DSN Normalization

Application of Duplex-Specific Thermostable Nuclease (DSN) to normalize RNA samples for Illumina sequencing

For Research Use Only

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Introduction

This protocol explains how to normalize Illumina® RNA-seq sample preparation based on the use of the Duplex-Specific thermostable nuclease (DSN) enzyme, purified from Kamchatka crab hepatopancreas and manufactured by Evrogen (www.evrogen.com). DSN normalization is performed after RNA-seq sample preparation and before cluster generation. It involves the degradation of abundant DNA molecules derived from rRNA, tRNA, and housekeeping genes while preserving DNA molecules derived from less abundant transcripts. This method can be useful in a wide range of applications, including transcriptome discovery and annotation, the analysis of bacterial transcriptomes that lack poly-A tails, and the analysis of highly degraded RNA from sources such as FFPE.

CAUTION

This document describes an experimental application of Illumina technology, which is being shared with the Illumina community as a courtesy. This application is not a standard Illumina kit. Illumina Technical Support scientists and Field Application scientists may not be able to answer questions. Customers seeking to use this protocol should be aware that even experienced users may face significant challenges in achieving consistent results.

Sample Prep Workflow

Depending on the intended application of DSN normalization, first perform Illumina's mRNA-Seq Sample Preparation or Directional mRNA-Seq Sample Preparation, modifying the protocols as specified in Table 1. Following sample preparation, perform DSN normalization prior to cluster generation.

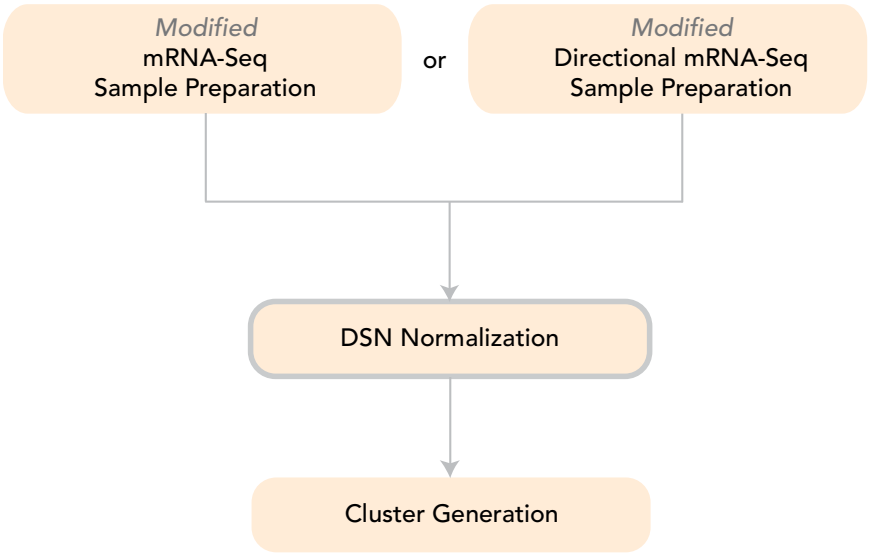

Figure 1 RNA-Seq Sample Prep Workflow with DSN Normalization

> **NOTE**
> See the *mRNA-Seq Sample Preparation Guide or Directional mRNA-Seq Library Prep Pre-Release Protocol* available at http://www.illumina.com/rna.

Table 1 RNA Sample Preparation Protocol Modifications

Application/Modification	Conventional cDNA Normalization	Prokaryotic or Eukaryotic Transcriptome Discovery and Annotation	FFPE
No modification	X		
No Poly-A Selection		X	
No Poly-A Selection or Fragmentation			X
Total RNA Input	1 µg	100 ng	100 ng

> **NOTE**
> If you are modifying the mRNA-Seq or Directional mRNA-Seq sample preparation protocol for no poly-A selection or no poly-A selection or fragmentation, then the input total RNA should be reduced to 100 ng.

