

Preparing Samples for Analysis of Small RNA

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Introduction

This protocol explains how to prepare libraries of small RNA for subsequent cDNA sequencing on the Illumina Cluster Station and Genome Analyzer.

You will physically isolate small RNA, ligate the adapters necessary for use during cluster creation, and reverse-transcribe and PCR to generate the following template format:

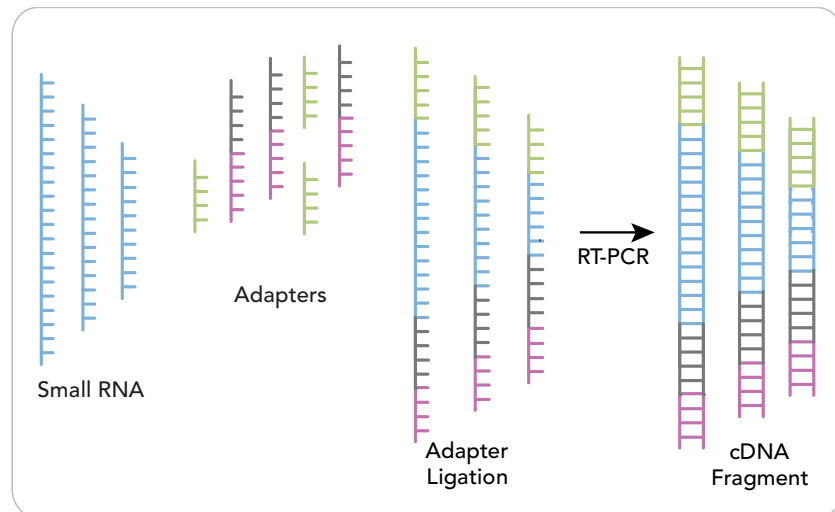


Figure 1 Fragments after Sample Preparation

The 5' small RNA adapter is necessary for reverse transcription and amplification of the small RNA fragment. This adapter also contains the DNA sequencing primer binding site. The 3' small RNA adapter corresponds to the surface bound amplification primer on the flow cell used on the Cluster Station.

Workflow

You will need a minimum of 4 days to complete this protocol.

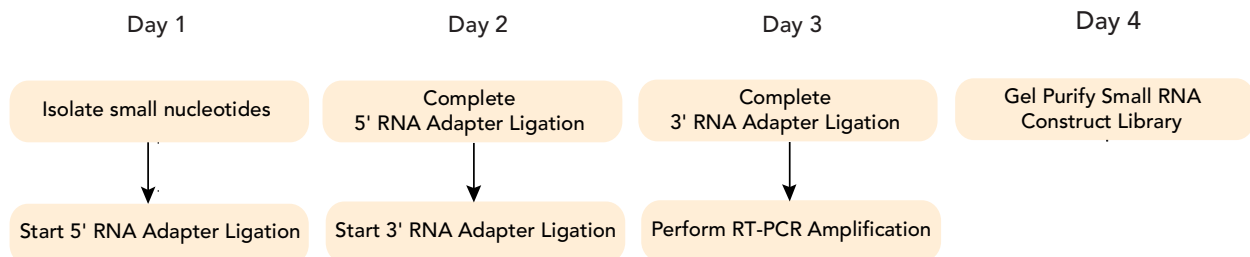


Figure 2 Sample Preparation Workflow

Kit Contents and Equipment Checklist

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

Small RNA Sample Prep Kit, Box 1

Store at -20°C

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

illumina Part #	Description
1000467	Ultra Pure Water (store at 4°C)
1000560	RNase OUT
1000571	10X Gel Elution Buffer
1000573	SRA 0.3 M NaCl
1000584	Phusion* Polymerase (Finnzymes Oy)
1000585	5X Phusion* HF Buffer (Finnzymes Oy)
1000587	T4 RNA Ligase
1000588	10X T4 RNA Ligase Buffer
1000591	Primer GX1
1000592	Primer GX2
1000597	SRA RT Primer
1001338	Resuspension Buffer
1001661	SRA Gel Loading Dye
1001662	25 bp Ladder
1001663	25 mM dNTP Mix
1001664	Glycogen
1001665	SRA Ladder
1003029	6X DNA Loading Dye

Small RNA Sample Prep Kit, Bag 1

Store at -80°C

These reagents are shipped at -80°C. As soon as you receive it, store the following components at -80°C.

ILLUMINA PART #	DESCRIPTION
1000595	SRA 5' Adapter
1000596	SRA 3' Adapter

Small RNA Sample Prep Kit, Box 2

Store at Room Temperature

These components are shipped at room temperature.

ILLUMINA PART #	DESCRIPTION
1001673	Spin X Cellulose Acetate Filter



NOTE

Briefly centrifuge the 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 small RNA sample preparations. It is normal to have leftover reagents following the preparation of eight samples.

