RNA purification, cDNA synthesis and preparation for Illumina microread sequencing in *Brachypodium distachyon*

Materials:

Stock Solutions

Special precautions should be taken to minimize RNA degradation with ribonucleases and to obtain libraries with high proportion of full-length cDNAs. To minimize RNase contamination, the workspace, centrifuge rotor, pipettors, and other equipment should be treated with RNase decontamination agents such as RNaseZap (Ambion). Plastic ware such as pipette tips and microcentrifuge tubes should be RNase-free grade. All RNA manipulations at room temperature should be performed in the shortest possible time. Frozen tissue powder should be placed directly into the ice-cold Concert reagent (Invitrogen), and the RNA solution before the clean up step should be treated with RNAsecure reagent (Ambion). All stock solutions should be prepared using RNase-free deionized water. Handling of the Concert reagent, phenol, chloroform, diethylpyrocarbonate (DEPC) and β-mercaptoethanol should be done in a fume hood. All RNA manipulations should be performed at 4˚C, except when indicated otherwise.

Stock solutions
1. 80% Ethanol
2. 2-Propanol
3. 3 M Sodium acetate, pH 5.5
4. 1 M Tris-HCl, pH 8.0
5. 5 M NaCl
6. RNase-free deionized water treated with diethylpyrocarbonate
7. 10% SDS

Reagents and Equipment

- *RNA purification:*
Reagents
1. RNaseZap (Ambion, cat. # AM9780)
2. RNase-free DNase I (Ambion, cat. # AM2238)
3. Concert Plant RNA Reagent (Invitrogen cat. # 12322-012)
4. RNase-free DNase I (Ambion, cat. # AM2238)
5. RNAsecure reagent (Ambion, cat. # AM7005)
6. RNeasy plant mini RNA kit (Qiagen, cat. # 74904)
7. Poly(A) Purist kit (Ambion, cat. # 1919)

Equipment
1. Microcentrifuge
2. Vortex mixer, rotating platform
3. Heating block or PCR thermal cycler
4. Spectrophotometer (NanoDrop Technologies)
5. Bioanalyzer (Agilent Technologies)

- *cDNA synthesis using SMART protocol and DSN library normalization:*
Reagents
1. BD SMART cDNA Library Construction kit (BD Biosciences Clontech, cat. # 634901)
2. TRIMMERDIRECT cDNA Normalization kit (Evrogen, cat. # NK002)
3. Phenol:chloroform:isoamyl alcohol (25:24:1) mixture
4. TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA)
5. Qiagen PCR Purification kit (Qiagen, cat. # 28106)

Equipment
1. Microcentrifuge
2. PCR thermal cycler

3. Spectrophotometer (NanoDrop Technologies)
4. Horizontal agarose gel electrophoresis

- *SMART/DSN cDNA preparation for Solexa/Illumina sequencing:*
Reagents
1. DNA Polymerase I, Large (Klenow) Fragment (NEB, cat. # M0210S)
2. T4 DNA Polymerase (Invitrogen, cat. # 18005-025)
3. T4 Polynucleotide Kinase (NEB, cat. # M0201S)
4. Klenow Fragment (3´-5´ *exo*- minus) (NEB, cat. # M0212S)
5. Adenosine 5´-Triphosphate (ATP) (NEB, cat. # P0756S)
6. 100 mM dNTPs (Invitrogen, cat. # 10297-018)
7. Phusion Hot Start High-Fidelity DNA Polymerase (NEB, cat. # F-540S)
8. T4 DNA Ligase (Invitrogen, cat. # 15224-017)
9. NuSieve GTG Agarose (Lonza, cat. # 50081)
10. Qiagen kits: PCR Purification (Cat. # 28106); MinElute PCR Purification (cat. #28004); MinElute Reaction Clean-up (cat. # 28204); and MinElute Gel Extraction (cat. #28604)
11. Genomic DNA Sample Prep Oligo Only kit (Solexa/Illumina cat. # FC-102-1003/1002579).
Equipment
1. Microcentrifuge
2. PCR thermal cycler
3. Spectrophotometer (NanoDrop Technologies)
4. Nebulizers (Invitrogen, cat. # K7025-05)
5. Tank with compressed nitrogen
6. Horizontal agarose gel electrophoresis system

