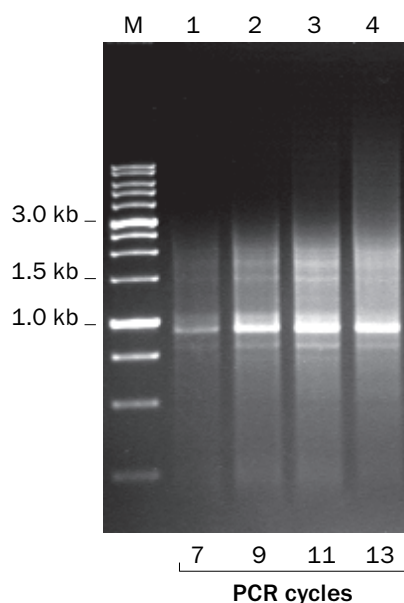
## VII Normalization protocol

31. Mix contents by gently flicking the tubes. Spin the tubes briefly in a microcentrifuge.
32. If the thermal cycler used is not equipped with a heated lid, overlay each reaction with a drop of mineral oil. Close the tubes, and place them into a thermal cycler.
33. Subject the tubes to PCR cycling using the following program:

Initial denaturation		95°C	1 min
Cycling	7 cycles	95°C	15 sec
		66°C	20 sec
		72°C	3 min

---

34. Put the Experimental tubes on ice. Use the Control tube to determine the optimal number of PCR cycles, as follows:
  - (a) Aliquot 12  $\mu$ L from the seven-cycle control tube into a clean microcentrifuge tube (for agarose/EtBr gel analysis).
  - (b) Run two additional cycles (for a total of nine cycles) with the remaining 38  $\mu$ L of the control PCR mixture.
  - (c) Aliquot 12  $\mu$ L from the nine-cycle control tube into a clean microcentrifuge tube.
  - (d) Run two additional cycles (for a total of 11) with the remaining 26  $\mu$ L of the control PCR mixture.
  - (e) Aliquot 12  $\mu$ L from the 11-cycle control tube into a clean microcentrifuge tube.
  - (f) Run two additional cycles (for a total of 13) with the remaining 14  $\mu$ L of the control PCR mixture.
35. Analyze 5  $\mu$ L aliquots of each control PCR reaction (seven, nine, eleven, and thirteen cycles; from step 34) alongside 0.1  $\mu$ g of 1 kb DNA size marker on a 1.5% (w/v) agarose/EtBr gel, run in 1X TAE buffer. Store the remaining materials on ice.
36. Determine the optimal number of cycles required for amplification of the control cDNA, as follows:



**Fig. 6. Analysis for optimizing PCR parameters.** 5  $\mu$ l of each aliquot from the Control tube (from step 34) 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  $\mu$ g loaded.

When the PCR product yield stops increasing with an additional cycle, the reaction has reached a plateau. The optimal number of cycles should be one or two cycles less than that needed to reach the plateau.

A typical electrophoresis result indicating an optimal number of PCR cycles should appear as a moderately strong cDNA smear of the expected size distribution, with several bright bands corresponding to abundant transcripts. For cDNA prepared from most mammalian RNAs, the overall signal intensity (relative to that of 0.1  $\mu$ g of 1 kb DNA size marker run on the same gel) should be roughly similar to that shown in lane 2 of Fig. 6.

If the cDNA smear appears in the high-molecular-weight region of the gel (e.g., as shown in lane 4 of Fig. 6), especially if no bright bands are distinguishable, your PCR parameters may be suboptimal. If the smear is faint, such as that shown in lane 1 of Fig. 6, this indicates that too few PCR cycles were used for amplification (see «Troubleshooting», subsection XI. B).

- ▶ **Note:** *The optimal number of PCR cycles must be determined individually for each experimental sample. Using the optimal number of PCR cycles ensures that the ds cDNA remains in the exponential phase of amplification. PCR overcycling is extremely undesirable, as it yields nonspecific PCR products. Therefore, it is better to use fewer cycles than too many.*

## VII Normalization protocol

37. Retrieve the seven-cycle experimental tubes from ice, return them to the thermal cycler, and (if necessary) subject them to additional PCR cycles to reach the optimal number indicated in the control cDNA experiment. Next, immediately subject the tubes to an additional nine cycles of PCR.

► **Note:** *In total, the 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 Fig. 6, the optimal number of PCR cycles determined using the control tube is nine. Thus, in this example  $X=9$ , and the seven-cycle experimental tubes should be subjected to  $2+9$  additional PCR cycles.*

38. Analyze 5  $\mu$ L of each experimental PCR reaction alongside 5  $\mu$ L of the control PCR reaction representing the optimal number of PCR cycles, and 0.1  $\mu$ g of 1 kb DNA size marker on a 1.5% (w/v) agarose/EtBr gel run in 1X TAE buffer.

39. Store remaining control cDNA representing the optimal number of PCR cycles at  $-20^{\circ}\text{C}$ .

40. Compare the banding pattern intensity of the PCR products from the experimental and control tubes, as follows:

- If the overall signal intensity of PCR products from the experimental tubes is similar to that of the control, proceed to step 41.
- If the smear from the experimental tubes is much fainter than that of the control, PCR undercycling may be an issue. Subject the experimental tubes to two or three more PCR cycles and repeat the electrophoresis. If there is still a strong difference between the overall signal intensity of all experimental PCR products and the control, the normalization process might have been too strong. (see «Troubleshooting», subsection XI. C).
- If the overall signal intensity of the experimental PCR products is much stronger than the control, especially if there are distinct bright bands present, the normalization process might have been unsuccessful (see «Troubleshooting», subsection XI. C).

41. Select the tube(s) showing efficient normalization. For comparison, Fig. 7 shows a characteristic gel profile of normalized human cDNA. A typical result, indicative of efficient normalization, should have the following characteristics:
- PCR products from experimental tube(s) containing efficiently normalized cDNA appear as a smear without clear bands, whereas those from the non-normalized controls usually present a number of distinct bands.
  - The average length of the efficiently normalized cDNA is congruous with the average length of cDNA from the non-normalized control tube.
  - ▶ **Note:** *The upper boundary of the normalized cDNA smear usually does not exceed 5 kb. If the normalized cDNA appears as a uniform smear stretching from the input well to the low-molecular-weight region or bands are visible in the normalized cDNA sample, see «Troubleshooting», subsection XI. D.*
42. If cDNA from two or more Experimental tubes (step 41) appears 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.

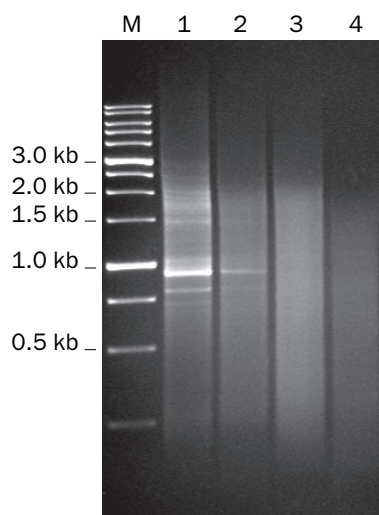
---

*Now you have obtained normalized ds cDNA. The resulting amount of ds cDNA per reaction with total volume of 50  $\mu$ L is anticipated to be in a range of 0.75 -1.35  $\mu$ g. This normalized cDNA can be stored at -20°C up to one month and used afterwards to prepare more normalized cDNA.*

---

43. Please refer to Section IX "Recommendations for further processing of normalized cDNA" to choose the protocol for further processing of amplified ds cDNA before use in intended downstream applications.
- ▶ **Note:** *Before cDNA processing you can estimate normalization efficiency using quantitative PCR or Virtual Northern blot with marker genes of known abundance. Please refer to the Section VIII "Analysis of normalization efficiency".*

## VII Normalization protocol



**Fig. 7. Analysis of cDNA normalization results**

5- $\mu$ l aliquots of the PCR products were loaded on a 1.5% agarose/EtBr gel. Lane M: 1-kb DNA size markers, 0.1  $\mu$ g 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.

