Preparing Samples for Digital Gene Expression-Tag Profiling with *Dpn*II Using the Oligo Only Kit

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Topics

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Introduction

This protocol explains how to prepare libraries of mRNA for subsequent cDNA tag sequencing on the Illumina Cluster Station and Genome Analyzer. You will isolate mRNA and create 20 bp tags with adapter sequences ligated onto the ends of the cDNA fragment to generate the following template format:

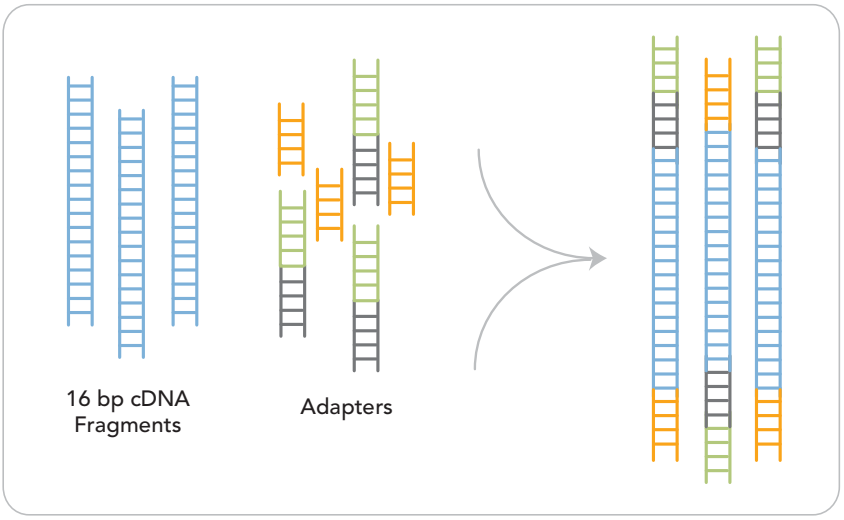

Figure 1 *Constructing Templates*

Adapter 1 introduces a correctly positioned *Mme*I restriction site and sequencing primer binding site, shown in gray. The Adapter 2 sequence corresponds to the surface-bound amplification primer on the flow cells used on the Cluster Station.

The method described in this protocol generates a unique 16 bp for each transcript, anchored with the restriction enzyme *Dpn*II. The 16 bp sequencing data combined with the known *Dpn*II restriction site data generates the unique 20 bp tag used for annotation. The sample prep protocol has been optimized for sequencing on the Illumina Genome Analyzer. The quantitative expression level of the unique transcripts is demonstrated by the number of times the sequence is detected.

An outline of the sample prep protocol is shown in Figure 2. You will need a minimum of three days to complete this protocol. The starting material for this protocol is 1–2 µg of total RNA. Use the method of your choice to isolate total RNA prior to using this kit. Please confirm the quality of your RNA as described on page 8. This is critical to sample preparation.