Equipment Checklist

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

- ▶ 4°C microcentrifuge
- ▶ Benchtop microcentrifuge
- ▶ Dark Reader transilluminator (Clare Chemical Research, Part # D195M)
- ▶ Electrophoresis power supply
- ▶ Room temperature tube rotator
- ▶ Savant Speed Vac
- ▶ Thermal cycler
- ▶ XCell Sure Lock Mini-Cell electrophoresis unit (Invitrogen, Part # EI0001)

Isolate Small RNA by Denaturing PAGE Gel

This protocol purifies small RNA from total RNA by separating them based on nucleotide length and removing a band from the denaturing gel that corresponds to the nucleotide length of interest. This protocol discusses methods for studying small RNA in the range of 18–30 nucleotides. RNA of other lengths can be queried by altering the size range of the nucleotides isolated in this initial step.

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.

Consumables **Illumina-Supplied**

- ▶ Ultra pure water, thawed and stored at 4°C
- ▶ SRA gel loading dye
- ▶ SRA ladder
- ▶ SRA 0.3 M NaCl
- ▶ Spin X cellulose acetate filters
- ▶ Glycogen

User-Supplied

- ▶ 15% Novex TBE-urea PAGE gel, 1.0 mm, 10 well
- ▶ 5X Novex TBE buffer
- ▶ Ultra pure ethidium bromide 10 mg/ml
- ▶ Room temperature 100% ethanol
- ▶ Room temperature 75% ethanol
- ▶ Purified total RNA (10 µg) in 10 µl volume
- ▶ Clean scalpels
- ▶ 21-gauge needles

Procedure **Prepare Gel Electrophoresis Reagents and Apparatus**

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 of libraries. This holds true for every gel purification step in this protocol.

1. Determine the volume of 1X TBE buffer needed. Dilute the 5X TBE buffer to 1X for use in electrophoresis.



Use nuclease free water for all electrophoresis buffer dilutions.

2. Assemble the gel electrophoresis apparatus per the manufacturer's instructions.
3. Pre-run the 15% TBE-urea gel for 15–30 minutes at 200 V and wash the wells using 1X TBE.

Sample Electrophoresis

1. While the gel is pre-running, mix 2 μ l of SRA ladder with 2 μ l of SRA gel loading dye in a sterile, RNase-free, 200 μ l microfuge tube.
2. Mix 10 μ l (10 μ g) of total RNA with 10 μ l of SRA gel loading dye in a sterile, RNase-free 200 μ l microfuge tube.
3. Heat the sample and ladder tubes at 65°C for 5 minutes in a thermal cycler.
4. Centrifuge the heated tubes to collect the entire column of the tube.
5. Load both the entire SRA ladder and sample RNA on the same gel with several lanes between them.
6. Run the gel at 200 V for 1 hour.
7. Remove the gel from the apparatus.

Recover the Isolated RNA

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 TBE/ethidium bromide in a clean container for 2 minutes.
4. View the gel on a Dark Reader transilluminator to avoid being exposed to UV light. The SRA ladder is in 20 bp steps up to 100 bp.



Prolonged personnel exposure to UV light can damage your DNA.

5. Using a clean scalpel, cut out a band of gel corresponding to the 18–30 nucleotide bands in the marker lane.
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 300 μ l of SRA 0.3 M NaCl to the gel debris in the 2 ml tube and elute the DNA by rotating the tube gently at room temperature for 4 hours.
9. Transfer the eluate and the gel debris to the top of a Spin X cellulose acetate filter. Centrifuge the filter in the microfuge for 2 minutes at full speed.
10. Add 3 μ l of glycogen and 750 μ l of room temperature 100% ethanol.
11. Incubate at -80°C for 30 minutes.
12. Immediately centrifuge to 14K for 25 minutes on a 4°C microcentrifuge.
13. Remove the supernatant and discard it.
14. Wash the pellet with 750 μ l of room temperature 75% ethanol.
15. Remove the supernatant and discard it.
16. Allow the RNA pellet to air dry.
17. Resuspend the RNA pellet in 5.7 μ l of ultra pure water.

Ligate 5' RNA Adapters

This protocol ligates adapters to the 5' end of the isolated small RNA.

Consumables

Illumina-Supplied

- ▶ Ultra pure water
- ▶ SRA 5' adapter
- ▶ 10X T4 RNA ligase buffer
- ▶ T4 RNA ligase
- ▶ RNase OUT
- ▶ SRA gel loading dye
- ▶ SRA ladder
- ▶ SRA 0.3 M NaCl
- ▶ Glycogen

User-Supplied

- ▶ Isolated small RNA
- ▶ 15% Novex TBE-urea PAGE gel, 1.0 mm, 10 well
- ▶ 5X Novex TBE buffer
- ▶ Ultra pure ethidium bromide 10 mg/ml
- ▶ Room temperature 100% ethanol
- ▶ Room temperature 75% ethanol
- ▶ Clean scalpels
- ▶ 21-gauge needles

Procedure

Ligate the Adapter

1. Add the following in the indicated order to each tube of PAGE gel isolated small RNA. Starting volume of small RNA is 5.7 μ l.
 - SRA 5' adapter (1.3 μ l)
 - 10X T4 RNA ligase buffer (1 μ l)
 - RNase OUT (1 μ l)
 - T4 RNA ligase (1 μ l)The total volume should be 10 μ l.
2. Incubate at 20°C for 6 hours in a thermal cycler and hold overnight at 4°C.
3. Stop the reaction by adding 10 μ l of SRA gel loading dye.