## VIII Analysis of normalization efficiency

cDNA normalization considerably decreases the concentrations of highly abundant transcripts in a cDNA population (by about 1000-fold for the most abundant transcripts), but typically doesn't change the concentrations of medium abundance transcripts. The concentrations of rare molecules may slightly increase, or they may remain the same. As a result, the normalized cDNA is enriched for rare transcripts, but also includes medium and high abundance transcripts.

Either quantitative PCR (qPCR) or virtual Northern blot [8] can be used to estimate the efficiency of normalization prior to cDNA cloning or sequencing. Comparing the abundance levels of already studied transcripts before and after normalization, one can see a relative reduction in the representation level of abundant transcripts in a normalized cDNA sample (in comparison with a non-normalized one).

Alternatively, clones may be randomly picked and sequenced from normalized and non-normalized cDNA libraries, and the gene discovery rates may be compared between the libraries. A successfully normalized cDNA library will have a higher gene discovery rate than a non-normalized library; however, the particular characteristics of a given library will depend on the initial cDNA redundancy, the cDNA GC-content, the number of clones tested, etc.

- *Some problems that may occur during an analysis of normalization efficiency are discussed in the "Troubleshooting", subsection XI. E).*

## Analysis of normalization efficiency by qPCR

### **Important notes:**

- ▶ *Trimmer-2 kit provides GAPD primer mix allowing qPCR-testing of normalization efficacy in human and mouse cDNA samples on the example of glyceraldehyde-3-phosphate dehydrogenase (GAPD) that is expressed at high levels in most mammalian tissues and cell lines.*

*For cDNA from other sources (non-human and non-mouse), please select and design primers specific for source-specific high abundance transcripts.*

### **Reagents required**

- Ready-to-use qPCR Master Mix containing SYBR Green I dye and ROX reference dye

### **qPCR protocol**

1. Make sure that the difference between concentrations of normalized cDNA (from step 42 of the Normalization protocol) and control cDNA (from step 39 of the Normalization protocol) does not exceed 2-times. If necessary, equalize cDNA concentrations.
  - ▶ **Note:** *cDNA concentration should be estimated using agarose gel-electrophoresis or spectrophotometric analysis.*
2. Aliquot 1  $\mu\text{L}$  of normalized cDNA (from step 42 of the Normalization protocol) into a sterile 1.5 mL tube; add 39  $\mu\text{L}$  of sterile RNase-free water to the tube, mix well by vortexing and spin the tubes briefly in a microcentrifuge.
  - ▶ **Note:** *If the normalized cDNA sample was stored at  $-20^{\circ}\text{C}$ , pre-heat it at  $65^{\circ}\text{C}$  for 1 min and mix by gently flicking the tubes before taking aliquots. Store the remaining cDNA at  $-20^{\circ}\text{C}$ .*
3. Aliquot 1  $\mu\text{L}$  of control cDNA (from step 39 of the Normalization protocol) into another sterile 1.5 mL tube; add 39  $\mu\text{L}$  of sterile RNase-free water to the tube, mix well by vortexing and spin the tubes briefly in a microcentrifuge.
  - ▶ **Note:** *If the control cDNA sample was stored at  $-20^{\circ}\text{C}$ , pre-heat it at  $65^{\circ}\text{C}$  for 1 min and mix by gently flicking the tubes before taking aliquots. Store the remaining cDNA at  $-20^{\circ}\text{C}$ .*



## VIII Analysis of normalization efficiency

4. Prepare qPCR reactions with primers specific for high abundance transcripts in the experimental cDNA samples and 1  $\mu$ L aliquots of the diluted cDNA from steps 2 and 3.
5. Perform PCR cycling as described in the instructions (or instruction booklet) provided with the ready-to-use qPCR Master Mix. Three-step cycling protocol is recommended.

▶ **Note:** *Appropriate annealing temperature for the GAPD primer mix provided in the Trimmer-2 kit is 60°C.*

6. When cycling is completed, use thermal cycler software to identify Ct for each PCR reaction. Calculate mean value Ct for each cDNA sample. Mean Ct for the control cDNA should be less than 20.

▶ **Note:** *If mean Ct for the control cDNA is 21 or more, this indicates that the transcript tested is not in high abundance in the cDNA sample. Thus, its concentration may remain unchanged during normalization. In this case, repeat qPCR with primer pair specific to another high abundance transcript.*

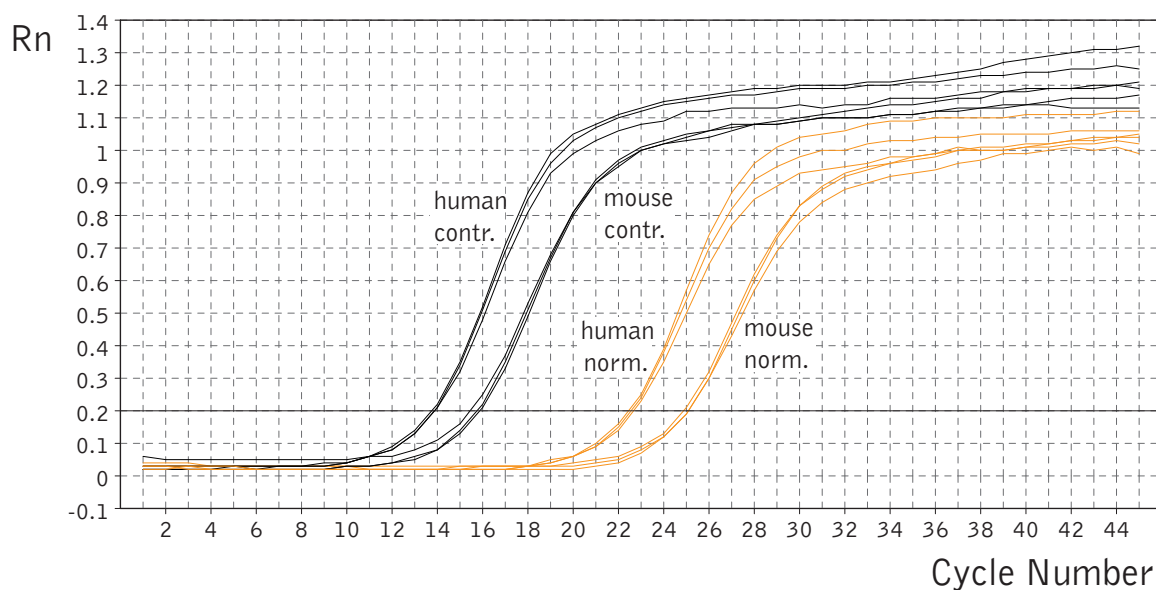
▶ **Note:** *GAPD is expressed at high levels in most human and mouse tissues and cell lines, however there could be some exceptions. In some samples, GAPD transcripts belong to intermediate or low abundance groups, and unchanged or slightly increased concentration of these transcripts in normalized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

7. Calculate  $\Delta$ Ct as follows:

$$\Delta\text{Ct} = \text{Ct}_C - \text{Ct}_N,$$

where  $\text{Ct}_C$  is the mean Ct for the control cDNA sample and  $\text{Ct}_N$  is the mean Ct for the normalized cDNA sample.

$\Delta\text{Ct} \geq 5$  indicates effective normalization.  $\Delta\text{Ct} \leq 4$  indicates unsuccessful normalization.



**Fig. 8. Analysis of cDNA normalization results by qPCR.** Efficiency of normalization of human and mouse brain cDNA was tested using quantitative PCR with GAPD primer mix.  $\Delta Ct = 9$  indicates successful normalization in both cases.