- *cDNA synthesis using random priming protocol:*
Reagents
1. Superscript III First Strand Synthesis kit (Invitrogen, cat. # 11904-018)
2. 100 mM dNTPs (Invitrogen, cat. # 10297-018)
3. DNA Polymerase I, Large (Klenow) Fragment (NEB, cat. # M0210S)
4. Qiagen PCR Purification kit (Cat. # 28106)
Equipment
1. PCR thermal cycler

- *Sequencing using Solexa/Illumina 1G Genome Analyzer:*
Reagents
Illumina Standard Cluster Generation kit (cat. # FC-103-1001/0801-0304)
36 Cycle Solexa/Illumina Sequencing kit (cat. # FC-104-1003 /1001461)
Equipment
Illumina Cluster Station (cat. # SY-301-2001/0100-0004)
Illumina Genome Analyzer (cat. # SY-301-1001/0100-0005)

Methods:

Total RNA isolation:
This protocol has been used successfully for *Arabidopsis*, rice, poplar, and *Brachypodium* and yields high quality intact RNA suitable for a synthesis of cDNA libraries enriched with full length cDNAs. Approximately 200 mg of ground tissue yields up to 60-100 µg of total RNA. To prevent contamination with genomic DNA, RNA should be digested with DNase I followed by a clean up on Qiagen mini-column. The procedure can be scaled up without changing tissue/reagents ratios if higher amounts of the total RNA are desired.

Extraction of the total RNA and genomic DNA digestion:
1. Grind flash-frozen tissues in liquid nitrogen using mortar and pestle or in stainless steel jars using Mixer Mill MM 301 (Retsch, cat. # 20.741.0001).
2. Transfer approximately 200 mg of frozen tissue powder directly into 1 mL of ice-cold Concert Plant RNA Reagent, immediately vortex for ~20 seconds and shake for 5 min. at room temperature (RT).
3. Centrifuge at ~21,000 x g for 2 min. Transfer the supernatant to a new tube on ice.
4. Add 200 µL of cold 5M NaCl and centrifuge at ~21,000g for 2 min.
5. Transfer the supernatant to new tube, add 500 µL of chloroform and mix by inverting. Centrifuge at ~21,000 x g for 2 min. and transfer the aqueous/top layer to a pre-chilled 2 mL tube. Repeat the chloroform extractions 2-3 times until the aqueous phase is clear.
6. After the final chloroform extraction step, transfer the aqueous layer to a pre-chilled tube, add 0.8 volumes of 2-propanol and precipitate RNA for 10 min. at RT.
7. Centrifuge at ~21,000 x g for 10 min., remove supernatant and wash RNA pellet with cold 80% ethanol.
8. Air dry the pellet for approximately 5 min. and re-suspend RNA in 178 µl of 1 x RNAsecure reagent. To inactivate RNases, incubate for 10 min at 65°C.
9. Add 20 µl of 10 x Turbo-DNase buffer, 2 µl of Turbo-DNase and digest the DNA at 37°C for 10 min.

RNA clean-up:
1. Add 700 µl of RLT buffer from RNeasy Plant Mini RNA kit (Qiagen) to the digestion reaction.
2. Mix with 500 µL of 95% ethanol and proceed with RNA clean up according to the manufacture's protocol.
3. Retain 2 µl for NanoDrop spectrophotometer and 100-500 ng in 2 µl of water to check RNA integrity using Agilent 2100 Bioanalyzer (*see* **Note 1)**.

Purification of poly(A) RNA:
Poly(A) mRNA purification is necessary for randomly primed cDNA synthesis, but not necessary for SMART prepared cDNA synthesis. The reason is that the random hexamer primers used in random priming can bind to ribosomal RNA thereby necessitating there complete removal. In order to obtain high quality mRNA essentially free of other cellular RNAs, we have found that two cycles of oligo d(T) purification using Ambion's Poly(A) Purification kit are required.