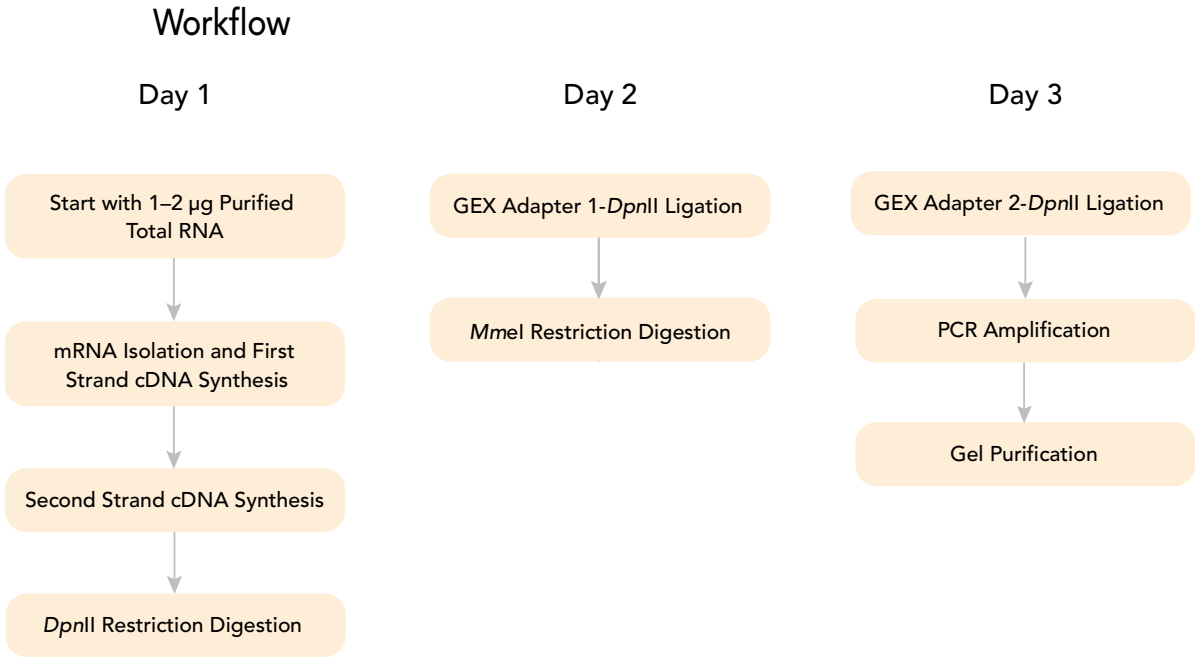

Figure 2 Sample Preparation Workflow

Kit Contents, User-Supplied Consumables, and Equipment Checklist

Check to ensure that you have all of the reagents and equipment identified in this section before proceeding to sample preparation.

Gene Expression *Dpn*II Tag Oligo Only Sample Prep Kit

Store at -20°C

This box is shipped at -80°C. As soon as you receive it, store the following components at -20°C.

- GEX *Dpn*II Adapter 1, 35 µl, 10 µM, part # 1000589
- GEX Adapter 2, 10 µl, 10 µM, part # 1000590
- Primer GX1, 10 µl, 25 µM, part # 1000591
- Primer GX2, 10 µl, 25 µM, part # 1000592

Briefly centrifuge all tubes before use, as the contents may have settled on the sides.

All reagents are supplied in excess to guarantee you have the quantity necessary to perform eight digital gene expression-tag profiling sample preparations. It is normal to have leftover reagents following the preparation of eight samples.

User-Supplied Consumables

The following user-supplied consumables can be made in-house or are available from vendors such as Invitrogen, New England Biolabs, Thermo Fisher Scientific, or Sigma:

- Ultra Pure Water
- Sera-Mag Magnetic oligo(dT) beads
- Binding Buffer (20 mM Tris-HCl pH 7.5, 1.0 M LiCL, 2 mM EDTA)
- Washing Buffer(10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA)
- 5X First Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 0.1 M DTT)
- 10 mM 5mC dNTP Mix
- RNase OUT (40 U/µl)
- SuperScript II Reverse Transcriptase (200 U/µl)
- 100 mM DTT
- Second Strand Buffer (500 mM Tris-HCl pH 7.8, 50 mM $MgCl_2$, 10 mM DTT)
- RNase H (2 U/µl)
- DNA Polymerase I (10 U/µl)
- Buffer C (1X PBS, 0.01% Tween 20)
- Cleaning Solution (1X PBS, 1 mM $CaCl_2$)
- Cleaning Solution Additive (10 mg/ml Pronase)
- Buffer D (1X TE, 0.01% Tween 20)