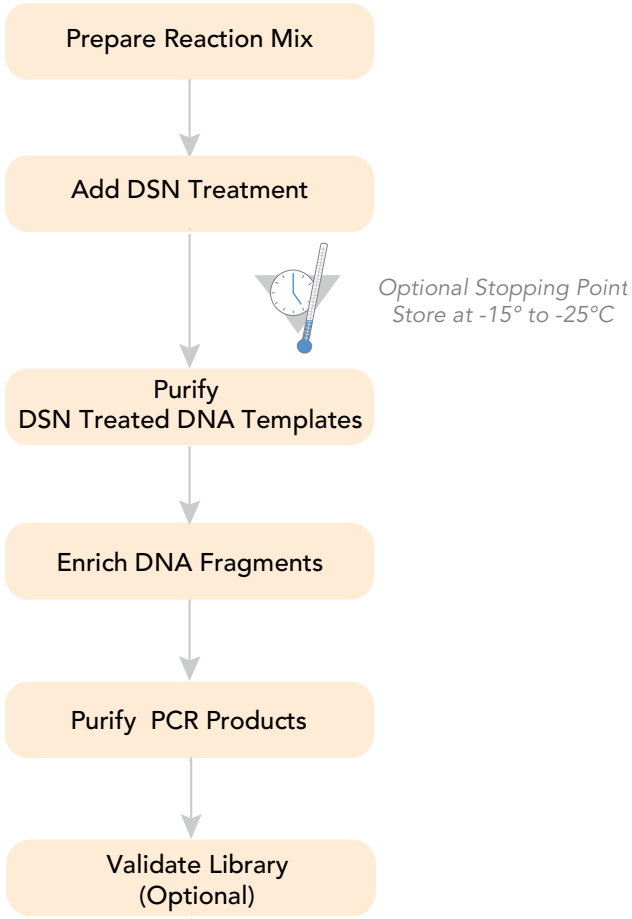

Figure 2 DSN Normalization Workflow

User-Supplied Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to DSN normalization. The requirement of some supplies are dependant upon the intended application of DSN normalization and are specified where necessary.

Table 2 User-Supplied Consumables

Consumable	Supplier
1 M HEPES buffer solution	Invitrogen, part # 15630-080
25 mM dNTP	General lab supplier
5 M NaCl solution	Ambion, part # AM9760G
5X Phusion™ Buffer and Phusion Polymerase (Finnzymes Oy)	NEB, part # F-530
96-well V-bottom plate	Axygen, part # P96450V-C
[Optional] DNA 1000 LabChip Kit	Agilent, catalog # 5067-1504
DSN Kit	Evrogen, part # EA001
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
[Optional] GAPDH Probes	AB, part # 4333764F
MicroAmp clean adhesive seal	AB, part # 4306311
MinElute PCR Purification Kit	QIAGEN, part # 28004
Nuclease-free water	Ambion, part # AM9937
[for mRNA-Seq applications] PCR Primer PE 1.0 PCR Primer PE 2.0	Illumina 100 RXN Paired End DNA Sample Prep Oligo Only Kit, catalog # PE-102-1003 (100 samples), or Illumina 400 RXN Paired End DNA Sample Prep Oligo Only Kit, catalog # PE-102-1004 (400 samples)
[for Directional mRNA-Seq applications] Primer GX1 Primer GX2	Illumina DGE-Small RNA Sample Prep Oligo Only Kit, catalog # FC-102-1013 (100 samples)
[Optional] S18 Probes	AB, part # 4333760F
Sample library (80–100 ng in 13.5 µl)	Output from your Illumina mRNA-Seq sample preparation
SPRI beads	Agencourt AMPure, part # 29152

Table 3 User-Supplied Equipment

Consumable	Supplier
[Optional] 2100 Bioanalyzer	Agilent
Heat block	General lab supplier
Magnetic stand-96	Ambion, part # AM10027
Thermal cycler	General lab supplier

NOTE
During the *Add DSN Treatment* process, Illumina recommends having no more than four tubes in the thermal cycler. If you are using more that four tubes, ensure that you have an additional thermal cycler.

Prepare Reaction Mix

This process denatures double-stranded (ds) DNA molecules to form single-stranded (ss) DNA molecules, followed by their subsequent renaturation. There is a correlation between the time to anneal and the relative concentration in a solution, so DNA molecules derived from highly abundant sources, such as rRNA, tRNA, and housekeeping genes, will re-anneal at a faster rate than those that are derived from less abundant transcripts. At the end of this procedure, the reaction mixture will contain both dsDNA and ssDNA.