1. Bring the sample volume to 250 µL with nuclease-free water, add 250 µL of 2x binding solution and mix thoroughly.
2. Add each sample to the oligo(dT) cellulose tube, mix well and incubate the at 72°C for 5 min. Incubate the sample on a rocker for 60 min. at RT with periodic 'flick-mixing'.
3. Centrifuge the sample at 4000 x g at room temperature for 3 min. and remove the supernatant. Add 500 µL of Wash Solution I, mix by vortexing and transfer to the column in provided tube.
4. Centrifuge the sample/column at 4000 X g at RT for 3 min., discard the supernatant and repeat the process with Wash Solution I.
5. Add 500 µL of Wash Solution II to the column, vortex briefly, and centrifuge at 4000 x g at RT for 3 min., discard the supernatant and repeat the process with another 500 µL of Wash Solution II.
6. Place the spin column into new collection tube, add 100 µL of preheated RNA Storage Solution (to 72°C). Vortex briefly and centrifuge at 5,000 x g at RT for 2 min.
7. Add a second 100 µL volume of RNA Storage Solution to column and repeat the RNA elution.
8. Transfer the sample to 1.5 mL microcentrifuge tube. Add 20 µL of 5M ammonium acetate, 1 µL of 5 mg/mL glycogen and 550 µL of 100% ethanol to the eluted mRNA. Mix by inverting and precipitate at -80°C for at least 1 hr.
9. Centrifuge at maximum speed for 30 min. at 4°C. Carefully remove supernatant, add 1 mL of 80% cold ethanol, vortex briefly and centrifuge for 10 min. at 4°C. Discard the supernatant and centrifuge for 2 min. to remove all traces of ethanol.
10. Allow the pellet to air dry for no longer than 5 min. Dissolve pellet in ~15-50 µL of preheated RNA Storage Solution.
11. Check the RNA quantity and integrity using NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer.

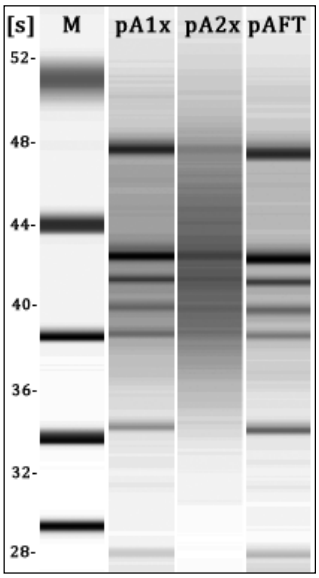

Fig. 1. Bioanalyzer analysis of polyadenylated mRNA fractions. M - RNA markers ([s] indicates relative RNA sizes); pA1x – poly(A) RNA purified once using oligo d(T) cellulose (Ambion RNA Purist kit); pA2x – poly(A) RNA purified twice using oligo d(T) column; pAFT – flow through fraction of the pA1x sample through oligo d(T) column. pA2x fraction is suitable for random primed cDNA synthesis protocol for the Illumina sequencing. RNA was analyzed using Agilent 2100 Bioanalyzer.

12. Pool approximately 4 µg of 1 x purified poly(A) RNA, bring sample volume to 250 µL with water and repeat the purification steps described above using the same oligo d(T) column.
13. Retain the flow through fraction for the Bioanalyzer analysis (**Fig. 1**). Typically, the second cycle of oligo d(T) purification starting from 4 µg of 1X purified poly(A) RNA yields about 1 µg of mRNA essentially free of cellular RNAs when starting from 4 µg of 1X purified poly(A) RNA.

Construction of cDNA libraries:

To obtain cDNA libraries suitable for Illumina sequencing, we have used two different approaches. The first method is based on amplification of the full-length enriched cDNA libraries using the SMART technology (Zhu, 2001; BD Biosciences Clontech). The second approach is to generate cDNA libraries from highly enriched poly(A) mRNA using random hexamer priming. The advantages of the SMART protocol include: a small amount of starting RNA material is required which is essential when the amount of tissue or RNA available is a limiting factor, an ability to generate full length cDNAs both from total or poly(A) RNA, and the ability to couple the procedure with library normalization using Duplex Specific Nuclease (DSN) treatment (Zhulidov, 2004). The DSN normalization corrects for the bias in rare transcript coverage observed in non-normalized cDNA libraries. Potential pitfalls of the SMART cDNA preparation for Illumina sequencing may be over-amplification of the most abundant or preferential amplification of the shorter molecules and/or under-representation of the 5' UTRs. In addition, microreads obtained from SMART libraries should be filtered for the sequences of SMART primers that flank both the 5' and the 3' cDNA ends. Some target mRNAs containing strong transcriptional pauses may also be under-represented or lost during the synthesis of the first strand of the full length cDNA. Randomly primed cDNA libraries have an advantage of unbiased representation of the 5' cDNA ends including 5' untranslated regions (UTRs). The average first cDNA strand fragment length can also be controlled by amount and/or by length (i.e., hexa-, hepta-, octamers, or their mixtures) of random primers. Therefore, the nebulization step can be omitted from the Illumina cDNA preparation. The disadvantages of random priming include a requirement for the high purity of poly(A) RNA (to avoid contamination with non-polyadenylated cellular RNAs) and a requirement for larger starting amounts of tissues to obtain highly purified mRNA in microgram quantities.

Construction of the SMART prepared full length enriched cDNA libraries:

This protocol is a modification of BD Clontech SMART cDNA Library Construction method and utilizes SMART adapter primers (Zhu, 2001). You may also construct cDNA libraries without using expensive kits by using your own primers and a high fidelity polymerase.

1. In a PCR tube, add 1 µL of each primer (CDS III/3' PCR primer to capture the poly(A) tail and 5' SMART IV Oligonucleotide). Add 250-500 ng of poly(A) RNA sample and bring volume to total of 5 µL with nuclease-free water.
2. Incubate at 72°C and place on ice for 2 min.
3. Add 2µL of 5X First-Strand Synthesis Buffer (Clontech kit) and 1 µL of 20 mM dithiothreitol (DTT) and 1 µL of 10 mM dNTPs and 1 µL of moloney murine leukemia virus reverse transcriptase (M-MLV RT).
4. Incubate at 42°C for 1 hr. in the thermal cycler and proceed to the amplification step or store at -20°C.
5. Prepare a PCR reaction with the following reagents: 80 µL sterile water, 10 µL of 10X Advantage 2 PCR Buffer, 2 µL of 50X dNTPs (10 mM each), 4 µL of 5' PCR primer II A, 2 µL of 50X Advantage 2

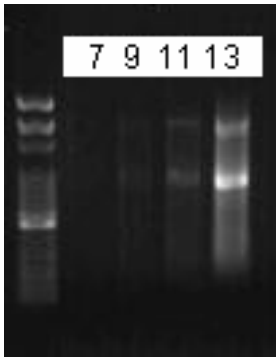

Fig. 2. Optimization of PCR cycling for a SMART cDNA library. 5 µL of PCR reaction were run on a 1% gel along with a 100 bp DNA marker.

PCR Polymerase Mix, and 2 µL of the control first-strand cDNA (provided with BD Biosciences Clontech kit).
6. PCR amplify in a thermocycler: {95°C for 5 min., (95°C for 20 sec., 65°C for 30 sec., 68°C for 6 min.) x 15 cycles, 68°C for 7 min.}.
7. Remove 5 µL aliquots at cycles 7, 9, 11, 13, and 15 for gel electrophoresis (*see* **Note 2**).
8. Separate PCR products on a 1% agarose gel to determine optimal cycle number for library amplification (**Fig. 2**).
9. Amplify the experimental cDNA library using the optimized cycling conditions (determined in step 7) and verify amplification products quality on a 1% agarose gel.
10. Purify the PCR products using QIAquick PCR Purification kit.