## IX Recommendations for further processing of normalized cDNA

Adapter pair used for ds cDNA preparation	Intended application	Recommendations
PlugOligo-1 and CDS-1 adapters OR SMARTer II A Oligonucleotide and 3' SMART CDS Primer II A	Non-directional cDNA library cloning and Sanger sequencing	see Appendix B
PlugOligo-3M and CDS-4M adapters OR SMART IV Oligonucleotide and CDS-4M adapter	Directional cDNA library cloning	see Appendix C
	SOLiD or Illumina sequencing	see Appendix D
	Roche/454 sequencing	see Appendix E
PlugOligo-3M and CDS-Gsu adapters OR SMART IV Oligonucleotide and CDS-Gsu adapter	Roche/454 sequencing	see Appendix F

## X Appendixes

### Appendix A cDNA synthesis and amplification using SMART-based kit (Clontech)

#### Reagents required

- Purified RNA for cDNA synthesis (at least 1-2 µg of total RNA or 0.5-1 µg of polyA+ RNA)
- ▶ *The RNA may be isolated using a number of suitable methods that yield stable RNA preparations from most biological sources; two examples are the TRIzol method (Gibco/Life Technologies) and the RNeasy kit (Qiagen). Total RNA can also be isolated as described in [9].*

*Following RNA isolation, RNA quality should be estimated using denaturing formaldehyde/agarose gel electrophoresis, as described by Sambrook [10]. The RNA length generally depends on the RNA source, however, if experimental RNA is not larger than 1.5 kb, we suggest you prepare fresh RNA after checking the quality of the RNA purification reagents. If problems persist, you may need to find another source of tissue/cells.*

*In general, genomic DNA contamination does not affect cDNA synthesis, meaning that DNase treatment is not required. When necessary, excess genomic DNA can be removed by LiCl precipitation or phenol:chloroform extraction.*

- SMART-based kit (Clontech)
- ▶ *Please refer to the section IV (cDNA preparation) to choose the kit suitable for your needs.*
- Encyclo PCR Kit (Evrogen, Cat.# PK001) or analogues
- QIAquick PCR Purification Kit (Qiagen)
- Sterile molecular biology grade water (sterile RNase-free water)
- Agarose gel electrophoresis reagents
- DNA size markers (1-kb DNA ladder)

## First-strand cDNA synthesis

### **Important notes:**

- ▶ *The following protocol describes the use of reagents provided in SMART-based kits (Clontech) and additional 3'-end adapters included in Trimmer-2 kit for synthesis of first-strand cDNA suitable for normalization procedure and allowing various downstream application of normalized cDNA. Please refer to the Section IV (cDNA preparation) to choose the adapter pair suitable for your needs.*
- ▶ *The sequence complexity and average length of the normalized cDNA library strongly depend on the quality and amount of the starting RNA material used to prepare the cDNA. For best results, at least 1-2 µg of total RNA or 0.5-1 µg of polyA+ RNA should be used at the beginning of first-strand cDNA synthesis. The minimum amount of starting RNA for cDNA synthesis is 250 ng of total RNA or 100 ng of polyA+ RNA.*
- ▶ *We strongly recommend that you perform a positive control cDNA synthesis with control RNA provided in the cDNA synthesis kit, that you use, simultaneously with experimental cDNA synthesis. This control is performed to verify that all reagents are working properly.*
  1. Immediately before taking the aliquot for the cDNA synthesis, heat the RNA samples at 65°C for 1-2 min, mix the contents by gently flicking the tube (to prevent RNA aggregation), and then spin the tube briefly in a microcentrifuge.
  2. For each RNA sample, combine the following reagents in a sterile PCR tube:
 

1-3 µL	RNA solution in sterile RNase-free water (1-2µg of total RNA or 0.5-1 µg of polyA(+) RNA) For the control reaction use 1 µL (1 µg) of the control RNA
1 µL	5'-end adapter*
1 µL	3'-end adapter*
x µL	Sterile RNase-free water
5 µL	
	Total volume

**\*Refer to Section IV cDNA preparation to choose the adapter pair that can be used with the SMART-based cDNA synthesis kit.**

## Appendix A

3. Gently pipette the reaction mixtures and spin the tubes briefly in a microcentrifuge. If the thermal cycler used is not equipped with a heated lid, overlay each reaction with a drop of molecular biology-grade mineral oil to prevent the loss of volume due to evaporation.
4. Incubate the mixture in a thermal cycler at 70°C for 2 min (use heated lid).
5. Decrease the incubation temperature to 42°C. Keep the tubes in the thermal cycler at 42°C while preparing the RT master mix (~ 1 to 3 min).
6. While steps 4 and 5 are ongoing, prepare an RT master mix for each reaction tube by combining the following reagents in the order shown:

2 $\mu$ L	5X First-strand buffer
1 $\mu$ L	DTT (20 mM)
1 $\mu$ L	50X dNTP
1 $\mu$ L	SMARTScribe MMLV Reverse Transcriptase
<hr/>	
5 $\mu$ L	Total volume
- ▶ **Note:** *Optionally, 0.5  $\mu$ L of RNase inhibitor (20 U/ $\mu$ L) can be added to the reaction to prevent RNA degradation during cDNA synthesis.*
7. Gently pipette the RT master mix and spin the tube briefly in a microcentrifuge.
8. Add 5  $\mu$ L RT master mix to each reaction tube from step 5. Gently pipette the reaction mix, and spin the tubes briefly in a microcentrifuge to deposit contents at the bottom.
  - ▶ **Note:** *Do not remove the reaction tubes from the thermal cycler except for the time necessary to add the RT master mix.*
9. Incubate the tubes at 42°C for 1.5 h.
10. After incubation, place the tubes on ice to terminate the first-strand cDNA synthesis.

---

*First-strand cDNA can be stored at -20°C for up to one month and used for ds cDNA amplification.*

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### cDNA amplification

11. For each first-strand cDNA sample from step 10 above, prepare a PCR mixture by combining the following reagents in the order shown:

80 µL	Sterile RNase-free water
10 µL	10X Encyclo PCR buffer*
2 µL	dNTP mix (10mM each)*
4 µL	PCR primer M1 (10 µM)
2 µL	First-strand cDNA (from step 10)
2 µL	50X Encyclo polymerase mix*

---

100 µL Total volume

\* The component is provided in the Encyclo PCR kit.

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

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

13. If the thermal cycler used is not equipped with a heated lid, overlay each reaction mixture with a drop of mineral oil. Close the tubes, and place them into a thermal cycler.

14. Subject the tubes to PCR cycling using the following program:

Initial denaturation		95°C	1 min
Cycling	X cycles*	95°C	15 sec
		66°C	20 sec
		72°C	3 min

---

\*X is the optimal number of PCR cycles for a given amount of total or poly(A)+ RNA used for the first-strand cDNA synthesis according to the Table 3 below.

**Table 3. PCR cycling parameters**

Total RNA ( $\mu\text{g}$ )	polyA+ RNA ( $\mu\text{g}$ )	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	0.1-0.25	18-21

The recommended parameters were tested using placenta and skeletal muscle total and poly(A)+ RNA and an 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 possible, 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 also Troubleshooting Guide, Section B).

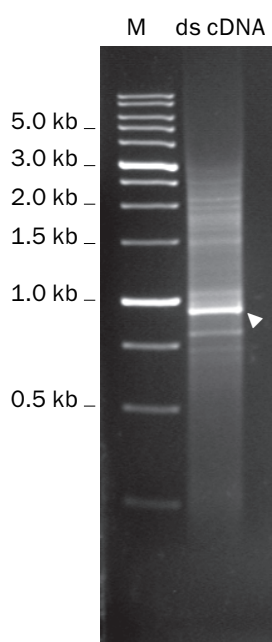
Please note, cDNA samples that require more than 25 PCR cycles to be amplified may not be representative. We do not recommend using such samples for normalization. Repeat cDNA amplification using larger amounts of first-strand cDNA.

15. When cycling is complete, place the tubes on ice.
16. Analyze 5  $\mu\text{L}$  of the PCR product alongside 0.1  $\mu\text{g}$  of 1 kb DNA size marker on a 1.5% (w/v) agarose/EtBr gel run in 1X TAE buffer to estimate cDNA quality and concentration.

A typical electrophoresis result indicating successful cDNA synthesis should appear as a moderately strong cDNA smear of the expected size distribution, with several bright bands corresponding to abundant transcripts. Compare the intensity of the banding pattern of the PCR product with the 1-kb DNA ladder (0.1  $\mu\text{g}$  run on the same gel). For cDNA from mammalian RNA sources, the overall signal intensity (relative to the DNA ladder) should be roughly similar to that shown in Fig. 9.