Consumables

User-Supplied

- 1 M HEPES buffer solution
- 5 M NaCl solution
- Nuclease-free water
- Sample library from mRNA-Seq or directional mRNA-Seq sample prep protocol (80–100 ng in 13.5 µl. DNA amount is measured by DNA-1000 chip.)

Preparation

- Prepare the following 4x Hybridization Buffer. Excess buffer can be prepared and stored for future use at -15° to -25°C.

Reagent	Volume (µl)
1 M HEPES buffer solution	200
5 M NaCl solution	400
Nuclease-free water	400
Total Volume Per Sample	**1,000**

> NOTE
> Remove the frozen 4x Hybridization Buffer from storage and allow it to come to room temperature for at least 15–20 minutes. Ensure that there is no visible pellet or precipitate in the buffer before use.

- Ensure that the thermal cycler and heat block are located near each other.
- Pre-heat the heat block to 68°C.

Procedure

1 Prepare the following reaction mix in a separate, sterile, nuclease-free 200 µl PCR tube on ice for each sample to be normalized.

Reagent	Volume (µl)
Sample library (80–100 ng)	13.5
4X Hybridization buffer	4.5
Total Volume Per Sample	**18**

> NOTE
> The sample library volume is optimized for the removal of rRNA from a non-poly-A selected library. For a reduction of highly expressed transcripts from a poly-A selected library, more sample library may be needed for optimal results.

2 Gently pipette the entire volume up and down 10 times, then centrifuge briefly to mix.

3 Transfer the entire volume of reaction mix directly to the bottom of a new, sterile, nuclease-free 200 µl PCR tube, using a pipette. Do not let the sample contact the side of the tube during the process.

4 Incubate the reaction mix tube on the thermal cycler using the following PCR cycling conditions:
   a 2 minutes at 98°C
   b 5 hours at 68°C

> CAUTION
> Following incubation, keep the thermal cycler lid closed and the temperature held at 68°C. Do not remove the reaction mix tube from thermal cycler prior to and during DSN treatment.

5 Proceed immediately to *Add DSN Treatment*.

Add DSN Treatment

This process removes dsDNA, representing highly abundant transcripts, from the reaction mixture by treatment with DSN. DSN is a thermal stable double-stranded nuclease that has been isolated from the Kamchatka crab. DSN will remove the dsDNA while preserving the ssDNA.

Consumables

User-Supplied

- DSN Kit

Preparation

- Dilute the 10X DSN Master buffer supplied in the DSN kit to 2X with nuclease-free water
- Prepare the DSN Enzyme according to the manufacturers instructions

Procedure

1. Pre-heat the 2X DSN buffer on the pre-heated heat block at 68°C.

   NOTE
   Do not remove the 2X DSN buffer from the heat block during DSN treatment.

2. Quickly add 20 µl of pre-heated 2X DSN buffer to the first reaction mix tube.
3. With the reaction mix tube remaining within the thermal cycler, gently pipette the entire volume up and down 10 times to mix thoroughly using a pipette set to 40 µl.

   CAUTION
   Pipette the solution directly to the bottom of the PCR tube and do not let the sample contact the side of the tube during the process.

   NOTE
   It is important to keep the thermal cycler closed, except for the time necessary to add the 2X DSN buffer and mix. When preparing more than one reaction mix tube, keep the thermal cycler lid closed when extracting the 2X DSN buffer from its tube, then open the thermal cycler lid only for the time necessary to add and mix the 2X DSN buffer.

NOTE
Illumina recommends having no more than four tubes in the thermal cycler. If you are using more that four tubes, use an additional thermal cycler.

4 Repeat steps 2 and 3 for each reaction mix tube.

5 Incubate the reaction mix tubes on the thermal cycler at 68°C for 10 minutes.

6 Quickly add 2 µl of DSN enzyme to the first reaction mix tube using a 2 µl pipette.

7 With the reaction mix tube remaining within the thermal cycler, gently pipette the entire volume up and down 10 times to mix thoroughly using a pipette set to 40 µl.

CAUTION
Pipette the solution directly to the bottom of the PCR tube and do not let the sample contact the side of the tube during the process.

NOTE
It is important to keep the thermal cycler closed, except for the time necessary to add the DSN enzyme and mix. Failure to do so may decrease the normalization efficiency due to the non-specific digestion of secondary structures formed by ss-DNA.