Library normalization by DSN treatment:
This normalization method uses a modified protocol for double strand cDNA removal by treatment with duplex-specific nuclease (DSN) isolated from the Kamchatka crab (Evrogen). The ds-DNA is first denatured, then re-annealed, followed by the digestion with DSN (Zhulidov, 2004). The key to the DSN treatment is optimization. We have worked out precise methods with several modifications for the optimization of this normalization procedure (discussed below). *Note*: The two most important factors to consider when conducting this technique is the time allowed for DNA re-annealing and the dilution of the DSN enzyme. The different lots provided by the company may have varying enzyme activity, thereby requiring an optimization of the DSN dilutions for each lot purchased. We have found that several key factors play a role in the optimization of the DSN procedure. The annealing time is a critical parameter. We use 1.5, 2, 3, and 4 hrs. for cDNA re-association before nuclease treatment. Additional optimization can be achieved by increasing dilutions of the DSN enzyme. We suggest using 1/4, 1/8, 1/16, and 1/64 dilutions of the enzyme to optimize DSN treatment (*see* **Note 3**).

1. Combine 1100-1200 ng of cDNA in 12 µL, 4 µL of 4 x hybridization buffer (200 mM HEPES, pH 7.5, 2 M NaCl). Divide each sample into 2, 8 µL aliquots in PCR tubes (treatment and control).
2. Incubate the tubes at 98°C for 2 min., then at 68°C for 1.5, 2, 3, and 4 hrs to optimize re-association conditions.
3. While the cDNA is incubating, prepare the DSN dilutions using 50 mM Tris-HCl, pH8.0, and pre-heat the 2 x DSN master buffer (100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM DTT) at 68°C.
4. Following the incubation (re-annealing time), add 10 µL preheated master buffer and incubate at 68°C for 10 min.
5. Quickly add 2 µL of the diluted DSN enzyme to the sample tube and incubate at 68°C for 25 min.
6. Stop the reaction by add 20 µL of 5 mM EDTA and bring the final volume to 100 µL with 60 µL of sterile water.
7. Extract the normalized cDNA with an equal volume of phenol:chloroform and precipitate the DNA by adding $1/10^{th}$ volume of 3 M sodium acetate, and 2.5 volumes of 100% ethanol.
8. Re-suspend DNA pellet in 12 µl of sterile water. Purify the DSN products using QIAquick PCR Purification kit and use 2 µL to determine DNA quantity.
9. Amplify the normalized cDNA with the Advantage 2 Polymerase mix. Add the following reagents to a PCR tube: 39 µL of sterile water, 5 µL 10X Advantage 2 PCR Buffer, 1 µL of 50X dNTPs (10 mM each), 2 µL of 5' PCR primer II A (provided in Clontech SMART cDNA Synthesis Kit), 1 µL of Advantage 2 Polymerase Mix and 2 µL of template.
10. Run 7 cycles with the SMART cDNA synthesis thermal cycler program described above and repeat the PCR optimization procedure.
11. Cycle the non-normalized (No DSN treatment) samples by increasing 2 additional cycles for a total of 7, 9, 11, 13, and 15 cycles (**Fig. 2**).
12. Cycle the experimental (DSN treated) samples to the optimized number of cycles. Normalized samples usually require 2 additional cycles as compared to the non-normalized sample (**Fig. 3**).

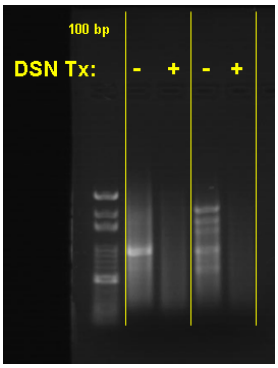

Fig. 3. cDNA libraries normalized by DSN treatment. 5 µL of non-normalized control (-) and normalized (+) cDNA libraries were separated on a 1% agarose gel. 100 bp DNA ladder.

Generation of random hexamer primed libraries:

1. Combine 1 µg of mRNA (*see* **Note 4**) in 4 µL of water and 6 µL of random hexamers (50 mg/mL).
2 Heat the mixture at 75°C for 5 min. and place on ice for 5 min.
3. Add the following components: 4µL of 5X Superscript III Buffer, 0.5 µL of RNAse inhibitor (40U/ml), 2 µL of 10 mM dNTPs, and 1 µL of Superscript III RT.
4. Incubate at 25°C for 10 min and then 42°C for 1 hr. Inactivate the reverse transcriptase by incubating at 70°C for 10 min.
5. Combine the following: 20 µL of the first strand reaction, 8 µL of 10X Klenow Buffer, 1 unit of RNase H, 68.8 µL of water and 3 µL of DNA Polymerase I (Klenow fragment).
6. Incubate at 15°C for 90 min. and stop the reaction by adding 5 µL of 0.5 M EDTA, pH 8.0.
7. Purify cDNA using Qiaquick PCR purification kit. Elute the sample into 30 µL of EB buffer.