- If the cDNA smear appears in the high-molecular-weight region of the gel, and especially if no bright bands are distinguishable, PCR overcycling may be an issue (see «Troubleshooting», subsection XI.F). Additional indication of PCR overcycling is preponderance of material in the lower part of the gel (i.e., <0.1 kb).
- If the smear is faint and the size distribution of PCR product is

less than expected, this indicates PCR undercycling (too few PCR cycles were used for amplification). Subject the tubes to two more PCR cycles and repeat the electrophoresis. If there is still a strong difference between the overall signal intensity of the PCR products obtained (relative to 0.1  $\mu\text{g}$  of DNA ladder) and PCR product shown in Fig. 9, see "Troubleshooting", subsection XI. F.



**Fig. 9. ds cDNA synthesized from poly(A)<sup>+</sup> placenta RNA using SMART kit.**

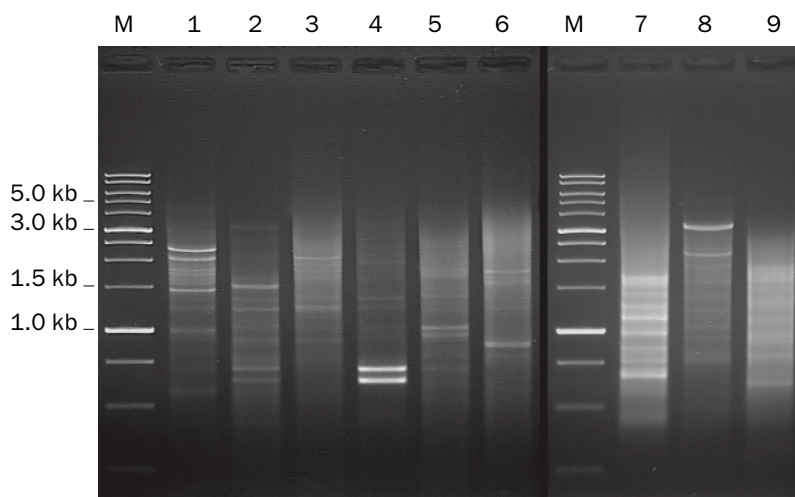
1  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA was used as starting material in a first-strand cDNA synthesis. 2  $\mu\text{l}$  of the first-strand cDNA was then used as template for SMART cDNA amplification in 100  $\mu\text{l}$  reaction volume. 16 PCR cycles were performed. 5  $\mu\text{l}$  of the PCR product was analyzed on a 1.5% agarose/EtBr gel. Lane M: 1-kb DNA ladder (0.1  $\mu\text{g}$  loaded). The arrow indicates a strong band at 900 bp typically seen for human placenta cDNA.

- ▶ **Note:** In general, for most mammalian tissues a visible smear of full-length-enriched cDNA should be within the range of 0.5-7 kb, while normal cDNA size for many non-mammalian species is less than 3 kb (Fig. 10). 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)<sup>+</sup> RNA.
- ▶ **Note:** cDNA with low molecular weight may 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.

17. Once the successful results for ds cDNA synthesis are achieved, purify amplified ds cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50  $\mu\text{L}$  of sterile RNase-free water. For normalization procedure please refer to Normalization protocol (Section VII).



## Appendix A



**Fig. 10. Agarose gel-electrophoresis of amplified cDNA from different sources.** 1 – mouse liver; 2 – mouse skeletal muscle; 3 – mouse brain; 4 – human leucocytes; 5 – human lung; 6 – human skeletal muscle; 7 – mosquito grub; 8 – copepod *Pontella* sp.; 9 – tomato *Lycopersicon esculentum*. M – 1 kb DNA ladder (SibEnzyme).

## Appendix B Processing of normalized cDNA before non-directional cDNA library cloning

### Reagents required

- Normalized cDNA (from step 42 of the Normalization protocol)
- Encyclo PCR kit (Evrogen, Cat.# PK001) or analogues
- QIAquick PCR Purification Kit (Qiagen)
- Sterile molecular biology grade water (sterile RNase-free water)

### Amplification of ds cDNA

- **Note:** *The normalized ds cDNA from step 42 of the Normalization protocol can be used for non-directional cloning into appropriate TA-cloning vector just after purification (step 8 of this Appendix). However, we recommend performing additional dilution and re-amplification of normalized cDNA with M1 primer, as described in steps 1-7 of this Appendix. The re-amplification allows to get rid from non-specific PCR products that are not flanked by M1 primer sequence. Small amounts of such fragments might be present in the samples after the first amplification.*

1. For each normalized cDNA sample, combine 2  $\mu$ L aliquot of normalized cDNA (from step 42 of the Normalization protocol) and 20  $\mu$ L of sterile RNase-free water in a new sterile tube, mix well by vortexing and spin the tubes briefly in a microcentrifuge.

- **Note:** *If the normalized cDNA samples were stored at  $-20^{\circ}\text{C}$ , pre-heat*

them at 65°C for 1 min and mix by gently flicking the tubes before taking aliquots. Store the remaining cDNA at -20°C.

- For each normalized cDNA sample, prepare a PCR mixture by combining the following reagents in the order shown:

80 µL	Sterile RNase-free water
10 µL	10X Encyclo buffer
2 µL	50X dNTP mix (10mM each)
4 µL	PCR primer M1 (10 µM)
2 µL	Diluted normalized cDNA from step 1 above
2 µL	50X Encyclo polymerase mix
<hr/>	
100 µL	Total volume

- Mix contents by gently flicking the tubes. Spin the tubes briefly in a microcentrifuge.
- If the thermal cycler used is not equipped with a heated lid, overlay each reaction mixture with two drops of mineral oil. Close the tubes, and place them into a thermal cycler.
- Subject the tubes to PCR cycling using the following program:

Initial denaturation		95°C	1 min
Cycling	12 cycles	95°C	15 sec
		66°C	20 sec
		72°C	3 min

- Analyze 5 µL aliquots of each ds cDNA sample alongside 0.1 µg of 1 kb DNA ladder on a 1.5% (w/v) agarose/EtBr gel run in 1X TAE buffer to estimate cDNA quality and concentration.
- If electrophoresis indicates poor yield of PCR products, subject the tubes to two more PCR cycles and repeat the electrophoresis.
  - **Note:** If low molecular weight, poor yield, or no PCR product is observed in the samples after PCR amplification, see «Troubleshooting», Section XI. G.

## Appendix B

8. Purify the amplified ds cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50  $\mu$ L of sterile RNase-free water.

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*The resulting normalized ds cDNA can be used for non-directional cloning of cDNA library into appropriate TA-cloning vectors.*

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## Appendix C Processing of normalized cDNA before directional cDNA library cloning

### Reagents required

- Normalized cDNA (from step 42 of the Normalization protocol) flanked by adapter sequences containing asymmetric SfiI A and SfiI B sites
- SfiI restriction endonuclease supplied with 10X reaction buffer
- Encyclo PCR kit (Evrogen, Cat# PK001) or analogues
- QIAquick PCR Purification Kit (Qiagen)
- Sterile molecular biology grade water (sterile RNase-free water)
- CHROMASPIN™-1000 columns (Clontech) or analogues / optional
- Agarose gel electrophoresis reagents
- DNA size markers (1-kb DNA ladder)

### Amplification of ds cDNA

- ▶ **Note:** *The normalized ds cDNA from step 42 of the Normalization protocol can be used for directional cloning into appropriate vector just after SfiI restriction endonuclease treatment (steps 8-12 of this Appendix). Re-amplification of the normalized cDNA (steps 1-7 of this Appendix) is optional, it is required only if higher amounts of product are needed for downstream applications.*

1. Combine 2  $\mu$ L of normalized cDNA (from step 42 of the Normalization protocol) and 20  $\mu$ L of sterile RNase-free water in a new sterile 1.5 mL tube; mix well by vortexing and spin the tubes briefly in a microcentrifuge.

- ▶ **Note:** *If the normalized cDNA samples were stored at -20°C, pre-heat*

them at 65°C for 1 min and mix by gently flicking the tubes before taking aliquots. Store the remaining cDNA at -20°C.