- 10X NEBuffer 4
- *Dpn*II Restriction Enzyme (10 U/µl)
- 10X *Dpn*II Buffer (10 U/µl)
- 5X T4 DNA Ligase Buffer
- T4 DNA Ligase (5 U/µl)
- 32 mM S-adenosylmethionine
- *Mme*I Restriction Enzyme (2 U/µl)
- CIAP (1 U/µl)
- Glycogen (20 µg/µl)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- 3 M NaOAc, pH 5.2
- -20C 100% Ethanol
- Room Temp 70% Ethanol
- 5X Phusion* HF Buffer (Finnzymes Oy)
- Phusion* DNA Polymerase (Finnzymes Oy) (2 U/µl)
- 25 mM dNTP mix
- 25 bp DNA ladder
- 10X NEBuffer 2
- 0.45 µm, 2 ml Spin-X Cellulose Acetate Filter
- Resuspension Buffer (10 mM Tris-HCl, pH 8.5)
- 6X DNA Loading Dye
- 6% Novex TBE PAGE Gel, 1.0 mm, 10 well
- 5X Novex TBE Buffer
- Ultra Pure Ethidium Bromide

Equipment Checklist

Check to ensure that you have all of the necessary user-supplied equipment before proceeding to sample preparation.

- Eppendorf thermomixers (part # 022670107 / 022670522)
  - 16°C Eppendorf thermomixer
  - 20°C Eppendorf thermomixer
  - 37°C Eppendorf thermomixer
  - 42°C Eppendorf thermomixer
  - 70°C Eppendorf thermomixer
- Benchtop microcentrifuge
- Clean scalpels
- Dark Reader transilluminator (Clare Chemical Research, part # D195M)
- Dynal MPC-S magnet (Invitrogen, part # 120-20D)
- Electrophoresis power supply
- Room temperature tube rotator
- Savant Speed Vac
- Thermal cycler

- Vortexer
- XCell Sure Lock Mini-Cell electrophoresis unit (Invitrogen, part # EI0001)
- 21-gauge needles
- Ice

Isolate mRNA and Synthesize First Strand cDNA

This protocol isolates mRNA from total RNA by binding the mRNA to a magnetic oligo(dT) bead. Using the mRNA attached to the bead as a template, oligo(dT) bound cDNA is synthesized to form a bead-bound mRNA/cDNA hybrid.

The starting material, total RNA, can be isolated by a number of techniques. Illumina recommends that you check total RNA integrity following isolation using an Agilent Technologies 2100 Bioanalyzer. Alternatively, a 1% agarose gel can be run and the integrity of RNA judged upon staining with ethidium bromide. High quality RNA will show a 28S rRNA band at 4.5 kb that should be twice the intensity of the 18S rRNA band at 1.9 kb. Both kb determinations are relative to a 1 kb ladder. The mRNA will appear as a smear from 0.5–12 kb.

Wear gloves and use sterile techniques when working with RNA. All plastic ware and reagents should be RNase-free.

Use 1.5 ml sterile, RNase-free, siliconized microtubes for all steps through *Mme*I digestion to prevent the magnetic beads from sticking to the tubes.

Consumables

User-Supplied

- Ultra pure water
- Sera-Mag Magnetic oligo(dT) beads
- Binding buffer
- Washing buffer
- 5X first strand buffer
- 10 mM 5mC-dNTP mix
- RNaseOUT
- Ice
- SuperScript II Reverse Transcriptase
- 100 mM DTT
- Purified total RNA (1–2 µg)

Procedure

Best Practice: Using the Magnetic Stand

Follow these guidelines throughout the sample preparation to prevent the beads from drying out.

Do not allow the beads to dry during the entire process. During all wash steps, add buffers to the tube containing the beads while the tube is on the magnetic stand.

1. Place the tube containing the beads on the magnetic stand for 1–2 minutes.
2. Add the buffer to the tube while the tube is on the magnetic stand.

Do not disturb the beads.

3. Cap the tube, remove it from the magnetic stand, and resuspend the beads. You can resuspend the beads by flicking the tube with your finger or use a vortexer set at 5–6.
4. Centrifuge briefly to collect any beads that may remain in the tube cap.
5. Repeat steps 1 through 4 as required.

Prepare the Bead Washing Buffers

- Dilute the 5X first strand buffer to 1X.

  Multiply each volume by the number of samples being prepared. Prepare 10% extra buffer if you are preparing multiple samples.

  - Ultra pure water (320 µl)
  - 5X first strand buffer (80 µl)

  The total volume should be 400 µl.