Preparation of cDNA for Solexa/Illumina sequencing:

We adapted a general procedure developed by Solexa/Illumina for genomic DNA preparation (Illumina Sample Preparation Protocol Version 2.3) with modifications described below.

1. Transfer the cDNA sample (~5 µg recommended) in a 50 µL volume to a nebulizer and add 750 µL of Illumina nebulization buffer (*see* **Note 5**).
2. Fragment the DNA using compressed nitrogen at 32-35 psi for 7 min. and centrifuge the nebulizers at 450 x g for 2 min. to collect the sample from the walls.
3. Purify the sheared DNA using a QIAquick PCR Purification Kit and elute into 32 µL of EB.
4. Mix the following in a PCR tube (*see* **Note 6**): 30 µL of hexamer/SMART cDNA (approximately 1 µg), 10 µL of 5X T4 DNA ligase buffer with 10 mM ATP (Invitrogen), 4 µL of 10 mM dNTPs mix, 2.5 µL of T4 DNA polymerase (3 U/µL), 1 µL of Klenow DNA pol. (5 U/µL) and 2.5 µL of T4 polynucleotide kinase (10 U/µL).
5. Incubate for 30 min at 20ºC. Purify the sample using the QIAquick PCR Purification kit and elute in 32 µl of EB.
6. To the 32 µL DNA from above, add 5 µL of 10X Klenow buffer, 10 µL of 1 mM dATP, and 3 µl of Klenow exo$^-$ (3' to 5' exo minus) polymerase (5 U/µL). Incubate for 30 min at 37°C (*see* **Note 7**).
7. Purify the DNA using a QIAquick MinElute Reaction Clean-up kit and elute into 12 µL of EB.
8. Prepare the following reaction mix (*see* **Note 8**): 10 µL of cDNA from above, 5 µL of 5X T4 DNA ligase buffer, 6 µL of adapter oligo mix, 4 µL of T4 DNA ligase.
9. Incubate for 15 min at room temperature.
10. Purify with a QIAquick MinElute PCR Purification Kit eluting in 10 µL of EB.
11. Prepare 3.5% (w/v) NuSieve agarose in 1X TBE buffer (*see* **Note 9**).
12. Run the gel electrophoresis at 5 V/cm and stain the gel in 1 µg/mL of ethidium bromide in water in the dark for 10 min.
13. Excise the area in the range of 120-200 bp quickly to limit the exposure to UV light to 30 or less seconds to minimize DNA damage.
14. Purify DNA from gel slice using QIAquick Gel Purification Kit and elute in 32 µL of EB buffer.
15. Prepare the following PCR reaction mix (*see* **Note 10**): 2 µL of DNA from above, 1 µL of PCR primer 1.1 (Illumina), 1 µL of PCR primer 2.1 (provided by Illumina), 1 µL of 10 mM dNTPs, 44 µL of water, and 1 µL of Phusion DNA polymerase.
16. Amplify using the following PCR protocol: 30 sec at 98°C, then [10 sec at 98°C, 30 sec at 65°C, 30 sec at 72°C] for 18 cycles, followed by 10 min. at 72°C.
17. Purify using the QIAquick PCR Purification Kit, elute in 30 µL of EB and run 5 uL of product on a 2% agarose gel (**Fig. 4**).
18. Measure the concentration of cDNA using a Nanodrop spectrophotometer.