- For each cDNA sample from step 1 above, prepare a PCR mixture by combining the following reagents in the order shown:

80 µL	Sterile RNase-free water
10 µL	10X Encyclo buffer
2 µL	50X dNTP mix (10mM each)
4 µL	PCR primer M1 (10 µM)
2 µL	Diluted normalized cDNA from step 1 above
2 µL	50X Encyclo polymerase mix
<hr/>	
100 µL	Total volume

- Mix contents by gently flicking the tubes. Spin the tubes briefly in a microcentrifuge.
- If the thermal cycler used is not equipped with a heated lid, overlay each reaction mixture with two drops of mineral oil. Close the tubes, and place them into a thermal cycler.
- Subject the tubes to PCR cycling using the following program:

Initial denaturation		95°C	1 min
Cycling	12 cycles	95°C	15 sec
		66°C	20 sec
		72°C	3 min
<hr/>			

- Analyze 5 µL aliquots of each ds cDNA sample alongside 0.1 µg of 1 kb DNA ladder on a 1.5% (w/v) agarose/EtBr gel run in 1X TAE buffer to estimate cDNA quality and concentration.
- If electrophoresis indicates poor yield of PCR products, subject the tubes to two more PCR cycles and repeat the electrophoresis.
  - **Note:** If low molecular weight, poor yield, or no PCR product is observed in the samples after PCR amplification, see «Troubleshooting», Section XI. G.

## Appendix C

8. Purify the amplified ds cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50  $\mu$ L of sterile RNase-free water.

### Digestion of the normalized cDNA with SfiI restriction endonuclease

9. For each cDNA sample from step 8 above, combine the following reagents in a sterile 0.5 mL tube:

44 $\mu$ L	Amplified ds cDNA (from step 8 above)
5 $\mu$ L	10X Reaction buffer
1 $\mu$ L	SfiI restriction endonuclease (10-20 U)
<hr/>	
50 $\mu$ L	Total volume

10. Incubate the tubes for 3 h at 50°C.
11. After digestion, purify cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50  $\mu$ L of sterile RNase-free water.
12. To enrich the cDNA samples with full-length sequences, perform size-selection of large cDNA molecules (>1350 bp) using CHROMASPIN™-1000.

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*The resulting ds cDNA can be used for directional cloning into vectors containing SfiI A and SfiI B sites (for example, pDNR-LIB or pTriplEx2 vectors from Clontech) linearized using SfiI restriction endonuclease.*

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## Appendix D Processing of normalized cDNA before SOLiD or Illumina sequencing

### Reagents required

- Normalized ds cDNA (from step 42 of the Normalization protocol) flanked by adapter sequences containing asymmetric SfiI A and SfiI B sites
- SfiI restriction endonuclease supplied with 10X reaction buffer
- Encyclo PCR Kit (Evrogen, Cat.# PK001) or analogues
- QIAquick PCR Purification Kit (Qiagen)

- Sterile molecular biology grade water (sterile RNase-free water)
- Agarose gel electrophoresis reagents
- DNA size markers (1-kb DNA ladder)

### Amplification of ds cDNA

► **Note:** *In order to obtain sufficient amount of normalized ds cDNA for direct application in high-throughput sequencing, we recommend setting-up PCR amplification with M1 primer in two identical 100  $\mu$ L reactions. The total amount of ds cDNA after amplification is anticipated to be in a range of 3 -4  $\mu$ g ( $\sim$  15 ng/ $\mu$ L).*

1. Aliquot 2  $\mu$ L of normalized cDNA (from step 42 of the Normalization protocol) into a sterile 1.5 mL tube; add 20  $\mu$ L of sterile RNase-free water to the tube, mix well by vortexing and spin the tubes briefly in a microcentrifuge.

► **Note:** *If the normalized cDNA samples were stored at -20°C, pre-heat them at 65°C for 1 min, then mix by gently flicking the tubes before taking aliquots. Store the remaining ds cDNA at -20°C.*

2. For each cDNA sample from step 1 above, prepare PCR mixture combining the following reagents in the order shown:

160 $\mu$ L	Sterile RNase-free water
20 $\mu$ L	10X Encyclo buffer
4 $\mu$ L	50X dNTP mix (10mM each)
8 $\mu$ L	PCR primer M1 (10 $\mu$ M)
4 $\mu$ L	Diluted normalized cDNA from step 1 above
4 $\mu$ L	50X Encyclo polymerase mix
<hr/>	
200 $\mu$ L	Total volume

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

4. Aliquot 100  $\mu$ L of PCR mixture into two sterile 0.2 ml PCR tubes.

5. If the thermal cycler used is not equipped with a heated lid, overlay each reaction with two drops of mineral oil. Close the tubes, and place them into a thermal cycler.

6. Subject the tubes to PCR cycling using the following program:

Initial denaturation		95°C	1 min
Cycling	12 cycles	95°C	15 sec
		66°C	20 sec
		72°C	3 min

---

7. When cycling is complete, analyze 5  $\mu$ L aliquots of each ds cDNA sample alongside 0.1  $\mu$ g of 1 kb DNA ladder on a 1.5% (w/v) agarose/EtBr gel run in 1X TAE buffer to estimate cDNA quality and concentration.
8. If electrophoresis indicates poor yield of PCR products, subject the tubes to two more PCR cycles and repeat the electrophoresis.
- **Note:** *If low molecular weight, poor yield, or no PCR product is observed in the samples after PCR amplification, see «Troubleshooting», Section XI. G.*
9. Pool the reaction mixtures from two identical tubes with amplified normalized cDNA into a new sterile tube.
10. Purify the amplified ds cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50  $\mu$ L of sterile RNase-free water.

### Digestion of the normalized cDNA with SfiI restriction endonuclease

11. For each cDNA sample from step 10 above, combine the following reagents in a sterile 0.5 mL tube:

44 $\mu$ L	Amplified ds cDNA (from step 10 above)
5 $\mu$ L	10X Reaction buffer
1 $\mu$ L	SfiI restriction endonuclease (10-20 U)
<hr/>	
50 $\mu$ L	Total volume

12. Incubate the tubes for 3 h at 50°C.
13. After digestion, purify cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50  $\mu$ L of sterile RNase-free water.

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*The resulting ds cDNA can be applied for ABI/SOLiD or Illumina/Solexa sequencing. Please contact your sequencing facility for further instruction on ds cDNA processing.*

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## **Appendix E Processing of normalized cDNA flanked at 3'-end with CDS-4M adapter before Roche 454 sequencing**

### **Reagents required**

- Amplified ds cDNA (from step 42 of the Normalization protocol) flanked by Plug- Oligo-3M and CDS-4M adapter sequences
- Encyclo PCR Kit (Evrogen, Cat.# PK001) or analogues
- QIAquick PCR Purification Kit (Qiagen)
- Sterile molecular biology grade water (sterile RNase-free water)
- Agarose gel electrophoresis reagents
- DNA size markers (1-kb DNA ladder)

### **cDNA amplification with 454 PCR Primer mix**

- ▶ **Note:** *In order to obtain sufficient amount of normalized ds cDNA for direct application in high-throughput sequencing, we recommend setting-up PCR amplification with 454 PCR primer mix in two identical 100  $\mu$ L reactions. The total amount of ds cDNA after amplification is anticipated to be in a range of 3-4  $\mu$ g ( $\sim$  15 ng/ $\mu$ L).*

1. Combine 2  $\mu$ L of the normalized cDNA (from step 42 of the Normalization protocol) with 38  $\mu$ L of sterile RNase-free water in a new sterile 1.5 mL tube. Mix the contents by gently flicking the tube. Spin the tube briefly in a microcentrifuge.