Prepare the Total RNA

1. Dilute 1–2 µg of total RNA with ultra pure water to 50 µl in a sterile RNase-free 200 µl microtube.
2. Heat the 50 µl of total RNA at 65°C in a thermal cycler for 5 minutes to disrupt any secondary structure.
3. Immediately place on ice.

Prepare the Magnetic Oligo(dT) Beads

1. While the RNA is denaturing, thoroughly resuspend the supplied oligo(dT) beads and transfer 50 µl to a 1.5 ml RNase-free, siliconized microtube.
2. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
3. Wash the beads by resuspending them in 100 µl of binding buffer.
4. Place the tube back on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
5. Wash with another 100 µl of binding buffer.
6. Place the tube back on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
7. Resuspend the beads in 50 µl of binding buffer.

Isolate the mRNA

1. Add 50 µl of diluted total RNA to the tube containing oligo(dT) beads in 50 µl of binding buffer.
2. Using the room temperature tube rotator, rotate the tube at room temperature for 5 minutes.
3. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
4. Wash beads by resuspending them in 200 µl of washing buffer.
5. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
6. Repeat steps 4 and 5 with another 200 µl of washing buffer.
7. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
8. Wash the beads by resuspending them in 100 µl of freshly prepared 1X first strand buffer.
9. Repeat steps 7 and 8 three additional times for a total of four washes in 100 µl of 1X first strand buffer.
10. Upon completion of the four washes you should have a tube of beads resuspended in 1X first strand buffer.

Synthesize the First Strand cDNA

1. Premix the following reagents in the order listed in a separate tube.

   Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.

   - Ultra pure water (29.5 µl)
   - 5X first strand buffer (10 µl)
   - 100 mM DTT(5 µl)
   - 10 mM 5mC-dNTP mix (2.5 µl)
   - RNaseOUT (1 µl)

   The total volume should be 48 µl.
2. Place the tube of beads resuspended in 1X first strand buffer on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
3. Resuspend the beads in 48 µl of the first strand cDNA synthesis premix outlined in step 1. Mix well.
4. Heat the bead/premix tube at 42°C in a thermal cycler for 2 minutes.
5. Add 2 µl of SuperScript II Reverse Transcriptase.
6. Incubate at 42°C in a thermomixer that is constantly mixing at 1400 rpm for 1 hour.
7. Transfer the tube to a 70°C thermomixer that is programmed to mix at 1400 rpm for 15 seconds and then standing for 2 minutes, for a total of 15 minutes. Place the tube on ice.

Synthesize the Second Strand cDNA

This protocol removes the strand of mRNA and synthesizes a replacement strand generating double-stranded cDNA bound to the oligo(dT) bead.

Consumables

User-Supplied

- Ultra pure water
- Second strand buffer
- 10 mM 5mC-dNTP mix
- RNase H
- DNA Polymerase I
- Buffer C
- Cleaning solution
- Cleaning solution additive
- Buffer D
- 10X *Dpn*II buffer
- mRNA/cDNA hybrid (50 µl)
- Ice

Procedure

Prepare the Bead Washing Reagents

- Dilute the 10X *Dpn*II buffer to 1X.

  Multiply each volume by the number of samples being prepared. Prepare 10% extra buffer if you are preparing multiple samples.
  - Ultra pure water (180 µl)
  - 10X *Dpn*II buffer (20 µl)

  The total volume should be 200 µl.

Prepare Fresh Working Cleaning Solution

- Add cleaning solution additive to the cleaning solution to create fresh working cleaning solution.

  Multiply each volume by the number of samples being prepared. Prepare 10% extra buffer if you are preparing multiple samples.
  - Cleaning solution (98.6 µl)
  - Cleaning solution additive (1.4 µl)

  The total volume should be 100 µl.