8 Repeat steps 6 and 7 for each reaction mix tube.

9 Incubate the reaction mix tubes on the thermal cycler at 68°C for 25 minutes.

10 Add 40 µl of 2X DSN stop solution to each reaction mix tube. Gently pipette the entire volume up and down to mix thoroughly, then place the tubes on ice.

This is a safe stopping point. If you are stopping, store your sample at -15° to -25°C.

Purify DSN Treated DNA Templates

This process purifies DSN treated DNA with SPRI beads.

Consumables

User-Supplied

- 96-well V-bottom plate
- Freshly prepared 80% Ethanol (EtOH)
- MicroAmp clean adhesive seal
- QIAGEN EB (provided in the MinElute PCR Purification Kit)
- SPRI beads

Procedure

1. Transfer 80 µl of each of the samples from step 10 of the *Add DSN Treatment* procedure to a separate well of a new, 96-well V-bottom plate.
2. Vortex the SPRI beads until they are well dispersed, then add 128 µl of well-mixed SPRI beads to each well of the 96-well V-bottom plate that contains the samples.
3. Gently pipette the entire volume up and down 8 times to mix thoroughly.
4. Incubate the plate for 5 minutes at room temperature.
5. Place the plate on the magnetic stand for 8 minutes at room temperature until the liquid appears clear. Do not remove the plate from the magnetic stand.

   NOTE
   To verify that the solution is clear, use a pipette to extract some supernatant to more closely view the beads. If beads are still visible in this supernatant, carefully return the supernatant to the well without disturbing the pellet.

6. Remove and discard the supernatant from the plate with a pipette. Take care not to disturb the SPRI beads.
7. Add 180 µl of freshly prepared 80% EtOH to each well of the plate that contains the samples, without disturbing the beads. Do not remove the plate from the magnetic stand.
8. Incubate the plate for 30 seconds at room temperature, then remove and discard the supernatant from each well using a multichannel pipette.
9. Repeat steps 6 and 7 once for a total of two 80% EtOH washes.
10. Seal the plate with a MicroAmp Clean Adhesive Seal.
11. Remove the plate from the magnetic stand and briefly centrifuge at 1,000 rpm for 10 seconds.
12. Remove the MicroAmp Clean Adhesive Seal.

13 Place the plate on the magnetic stand for 30 seconds at room temperature, then remove and discard the EtOH using a 10 µl pipette.

14 Heat the plate on the pre-heated heat block for 1–2 minutes at 37°C until the pellet appears dry.

> NOTE
> Over-dried beads will contain many cracks in the pellet. If this is the case, place the plate in the magnetic stand. The bead pellet is very light when over-dried and will be easily blown out of the well.
> If the bead is not dry enough, the DNA recovery rate is lower due to the remaining EtOH.

15 Add 30 µl of QIAGEN EB to each pellet to elute the DNA. Gently pipette the entire volume up and down 5 times to mix thoroughly.

16 Incubate the plate for 2 minutes at room temperature, then gently pipette the entire volume up and down 10 times to mix thoroughly.

> NOTE
> If the beads are over-dried, you will need to incubate the plate a longer time.

17 Place the plate on the magnetic stand for 1 minute at room temperature.

18 Transfer all of each supernatant to a separate, new, nuclease-free, 200 µl PCR tube.

Enrich DNA Fragments

This process amplifies DSN treated DNA with PCR.

Consumables

User-Supplied

- 5X Phusion Buffer (Finnzymes Oy)
- Phusion Polymerase (Finnzymes Oy)
- 25 mM dNTP
- Nuclease-free Water
- For mRNA-Seq applications:
  - PCR Primer PE 1.0
  - PCR Primer PE 2.0
- For Directional mRNA-Seq applications:
  - Primer GX1
  - Primer GX2

Procedure

1 Prepare the following PCR reaction mix in a separate, sterile, nuclease-free tube for each of the samples being prepared.