- ▶ **Note:** *If the normalized cDNA samples were stored at -20°C, pre-heat them at 65°C for 1 min, then mix by gently flicking the tubes before taking aliquots. Store the remaining ds cDNA at -20°C.*

2. For each cDNA sample from step 1 above, prepare PCR mixture combining the following reagents in the order shown:



## Appendix E

160 $\mu$ L	Sterile RNase-free water
20 $\mu$ L	10X Encyclo buffer
4 $\mu$ L	50X dNTP mix (10mM each)
8 $\mu$ L	454 PCR Primer mix (10 $\mu$ M each)
4 $\mu$ L	Diluted normalized cDNA from step 1 above
4 $\mu$ L	50X Encyclo polymerase mix
<hr/>	
200 $\mu$ L	Total volume

- Mix the contents by gently flicking the tube. Spin the tube briefly in a microcentrifuge.
- Aliquot 100  $\mu$ L of PCR mixture into two sterile PCR tubes.
- If the thermal cycler used is not equipped with a heated lid, overlay each reaction with two drops of mineral oil. Close the tubes, and place them into a thermal cycler.
- Subject the tubes to PCR cycling using the following program:

Initial denaturation		95°C	1 min
Cycling	3 cycles	95°C	15 sec
		50°C	20 sec
		72°C	3 min
	11 cycles	95°C	15 sec
		63°C	20 sec
		72°C	3 min

---

- Analyze 4  $\mu$ L aliquots of each PCR product alongside 0.1  $\mu$ g of 1 kb DNA ladder on a 1.5% (w/v) agarose/EtBr gel run in 1X TAE buffer to estimate cDNA quality and concentration. If necessary, add 1-3 PCR cycles and repeat electrophoresis. Use the following PCR program for additional cycles:

Cycling	1-3 cycles	95°C	15 sec
		63°C	20 sec
		72°C	3 min

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- **Note:** *If low molecular weight, poor yield, or no PCR product is observed in the samples after PCR amplification, see «Troubleshooting», Section XI. G.*
8. Pool the reaction mixtures from two identical tubes with amplified normalized cDNA into a new sterile tube.
  9. Purify the amplified ds cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50 µL of sterile RNase-free water.

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*The resulting ds cDNA is suitable for Roche/454 sequencing. Please contact your sequencing facility for further instruction on ds cDNA processing.*

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## **Appendix F Processing of normalized cDNA flanked at 3'-end with CDS-Gsu adapter before Roche 454 sequencing**

### **Reagents required**

- Normalized ds cDNA (from step 42 of the Normalization protocol) flanked at 3'-end with an adapter sequence containing a GsuI site
- GsuI restriction endonuclease supplied with 10X reaction buffer
- Encyclo PCR Kit (Evrogen, Cat.# PK001) or analogues
- QIAquick PCR Purification Kit (Qiagen)
- Sterile molecular biology grade water (sterile RNase-free water)
- Agarose gel electrophoresis reagents
- DNA size markers (1-kb DNA ladder)

### **Amplification of ds cDNA**

- **Note:** *The following protocol describes second PCR amplification of normalized cDNA in a single reaction with total volume of 100 µL. The resulting amount of ds cDNA per reaction is anticipated to be in a range of 1.5 -2 µg (~ 15 ng/µL). In order to obtain higher amounts of the product the reaction volume (or number of reactions) can be increased accordingly.*

## Appendix F

1. Combine 2  $\mu\text{L}$  of normalized cDNA (from step 42 of the Normalization protocol) and 20  $\mu\text{L}$  of sterile RNase-free water in a new sterile 1.5 mL tube; mix well by vortexing and spin the tubes briefly in a microcentrifuge.

► **Note:** *If the normalized cDNA samples were stored at  $-20^{\circ}\text{C}$ , pre-heat them at  $65^{\circ}\text{C}$  for 1 min and mix by gently flicking the tubes before taking aliquots. Store the remaining cDNA at  $-20^{\circ}\text{C}$ .*

2. For each cDNA sample from step 1 above, prepare a PCR mixture by combining the following reagents in the order shown:

160 $\mu\text{L}$	Sterile RNase-free water
20 $\mu\text{L}$	10X Encyclo buffer
4 $\mu\text{L}$	50X dNTP mix (10mM each)
8 $\mu\text{L}$	PCR primer M1 (10 $\mu\text{M}$ )
4 $\mu\text{L}$	Diluted normalized cDNA from step 1 above
4 $\mu\text{L}$	50X Encyclo polymerase mix
<hr/>	
200 $\mu\text{L}$	Total volume

3. Mix the contents by gently flicking the tube. Spin the tube briefly in a microcentrifuge.
4. Aliquot 100  $\mu\text{L}$  of PCR mixture into two sterile 0.2 ml PCR tubes.
5. If the thermal cycler used is not equipped with a heated lid, overlay each reaction with two drops of mineral oil. Close the tubes, and place them into a thermal cycler.
6. Subject the tubes to PCR cycling using the following program:

Initial denaturation		95 $^{\circ}\text{C}$	1 min
Cycling	12 cycles	95 $^{\circ}\text{C}$	15 sec
		66 $^{\circ}\text{C}$	20 sec
		72 $^{\circ}\text{C}$	3 min

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7. When cycling is complete, analyze 5  $\mu\text{L}$  aliquots of each ds cDNA sample alongside 0.1  $\mu\text{g}$  of 1 kb DNA ladder on a 1.5% (w/v)

agarose/EtBr gel run in 1X TAE buffer to estimate cDNA quality and concentration.

8. If electrophoresis indicates poor yield of PCR products, subject the tubes to two more PCR cycles and repeat the electrophoresis.
  - ▶ **Note:** *If low molecular weight, poor yield, or no PCR product is observed in the samples after PCR amplification, see «Troubleshooting», Section XI. G.*
9. Pool the reaction mixtures from two identical tubes with amplified normalized cDNA into a new sterile tube.
10. Purify the amplified ds cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50  $\mu$ L of sterile RNase-free water.

#### Digestion of the ds cDNA with Gsul restriction endonuclease

9. For each cDNA sample from step 10 above, combine the following reagents in a sterile 0.5 mL tube:

43 $\mu$ L	Amplified ds cDNA (step 10 of this appendix)
5 $\mu$ L	10X Reaction buffer
2 $\mu$ L	Gsul restriction endonuclease (10 U)
<hr style="width: 25%; margin-left: 0;"/>	
50 $\mu$ L	Total volume

10. Incubate the tubes for 3 h at 30°C.
11. After digestion, purify ds cDNA using QIAquick PCR Purification Kit. Elute ds cDNA with 50  $\mu$ L of sterile RNase-free water.

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*The resulting ds cDNA is suitable for Roche/454 sequencing. Please contact your sequencing facility for further instruction on ds cDNA processing.*

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## XI Troubleshooting

### XI. A DSN activity testing (step 9, Section VI. B)

Gel analysis shows that DNA in C-tube is fully or partially degraded.

Possible cause	Solution
DSN control template is fully or partially degraded during storage or delivery.	Analyze 1 $\mu$ L of DSN control template alongside 0.1 $\mu$ g of 1-kb DNA ladder on a 1.5% agarose/EtBr gel in 1X TAE buffer. If DSN Control template is fully or partially degraded, use another DNA to test DSN activity. You can use any purified plasmid DNA with a concentration of approximately 100 ng/ $\mu$ L.
Your working area, equipment, or solutions are contaminated by nucleases.	If the DSN control template is not degraded, but the DNA in C-tube is fully or partially degraded, it indicates that your working area, equipment, or solutions are contaminated by nucleases. Check that your work area, equipment, and solutions are free from nuclease contamination.

Gel analysis shows that DNA in E-tube is not completely degraded.

Possible cause	Solution
DSN enzyme is fully or partially inactive.	Use another DSN enzyme package.

### XI. B Amplification of the control cDNA (step 36, Section VII)

Gel analysis of PCR products from the Control tube reveals low-molecular-weight products, poor yield, or no products.

Possible cause	Solution
cDNA was synthesized with inappropriate 5'-end and/or 3'-end adapters.	Please refer to the subsection IV (cDNA preparation) to choose adapters suitable for cDNA synthesis.