Synthesize the Second Strand

1. Add 31 µl of ultra pure water to the 50 µl of mRNA/cDNA hybrid mix on ice.
2. Add the following reagents:
   - Second strand buffer (10 µl)
   - 10 mM 5mC-dNTP mix (3 µl)

3. Mix well and incubate on ice for 5 minutes.
4. Add the following reagents:
   - DNA Polymerase I (5 µl)
   - RNase H (1 µl)
5. Mix well and incubate at 16°C in a thermomixer, programmed to mix at 1400 rpm for 15 seconds and stand for 2 minutes, for a total of 2.5 hours.
6. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
7. Wash the beads by resuspending them in 750 µl of buffer C.
8. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
9. Resuspend the beads in 100 µl of fresh working cleaning solution.
10. Incubate at 37°C in a thermomixer, programmed to mix at 1400 rpm for 15 seconds and stand for two minutes, for a total of 15 minutes.
11. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
12. Resuspend the beads in 750 µl of buffer D.
13. Repeat steps 11 and 12 three additional times for a total of four washes in 750 µl of buffer D.
14. Upon completion of the four washes you should be left with a tube of beads resuspended in 750 µl of buffer D.
15. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
16. Resuspend the beads in 100 µl of 1X *Dpn*II buffer.
17. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
18. Resuspend the beads in 100 µl of 1X *Dpn*II buffer.
19. Transfer the bead and 1X *Dpn*II buffer solution to a sterile, RNase-free, siliconized 1.5 ml microtube.

Restriction Digest with *Dpn*II

This protocol cleaves the double stranded cDNA at every *Dpn*II site. All fragments other than the 3' fragment attached to the oligo(dT) bead are washed away.

Consumables

User-Supplied

- Ultra pure water
- 10X *Dpn*II buffer
- *Dpn*II
- Buffer C
- Cleaning solution
- Cleaning solution additive
- Buffer D
- Bead-attached cDNA resuspended in 100 µl of 1X *Dpn*II buffer

Procedure

Prepare the *Dpn*II Digestion Pre-Mix

- Premix the reagents in the following order in a separate tube.

  Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent if you are preparing multiple samples.
  - Ultra pure water (89 µl)
  - 10X *Dpn*II buffer (10 µl)

  The total volume should be 99 µl.

Prepare Fresh Working Cleaning Solution

- Add cleaning solution additive to the cleaning solution to create fresh working cleaning solution.

  Multiply each volume by the number of samples being prepared. Prepare 10% extra buffer if you are preparing multiple samples.
  - Cleaning solution (98.6 µl)
  - Cleaning solution additive (1.4 µl)

  The total volume should be 100 µl.

Set Up the *Dpn*II Digestion Mix

1. Place the tube of cDNA-attached beads resuspended in 1X *Dpn*II buffer on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
2. Resuspend the beads in 99 µl of the *Dpn*II digestion pre-mix.
3. Add 1 µl of *Dpn*II enzyme.
4. Incubate at 37°C in a thermomixer, programmed to mix at 1400 rpm for 15 seconds and stand for 2 minutes, for a total of 1 hour.

5. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
6. Wash the beads by resuspending them in 750 µl of buffer C.
7. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
8. Resuspend the beads in 100 µl of fresh working cleaning solution.
9. Incubate at 37°C in a thermomixer, programmed to mix at 1400 rpm for 15 seconds and stand for 2 minutes, for a total of 15 minutes.
10. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
11. Resuspend the beads in 750 µl of buffer D.
12. Repeat steps 10 and 11 three additional times for a total of four washes in 750 µl of buffer D.
13. Upon completion of the four washes you should be left with a tube of beads resuspended in 750 µl of buffer D.
14. Store the resuspended beads overnight at 4°C.

Ligate GEX *Dpn*II Adapter 1

This protocol ligates a defined gene expression adapter (GEX *Dpn*II Adapter 1) at the site of *Dpn*II cleavage. In addition, GEX *Dpn*II Adapter 1 contains the sequence for the restriction enzyme *Mme*I, which is necessary for future steps in sample preparation.

Consumables

Illumina-Supplied

- GEX *Dpn*II Adapter 1

User-Supplied

- Ultra pure water
- 5X T4 DNA ligase buffer
- T4 DNA ligase
- Buffer C
- Cleaning solution
- Cleaning solution additive
- Buffer D
- 10X NEBuffer 4
- Bead-attached cDNA resuspended in 100 µl of buffer D

Procedure

Prepare the Bead Washing Reagents

1. Dilute the 5X T4 DNA ligase buffer to 1X.

   Multiply each volume by the number of samples being prepared. Prepare 10% extra buffer if you are preparing multiple samples.