Reagent	Volume (µl)
DSN treated reaction mix (from step 18 of the *Purify DSN Treated DNA Templates* procedure)	30
5X Phusion buffer	10
Phusion polymerase	0.5
25 mM dNTP	0.5
Nuclease-free water	8
For mRNA-Seq applications: PCR Primer PE 1.0 PCR Primer PE 2.0	 0.5 0.5
For Directional mRNA-Seq applications: Primer GX1 Primer GX2	 0.5 0.5
Total Volume Per Sample	**50**

2 Amplify the PCR on the thermal cycler using the following PCR cycling conditions, depending upon the application:

- For mRNA-Seq applications:
  - a 30 seconds at 98°C
  - b 12 cycles of:
    10 seconds at 98°C
    30 seconds at 65°C
    30 seconds at 72°C
  - c 5 minutes at 72°C
  - d Hold at 10°C
- For Directional mRNA-Seq applications:
  - a 30 seconds at 98°C
  - b 12 cycles of:
    10 seconds at 98°C
    30 seconds at 60°C
    15 seconds at 72°C
  - c 10 minutes at 72°C
  - d Hold at 4°C

Purify PCR Products

This process purifies PCR product with SPRI beads.

Consumables

User-Supplied

- 96-well V-bottom plate
- Freshly prepared 80% Ethanol (EtOH)
- MicroAmp clean adhesive seal
- QIAGEN EB (provided in the MinElute PCR Purification Kit)
- SPRI beads

Procedure

1. Transfer 50 µl of each of the samples from step 2 of the *Enrich DNA Fragments* procedure to a separate well of a new, 96-well V-bottom plate.
2. Vortex the SPRI beads until they are well dispersed, then add 80 µl of well-mixed SPRI beads to each well of the 96-well V-bottom plate containing samples. Gently pipette the entire volume up and down 8 times to mix thoroughly.
3. Incubate the plate for 5 minutes at room temperature.
4. Place the plate on the magnetic stand for 8 minutes at room temperature until the liquid appears clear. Do not remove the plate from the magnetic stand.

   NOTE
   To verify that the solution is clear, use a pipette to extract some supernatant to more closely view the beads. If beads are still visible in this supernatant, carefully return the supernatant to the well without disturbing the pellet.

5. Remove and discard the supernatant from the plate with a pipette. Take care not to disturb the SPRI beads.
6. Add 180 µl of freshly prepared 80% EtOH to each well of the plate containing samples, without disturbing the beads. Do not remove the plate from the magnetic stand.
7. Incubate the plate for 30 seconds at room temperature, then remove and discard the supernatant from each well using a multichannel pipette.
8. Repeat steps 6 and 7 once for a total of two 80% EtOH washes.
9. Seal the plate with a MicroAmp Clean Adhesive Seal.
10. Remove the plate from the magnetic stand and briefly centrifuge at 1,000 rpm for 10 seconds.
11. Remove the MicroAmp Clean Adhesive Seal.

12 Place the plate on the magnetic stand for 30 seconds at room temperature, then remove and discard the EtOH using a 10 µl pipette.

13 Heat the plate on the pre-heated heat block for 1–2 minutes at 37°C until the pellet appears dry.

> NOTE
> Over-dried beads will contain many cracks in the pellet. If this is the case, place the plate in the magnetic stand. The bead pellet is very light when over-dried and will be easily blown out of the well.
> If the bead is not dry enough, the DNA recovery rate is lower due to the remaining EtOH.

14 Add 20 µl of QIAGEN EB to each pellet to elute the DNA. Gently pipette the entire volume up and down 5 times to mix thoroughly.

15 Incubate the plate for 2 minutes at room temperature, then gently pipette the entire volume up and down 10 times to mix thoroughly.

> NOTE
> If the beads are over-dried, you will need to incubate the plate a longer time.

16 Place the plate on the magnetic stand for 1 minute at room temperature.

17 Transfer all of each supernatant to a separate, new, nuclease-free, 200 µl PCR tube. Store the tubes at -15° to -25°C.

The amplification of heavily expressed genes should be reduced in the normalized samples.

Validate Library (Optional)

Illumina recommends performing the following quality control analysis on your normalized sample library to quantify the DNA concentration.

Consumables

User-Supplied

- DNA-1000 chip
- GAPDH probes
- Nuclease-free water
- S18 Probes

Procedure

1. Measure the DNA concentration by running 1 µl of the sample on an Agilent Technologies 2100 Bioanalyzer using a DNA-1000 chip.
2. Dilute the DNA to 5 nM in nuclease-free water, then take 1 µl for the Taqman assay, with S18 and GAPDH probes. A ratio of GAPDH/S18 ≥1 indicates the efficient removal of rRNA.

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