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Possible cause	Solution
cDNAs may have degraded during storage and/or hybridization procedure.	Check that your work area, equipment, and solutions are free from DNase contamination. Check the quality of starting cDNA on agarose gel electrophoresis. Repeat ethanol precipitation of cDNA after column purification (see Subsection VII. A of Normalization protocol) and normalization using a fresh cDNA aliquot.
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 perform control normalization using control cDNA template provided in the Trimmer-2 kit. Repeat the normalization using fresh aliquots of experimental cDNA.
The concentration of starting cDNA is low, but the quality is good.	Repeat normalization using more cDNA.
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 from step 26 of the Normalization protocol. Optimization of PCR parameters may include: <ul style="list-style-type: none"> <li>(a) decreasing the annealing temperature in increments of 2-4°C;</li> <li>(b) optimizing the denaturation temperature by decreasing or increasing it in 1°C increments; and/or</li> <li>(c) increasing the extension time in 1-min increments.</li> </ul>
Some reaction components may have degraded during storage and/or delivery	To test that all components work properly, perform control normalization using control cDNA template provided in the Trimmer-2 kit.

PCR products from the Control tube are overamplified after 7 PCR cycles.

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

### XI. C Amplification of normalized cDNA (step 40, Section VII)

A low-molecular-weight product, a poor yield, or no PCR products is obtained from tubes containing normalized (DSN-treated) cDNA, but a high-quality PCR product is obtained from the control tube.

Possible cause	Solution
The DSN treatment may have been too excessive.	<p>Make sure that the DSN enzyme was thoroughly mixed with the storage buffer. Granules of non-diluted enzyme may dramatically change the DSN concentration in the experimental samples. If this is the issue, repeat the normalization using fresh ds cDNA and well-diluted DSN enzyme.</p> <p>Otherwise, repeat the normalization using fresh ds cDNA and the following modifications:  <b>Normalization Protocol, steps 18a-18b:</b>            In three sterile tubes prepare the following dilutions of DSN enzyme:            Combine 3 <math>\mu</math>L of DSN storage buffer and 1 <math>\mu</math>L of DSN stock solution. Mix by gently pipetting the reaction mixture up and down. Mark the tube as 1/4 DSN.            Combine 5 <math>\mu</math>L of DSN storage buffer and 1 <math>\mu</math>L of DSN stock solution. Mix by gently pipetting the reaction mixture up and down. Mark the tube as 1/6 DSN.            Combine 7 <math>\mu</math>L of the DSN storage buffer and 1 <math>\mu</math>L of DSN stock solution. Mix by gently pipetting the reaction mixture up and down. Mark the tube as 1/8 DSN.</p>

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Possible cause	Solution
	<p><b>Normalization Protocol, step 22:</b> Treat the cDNA with DSN that has been diluted four-, six-, and eight-fold instead of the one-, two-, and four-fold dilutions specified in the Normalization Protocol.</p>

## XI. D Selection of well-normalized cDNA (step 41, Section VII)

The PCR products from all experimental tubes appear to be over-amplified or non-normalized.

Possible cause	Solution
The DSN treatment may have been insufficient.	<p>Check whether the DSN enzyme was thoroughly mixed with the storage buffer; if not, repeat the normalization using fresh ds cDNA and well-diluted DSN enzyme.</p> <p>If the DSN enzyme was diluted sufficiently, test the DSN activity using the procedure described in Section VI. B.</p> <p>If the DSN activity is acceptable, it is possible that microscopic drops of the initial cDNA remained on the experimental tube walls or in the oil layer during hybridization or DSN treatment, and were therefore not exposed to the DSN treatment. After dilution of the experimental samples, this untreated (non-normalized) cDNA could have contaminated the experimental samples and been amplified during the subsequent PCR step. Repeat the normalization more carefully.</p>
The concentration of the DSN enzyme may be unacceptably low.	Repeat the normalization using 2 $\mu$ l of the stock DSN solution instead of the 1/4 DSN dilution, and 1.5 $\mu$ l of the stock DSN solution instead of the 1/2 DSN dilution (see Normalization protocol, step 22).



The normalized cDNA appears on a gel as a uniform smear stretching from the wells to the low-molecular-weight region (Fig. 11).

Possible cause	Solution
The 72°C elongation step may be too long. An extended elongation may promote concatemerization of the cDNA adapter sequences.	Concatemers may be confirmed by cDNA sequencing. If this is the case, repeat the cDNA synthesis using modified PCR parameters in which the 72°C elongation step is decreased by up to 2 min.

### XI. E Testing the normalization efficiency (Section VIII)

Quantitative PCR or Virtual Northern blot show that the transcript abundance remains unchanged after the normalization procedure, but the cDNA sample seems to have been efficiently normalized.

Possible cause	Solution
The concentrations of non-normalized and normalized cDNA used for comparison may not be equal.	Equalize the concentrations of these cDNAs and repeat the test.
The transcripts selected for testing may not be abundant in the samples of interest.	Make sure that the transcripts selected for testing are in high abundance. In non-normalized cDNA, high abundance transcripts should yield PCR products after 18-23 cycles. However, the representation levels of intermediate and rare transcripts may not change during the normalization procedure. In some cases, a slight increase in the representation level of such transcripts may occur.
The normalization process was unsuccessful.	Microscopic drops of the initial cDNA may have remained on the experimental tube walls or in the oil layer during hybridization or DSN treatment, thereby escaping DSN treatment and subsequently contaminating the experimental

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Possible cause	Solution
	<p>samples to generate non-normalized cDNA during the PCR step. Repeat normalization more carefully.</p> <p>Perform control cDNA normalization using control cDNA template provided in the Trimmer-2 kit and test normalization efficacy. If control normalization is unsuccessful too, please contact Evrogen technical support: <a href="mailto:customer-support@evrogen.com">customer-support@evrogen.com</a></p>

### Sequencing of the normalized cDNA reveals large concatamers of cDNA adapters.

Possible cause	Solution
The 72°C elongation step may be too long. An extended elongation may promote concatemerization of the cDNA adapter sequences.	Repeat the cDNA synthesis and normalization using PCR parameters that have been modified as follows: in all amplification steps, shorten the 72°C step by up to 2 min.

### Sequencing of the normalized cDNA reveals that certain sequences are highly prevalent, even though they do not represent genes that are known to be highly expressed. This issue complicates effective gene discovery.

Possible cause	Solution
Ineffective hybridization of some sequences (e.g., due to high TA contents and/or the formation of secondary structures) may have negatively impacted normalization.	Clone fragments (~ 100 bp) of the undesirably prevalent genes, re-amplify them from purified plasmid DNA, purify the PCR products using a PCR purification kit, and mix the amplified fragments together to a final concentrations of 10 ng/μL (each). Add 1 μL of this "driver" to the hybridization mixture (Normalization Protocol,

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Possible cause	Solution
	<p>step 12) and repeat the normalization.</p> <p>The addition of this driver leads to subtraction of undesired transcripts during normalization [11].</p>

## XI. F cDNA synthesis and amplification using a SMART-based kit (Clontech) (step 16, Appendix A)

Gel analysis of PCR products obtained from both control and experimental RNA samples reveals low-molecular-weight products, poor yield, or no products.

Possible cause	Solution
RNA may have degraded during storage and/or first-strand cDNA synthesis.	<p>Use gel electrophoresis to estimate the concentration and quality of the RNA.</p> <p>If RNA degradation during cDNA synthesis is suspected, add 0.5 <math>\mu</math>L RNase inhibitor (20 U/<math>\mu</math>L, Ambion) to the first-strand synthesis reaction.</p> <p>Check that your work area, equipment, and solutions are free from RNase contamination.</p>
Electrophoresis data might be incorrect because amplified cDNA was frozen before electrophoresis	<p>If amplified samples are frozen before electrophoresis, heat them at 72°C for 2 min and mix before loading onto the agarose gel.</p>
You may have made an error during the procedure, such as omitting an essential component.	<p>Carefully check the protocol and repeat the first-strand synthesis and PCR. One typical mistake is not mixing the RNA samples thoroughly after defrosting. We recommend that you heat the RNA samples (65°C for 2-3 min) prior to aliquotting.</p>
If the PCR reaches its plateau after 25 or more cycles, the PCR conditions may not be optimal.	<p>The optimal number of PCR cycles may vary with different PCR machines and RNA templates. Optimize the PCR parameters and repeat the PCR using a fresh aliquot of first-strand cDNA. Optimization of PCR parameters may include:</p>