   - Ultra pure water (160 µl)
   - 5X T4 DNA ligase buffer (40 µl)

   The total volume should be 200 µl.

2. Dilute the 10X NEBuffer 4 to 1X.

   Multiply each volume by the number of samples being prepared. Prepare 10% extra buffer if you are preparing multiple samples.

   - Ultra pure water (180 µl)
   - 10X NEBuffer 4 (20 µl)

   The total volume should be 200 µl.

Prepare Fresh Working Cleaning Solution

- Add cleaning solution additive to the cleaning solution to create fresh working cleaning solution.

  Multiply each volume by the number of samples being prepared. Prepare 10% extra buffer if you are preparing multiple samples.

  - Cleaning solution (98.6 µl)
  - Cleaning solution additive (1.4 µl)

  The total volume should be 100 µl.

Ligate GEX *Dpn*II Adapter 1

This protocol involves the use of both the 1X T4 DNA ligase buffer and 5X T4 DNA ligase buffer. Follow the instructions carefully to ensure you use the correct solution in each step.

1. Place the tube containing the bead-attached cDNA resuspended in buffer D on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
2. Resuspend the beads in 100 µl of 1X T4 DNA ligase buffer.
3. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
4. Resuspend the beads in 100 µl of 1X T4 DNA ligase buffer.
5. Transfer the resuspended beads to a fresh microtube.
6. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
7. Add the following in the indicated order to each tube of beads.
   - Ultra pure water (36 µl)
   - GEX *Dpn*II Adapter 1 (3 µl)
   - 5X T4 DNA ligase buffer (10 µl)
   - T4 DNA ligase (1 µl)

   The total volume should be 50 µl.
8. Incubate at 20°C in a thermomixer that is constantly mixing at 1400 rpm for 2 hours.
9. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
10. Wash the beads by resuspending them in 750 µl of buffer C.
11. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
12. Resuspend the beads in 100 µl of fresh working cleaning solution.
13. Incubate at 37°C in a thermomixer, programmed to mix at 1400 rpm for 15 seconds and stand for 2 minutes, for a total of 15 minutes.
14. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
15. Resuspend the beads in 750 µl of buffer D.
16. Repeat steps 13 and 14 three additional times for a total of four washes in 750 µl of buffer D.
17. Upon completion of the four washes you should have a tube of beads resuspended in 750 µl of buffer D.
18. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.

19. Resuspend the beads in 100 µl of 1X NEBuffer 4.
20. Place the tube on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
21. Resuspend the beads in 100 µl of 1X NEBuffer 4.
22. Transfer the bead and 1X NEBuffer 4 solution to a fresh, sterile, RNase-free, siliconized 1.5 ml tube.

Restriction Digest with *Mme*I

This protocol applies the restriction enzyme *Mme*I to create the 16 bp tag. The binding site for the enzyme is at the GEX *Dpn*II Adapter I cDNA junction. The enzyme cuts downstream from the binding site. The resulting construct is no longer attached to the oligo(dT) bead and is free in solution.

Consumables

User-Supplied

- Ultra pure water
- 10X NEBuffer 4
- 32 mM S-adenosylmethionine
- *Mme*I
- CIAP
- Glycogen
- Bead-attached cDNA resuspended in 1X NEBuffer 4
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- 3M NaOAc, pH 5.2
- -20°C 100% ethanol
- Room temperature 70% ethanol

Procedure

Prepare the 10X S-adenosylmethionine

1. Dilute the 32 mM S-adenosylmethionine to working 10X concentration.
   - Ultra pure water (320 µl)
   - 32 mM S-adenosylmethionine (5 µl)

   The total volume should be 325 µl.