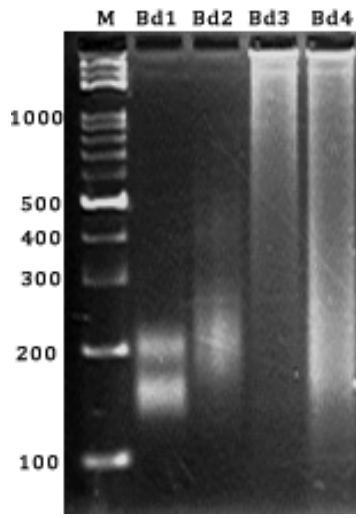

Fig. 4. Gel fractionation of hexamer-primed cDNA libraries. Lanes Bd1 and Bd2 - PCR enriched *Brachypodium* cDNA libraries of average sizes 160bp and 220 bp, respectively. Lanes Bd3 and Bd4 - cDNA libraries before gel fractionation and PCR enrichement. M = 100 bp DNA ladder markers (sizes in bp are indicated on left).

19. Dilute the cDNA to 10 nM final concentration by approximating the average MW of the fragments to ~160 bp (an average size of cDNA extracted from gel).
20. At this point, the cDNA may be used directly for Ilumina cluster generation or stored at -20^{o}C.

Notes:
1. A maximum of 100 µg of RNA can be bound to the Qiagen mini column, therefore multiple columns may be needed if the amount of RNA exceeds this limit. A 260:280 nm wavelength ratio for the RNA obtained by this method should be 2.0 or higher. Store RNA at -80°C.
2. During the *cDNA PCR amplification*, overcycling of the cDNA results in highly undesirable nonspecific PCR amplification. Therefore, it is necessary to optimize the number of cycles necessary to amplify a quality cDNA library.
3. Other important points to consider for optimal DSN normalization: start with a consistent 1100-1200 ng of cDNA; add all reagents/enzyme simultaneously via multi-channel pipette; treat the cDNA with the DSN enzyme for precisely 25 min.; perform a phenol/chloroform extraction followed by ethanol precipitation and Qiagen column purification of DSN-treated cDNA library to entirely eliminate the enzyme and salts.
4. The random hexamer approach requires isolation of poly(A) mRNA essentially free of other cellular RNAs. This could be achieved by an additional round of poly(A) mRNA purification on oligo(dT) cellulose (see above sections). To decrease an average size of fragments, the first cDNA strand is synthesized using a high ratio of hexamer primers (300 ng per each µg of poly(A) mRNA).
5. The SMART prepared cDNA must be sheared using a nebulizer in order to generate fragments less than 800 base pairs. The cDNA prepared by hexamer priming contains a significant population of double-stranded fragments in the range 120-220 bp and therefore, does not require an additional fragmentation via nebulization. For the random primed libraries proceed directly to step 7 below.
6. The nebulization/fragmentation process creates 5' and 3' overhangs. This step is implemented to convert the overhangs to blunt ends with phosphorylated 5' termini.
7. A single 'dA' nucleotide must be added to the 3' blunt end of the templates to accommodate the ligation of the adapters which have a single 'T' base overhang at their 3' ends. A single 'dA' is added to the ends of double-stranded cDNA molecules by employing the polymerase activity of exo minus (3' to 5') Klenow fragment.
8. The ligation reaction requires adapters supplied by Solexa/Illumina. The molar ratio of adapter to ds-cDNA fragments should be maintained approximately 10:1.
9. This gel purification step ensures proper size selection of cDNA fragments and removal of the excess of free adapters prior to Illumina sequencing.
10. This step allows for the selective enrichment and PCR amplification of cDNA fragments with adapter molecules attached to both ends. The PCR is performed with two primers provided by Illumina that anneal to the ends of the adapters. To avoid any skewing in the library representation PCR is limited to 18 cycles.

References:
Zhu, Y., Machleder, E., Chenchik, A. and Siebert, P. (2001) Reverse transcriptase template switching: a SMART approach for full-length cDNA library construction. Biotechniques, 30, 892-897.

Zhulidov, P.A., Bogdanova, E.A., Shcheglov, A.S., Vagner, L.L., Khaspekov, G.L., Kozhemyako, V.B., Matz, M.V., Meleshkevitch, E., Moroz, L.L., Lukyanov, S.A. et al. (2004) Simple cDNA normalization using kamchatka crab duplex-specific nuclease. Nucleic Acids Res, 32, e37.