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Possible cause	Solution
	<p>(a) decreasing the annealing temperature in increments of 2-4°C;</p> <p>(b) optimizing the denaturation temperature by decreasing or increasing it in 1°C increments; and/or</p> <p>(c) increasing the extension time in 1-min increments.</p>
Some reagents are not working properly.	<p>Perform control cDNA preparation using the reagents and protocol provided in the SMART-based kit.</p> <p>If cDNA synthesis using Clontech reagents is successful, it indicates that 3'-end adapter provided in the Trimmer-2 kit is degraded. Please contact Evrogen technical support: <a href="mailto:customer-support@evrogen.com">customer-support@evrogen.com</a></p> <p>If cDNA synthesis using Clontech reagents is not successful, it indicates that SMART-based kit used for cDNA preparation must be replaced.</p>

**Gel analysis of PCR products obtained from experimental RNA reveals low-molecular-weight products, poor yield, or no products; while high-quality PCR product is generated from the control RNA.**

Possible cause	Solution
The experimental RNA may be degraded (e.g. due to RNase contamination) or too diluted.	<p>Use gel electrophoresis to estimate the concentration and quality of the RNA. Then, check the stability of the RNA by incubating a small aliquot in water for 1 hr at 42°C and running it on a denaturing formaldehyde/agarose gel alongside an unincubated aliquot.</p> <p>If the RNA is degraded during the incubation, repeat the experiment using a fresh lot or preparation of RNA. Perform several rounds of phenol:chloroform extraction, as this can considerably increase RNA stability.</p>

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## XI Troubleshooting

Possible cause	Solution
	If RNA degradation during cDNA synthesis is suspected, add 0.5 $\mu\text{L}$ RNase inhibitor (20 U/ $\mu\text{L}$ , Ambion) to the first-strand synthesis reaction. Check that your work area, equipment, and solutions are free from RNase contamination.
The RNA may contain impurities that inhibit cDNA synthesis.	In some cases, ethanol or LiCl precipitation of RNA can remove impurities. If this does not help, re-isolate the RNA using another method.
If the PCR reaches its plateau after 25 or more cycles, the PCR conditions may not be optimal. The optimal number of PCR cycles may vary with different PCR machines and RNA templates.	Optimize the PCR parameters and repeat the PCR using a fresh aliquot of first-strand cDNA. Optimization of PCR parameters may include: (a) decreasing the annealing temperature in increments of 2-4°C; (b) optimizing the denaturation temperature by decreasing or increasing it in 1°C increments; and/or (c) increasing the extension time in 1-min increments.
RNA samples are from non-mammalian species with specific size distribution of RNA	If experimental RNA samples were isolated from 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 less than 2-3 kb.

**Gel analysis reveals that the concentration of the PCR product is low, but the quality is good.**

Possible cause	Solution
PCR undercycling may have resulted in a low yield of PCR product.	Subject the samples to two or three additional PCR cycles (plus one extra final extension cycle) and recheck the products. If a low yield of PCR product is still observed, this could indicate a low yield of first-strand cDNA.

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Possible cause	Solution
	<p>Repeat the experiment using more RNA.</p> <p>▶ <b>Note:</b> <i>We do not recommend that you use cDNA samples obtained after more than 25 PCR cycles because these samples may be not representative.</i></p>
The starting RNA concentration may have been low.	<p>Even if the total RNA concentration appears acceptable based on spectrophotometric analysis, a high content of tRNA may result in the mis-estimation of the mRNA concentration. If you have not already done so, use denaturing formaldehyde/agarose gel electrophoresis to estimate the concentration and quality of your RNA. If there is a high tRNA content, remove the low-molecular-weight RNA fraction using RNA purification columns.</p>

The PCR product is visualized as a very intense smear, none of the expected bright bands are distinguishable (see Fig. 11), and/or the smear appears in the high-molecular-weight region of the gel.

Possible cause	Solution
If bands are expected but not visible and the background smear is very intense, PCR overcycling may be an issue.	<p>Repeat PCR amplification with a fresh first-strand cDNA sample, using two or three fewer PCR cycles. Please note that cDNA prepared from some mammalian tissues (e.g., human brain, spleen, and thymus) may not show bright bands due to the very high complexity of the starting RNA.</p>
The 72°C elongation step may be too long. An extended elongation may promote concatemerization of the cDNA adapter sequences.	<p>Concatemers may be confirmed by cDNA sequencing. If this is the case, repeat the cDNA synthesis using modified PCR parameters in which the 72°C elongation step is decreased by up to 2 min.</p>

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Possible cause	Solution
The gel running parameters may alter band visibility.	Attempt to improve your electrophoretic results by testing the use of the following: 1X TAE buffer instead of 1X TBE; 1.1%-1.5% agarose concentration; and a running voltage up to 10 V/cm (10 V per each cm of space between the electrodes in the electrophoretic chamber). If amplified samples were frozen before electrophoresis, heat them at 72°C for 2 min and mix before loading onto the agarose gel.

### Gel analysis shows high content of low-molecular-weight (<0.1 kb) materials.

Possible cause	Solution
A preponderance of low-molecular-weight (<0.1 kb) materials in the raw PCR product could indicate PCR overcycling.	Repeat the PCR step with a fresh sample of first-strand cDNA, using 2-3 fewer cycles. <b>Note:</b> <i>The raw PCR product usually contains minor low-molecular-weight fraction, including unincorporated primers, adapters and very short PCR products. These small fragments are generally removed from the ds cDNA preparation in the purification step.</i>

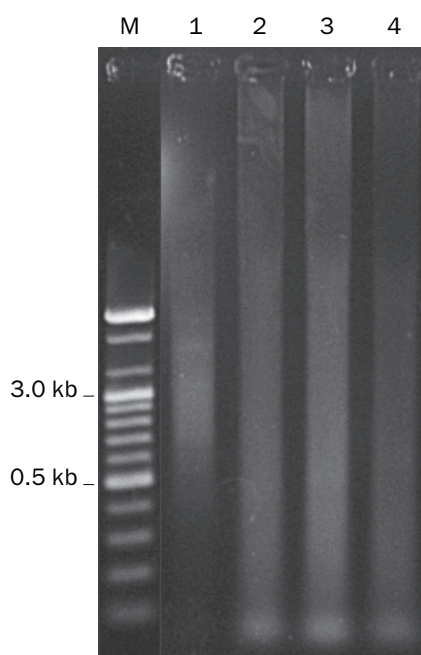
## XI. G Second amplification of normalized cDNA (Appendixes B-F)

### A low-molecular-weight product, a poor yield, or no PCR product is obtained after the second PCR amplification.

Possible cause	Solution
The cDNA or reagents may have degraded during storage.	Check that your work area, equipment, and solutions are free of nuclease contamination. Check the quality of the starting cDNA using agarose gel electrophoresis.

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Possible cause	Solution
	<p>Perform control cDNA amplification using control cDNA sample provided in the Trimmer-2 kit instead of experimental normalized cDNA.</p> <p>Repeat the amplification using a fresh aliquot of normalized cDNA from step 42.</p>
<p>If the concentration of PCR product is low, but the quality is good, the concentration of starting cDNA may be too low.</p>	<p>Repeat the PCR amplification using more cDNA.</p> <p>The PCR conditions may have not been optimal. The optimal number of PCR cycles may vary when different PCR machines, polymerases, and RNA templates are used. Optimization of PCR parameters may include:</p> <ul style="list-style-type: none"> <li>(a) decreasing the annealing temperature in increments of 2-4°C;</li> <li>(b) optimizing the denaturation temperature by decreasing or increasing it in 1°C increments;</li> <li>and/or</li> <li>(c) increasing the extension time in 1-min increments.</li> </ul>



**Fig. 11.** Agarose gel electrophoresis of non-normalized (lane 1) and normalized cDNA (lanes 2-4) that has been PCR amplified using a too-long extension step (6 min). No bright bands are visible, and the normalized cDNA appears as a smear starting from the high-molecular-weight region of the gel. Lane M shows a 1 kb DNA size marker.



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For notes...

## Endnotes

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