Prepare the Restriction Digest Reagents

1. Premix the reagent in the following order in a separate tube.

   Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent if you are preparing multiple samples.
   - Ultra pure water (76 µl)
   - 10X NEBuffer 4 (10 µl)
   - 10X S-adenosylmethionine (10 µl)

- *Mme*I (4 µl)

The total volume should be 100 µl.

Restriction Digestion with *Mme*I

1. Place the tube of bead-attached cDNA resuspended in the 1X NEBuffer 4 on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and discard it.
2. Resuspend the beads in 100 µl of the *Mme*I restriction digest pre-mix.
3. Incubate at 37°C in a thermomixer that is constantly mixing at 1400 rpm for 1.5 hours.
4. Place the tube of *Mme*I-digested cDNA and beads on the magnetic stand for 1–2 minutes. Carefully pipette off the supernatant and transfer it to a sterile, RNase-free, siliconized 1.5 ml microtube.

The construct is now in the supernatant. Retain the supernatant.

5. Discard the tube containing the beads.
6. Add 2 µl of CIAP to the retained supernatant.
7. Dephosphorylate for 1 hour at 37°C.
8. Extract once with 100 µl phenol/chloroform/isoamyl alcohol (25:24:1).
9. Extract once with 100 µl chloroform/isoamyl alcohol (24:1).
10. Add 1 µl of glycogen, 10 µl 3M NaOAc, and 325 µl of -20°C 100% ethanol.
11. Immediately centrifuge to 14K for 20 minutes.
12. Remove the supernatant and discard it.
13. Wash the pellet with 500 µl of room temperature 70% ethanol.
14. Remove the supernatant and discard it.
15. Dry the pellet using the speed vac.
16. Resuspend the pellet in 6 µl of ultra pure water.
17. Store overnight at -20°C.

Ligate GEX Adapter 2

This protocol ligates a defined gene expression adapter (GEX Adapter 2) at the site of *Mme*I cleavage. The GEX Adapter 2 contains sequences complementary to the oligos attached to the flow cell surface.

Consumables

Illumina-Supplied

- GEX Adapter 2

User-Supplied

- Ultra pure water
- 5X T4 DNA ligase buffer
- T4 DNA ligase
- cDNA construct resuspended in ultra pure water (6 µl)

Procedure

Ligate GEX Adapter 2

1. To each tube of 6 µl of *Mme*I-digested and resuspended cDNA, add the following in the order listed:
   - GEX Adapter 2 (1 µl)
   - 5X T4 DNA ligase buffer (2 µl)
   - T4 DNA ligase (1 µl)

   The total volume should be 10 µl.
2. Incubate at 20°C for 2 hours in the thermomixer.

Enrich the Adapter-Ligated cDNA Construct Using PCR

This protocol uses PCR to selectively enrich the DNA library with cDNA fragments that have adapter molecules on both ends. The PCR is performed with two primers that anneal to the ends of the adapters.

Consumables

Illumina-Supplied

- Primer GX1
- Primer GX2

User-Supplied

- Ultra pure water
- 5X Phusion* HF buffer (Finnzymes Oy)
- Phusion* DNA polymerase (Finnzymes Oy)
- 25 mM dNTP mix
- GEX Adapter 2 ligated cDNA (10 µl)

Procedure

Prepare the PCR Master Mix

- Premix the reagents in the following order in a separate tube:

  Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent if you are preparing multiple samples.

  - Ultra pure water (35.5 µl)
  - 5X Phusion* HF buffer (10 µl)
  - Primer GX1 (0.5 µl)
  - Primer GX2 (0.5 µl)
  - 25 mM dNTP mix (0.5 µl)
  - Phusion* DNA polymerase (0.5 µl)

  The total volume should be 47.5 µl.

PCR Amplification

1. Aliquot 47.5 µl of PCR master mix into a sterile, nuclease-free, 200 µl PCR tube.
2. Add 2.5 µl of GEX Adapter 2 ligated cDNA.
3. Amplify the PCR in the thermal cycler using the following protocol:
   - **a.** 30 seconds at 98°C
   - **b.** 15 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 the Amplified cDNA Construct

This protocol gel purifies the amplified cDNA construct in preparation for loading on the Illumina Cluster Station.

Consumables

User-Supplied

- Ultra pure water
- 25 bp ladder
- 10X NEBuffer 2
- Spin-X cellulose acetate filter
- Glycogen
- Resuspension buffer
- 6X DNA loading dye
- Amplified cDNA construct (50 µl)
- 6% Novex TBE PAGE gel, 1.0 mm, 10 well
- 5X Novex TBE buffer
- Ultra pure ethidium bromide
- 3 M NaOAc, pH 5.2
- -20°C 100% ethanol
- 70% ethanol (room temperature)

Procedure

It is important to follow this procedure exactly to ensure reproducibility. Illumina does not recommend purifying multiple samples on a single gel due to the risk of cross-contamination between libraries.

Prepare the Gel Electrophoresis Reagents and Apparatus

1. Determine the volume of 1X TBE buffer needed.
2. Dilute the 5X TBE buffer to 1X with Milli-Q water for use in electrophoresis.
3. Assemble the gel electrophoresis apparatus per the manufacturer's instructions.

Run the Gel Electrophoresis

1. Mix 5 µl of 25 bp ladder with 1 µl of 6X DNA loading dye.
2. Mix 50 µl of amplified cDNA construct with 10 µl of 6X DNA loading dye.
3. Load 5 µl of the 25 bp ladder and loading dye mix into one well of the 6% TBE PAGE gel.
4. Load 25 µl each of the PCR amplified construct and loading dye mix into two wells of the 6% TBE PAGE gel.

5. Run the gel for 30–35 minutes at 200 V.
6. Remove the gel from the apparatus.

Dilute the 10X NEBuffer 2

- Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.
  - Ultra pure water (90 µl)
  - 10X NEBuffer 2 (10 µl)

  The total volume should be 100 µl.

Recover the Purified Construct

1. Puncture the bottom of a sterile, nuclease-free, 0.5 ml microtube 4–5 times with a 21-gauge needle.
2. Place the 0.5 ml microtube into a sterile, round-bottom, nuclease-free, 2 ml microtube.
3. Pry apart the cassette and stain the gel with the ethidium bromide in a clean container for 2–3 minutes.
4. View the gel on a Dark Reader transilluminator, which is a safer alternative to a UV transilluminator.
5. Using a clean scalpel, cut out the 85 bp bands in the sample lanes.
6. Place the gel slice into the 0.5 ml microtube.
7. Centrifuge the stacked tubes at full speed for 2 minutes at room temperature to move the gel through the holes into the 2 ml tube.
8. Add 100 µl of 1X NEBuffer 2 to the gel debris in the 2 ml tube.
9. Elute the DNA by rotating the tube gently at room temperature for 2 hours.
10. Transfer the eluate and the gel debris to the top of a Spin-X filter.
11. Centrifuge the filter for 2 minutes at full speed.
12. Add 1 µl of glycogen, 10 µl of 3M NaOAc, and 325 µl of -20°C ethanol.
13. Immediately centrifuge to 14K for 20 minutes.
14. Remove and discard the supernatant, leaving the pellet intact.
15. Wash the pellet with 500 µl of room temperature 70% ethanol.
16. Remove and discard the supernatant, leaving the pellet intact.
17. Dry the pellet using the speed vac.
18. Resuspend the pellet in 10 µl resuspension buffer.

Validate the Library

Illumina recommends performing the following quality control analysis on your cDNA tag library.

Bioanalyzer Method

1. Load 1 µl of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer.
2. Check the size, purity, and concentration of the sample.

Manual Method

If a Bioanalyzer is not available, perform the following steps.

1. Determine the concentration of the library by measuring its absorbance at 260 nm. The yield from the protocol should be between 500 and 1000 ng of DNA.
2. Measure the 260/280 ratio. It should be approximately 1.8.
3. Load 10% of the volume of the library on a gel and check that the size range is as expected. It is similar in size to the size-range excised during the gel purification step (~85 bp).
4. Multiply this size by 650 (the molecular mass of a base pair) to get the molecular weight of the library. Use this number to calculate the molar concentration of the library.

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