Prepare Gel Electrophoresis Reagents and Apparatus

1. Determine the volume of 1X TBE buffer needed. Dilute the 5X TBE buffer to 1X for use in electrophoresis.
2. Assemble the gel electrophoresis apparatus per the manufacturer's instructions.
3. Pre-run the 15% TBE-urea gel for 15–30 minutes at 200 V.
4. Wash the wells using 1X TBE.

Sample Electrophoresis

1. While the gel is pre-running, mix 2 μ l of SRA ladder with 2 μ l of SRA gel loading dye in a sterile, RNase-free, 0.5 μ l microfuge tube.
2. Heat the ligated sample and ladder tubes at 65°C for 5 minutes in a thermal cycler just before loading on the gel.
3. Centrifuge the heated tubes to collect the entire column of the tube.
4. Load both the entire SRA ladder and sample RNA on the same gel with several lanes between them.
5. Run the gel at 200 V for 1 hour.
6. Remove the gel from the apparatus.

Recover the Isolated RNA

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 TBE/ethidium bromide in a clean container for 2 minutes.
4. View the gel on a Dark Reader transilluminator to avoid being exposed to UV light. The SRA ladder is in 20 bp steps up to 100 bp.



Prolonged personnel exposure to UV light can damage your DNA.

5. Using a clean scalpel, cut out a band of gel corresponding to the 40–60 nucleotide bands in the marker lane.
6. Place the band 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 300 μ l of SRA 0.3 M NaCl to the gel debris in the 2 ml tube.
9. Elute the DNA by rotating the tube gently at room temperature for 4 hours.

10. Transfer the eluate and the gel debris to the top of a Spin X cellulose acetate filter. Centrifuge the filter in the microfuge for 2 minutes at full speed.
11. Add 3 μ l of glycogen and 750 μ l of room temperature 100% ethanol.
12. Incubate at -80°C for 30 minutes.
13. Immediately centrifuge to 14K for 25 minutes on a 4°C microcentrifuge.
14. Remove the supernatant and discard it.
15. Wash the pellet with 750 μ l of room temperature 75% ethanol.
16. Remove the supernatant and discard it.
17. Allow the RNA pellet to air dry.
18. Resuspend the RNA pellet in 6.4 μ l of ultra pure water.

Ligate 3' RNA Adapters

This protocol ligates a defined RNA adapter to the 3' end of the isolated small RNA.

Consumables

Illustrina-Supplied

- ▶ Ultra pure water
- ▶ SRA 3' adapter
- ▶ 10X T4 RNA ligase buffer
- ▶ T4 RNA ligase
- ▶ RNase OUT
- ▶ SRA gel loading dye
- ▶ SRA ladder
- ▶ SRA 0.3 M NaCl
- ▶ Glycogen

User-Supplied

- ▶ 5' adapter-ligated small RNA in 6.4 μ l ultra pure water
- ▶ 10% Novex TBE-urea PAGE gel, 1.0 mm, 10 well
- ▶ 5X Novex TBE buffer
- ▶ Ultra pure ethidium bromide 10 mg/ml
- ▶ Room temperature 100% ethanol
- ▶ Room temperature 75% ethanol
- ▶ Clean scalpels
- ▶ 21-gauge needles

Procedure

Ligate the Adapter

1. Add the following in the indicated order to each tube of 5' RNA adapter-ligated small RNA. Starting volume of small RNA is 6.4 μ l.
 - SRA 3' adapter (0.6 μ l)
 - 10X T4 RNA ligase buffer (1 μ l)
 - RNase OUT (1 μ l)
 - T4 RNA ligase (1 μ l)The total volume should be 10 μ l.
2. Incubate at 20°C for 6 hours in a thermal cycler and hold overnight at 4°C.
3. Stop the reaction by adding 10 μ l of SRA gel loading dye.

Prepare Gel Electrophoresis Reagents and Apparatus

1. Determine the volume of 1X TBE buffer needed. Dilute the 5X TBE buffer to 1X for use in electrophoresis.
2. Assemble the gel electrophoresis apparatus per the manufacturer's instructions.
3. Pre-run the 10% TBE-urea gel for 15–30 minutes at 200 V.
4. Wash the wells using 1X TBE.

Sample Electrophoresis

1. While the gel is pre-running, mix 2 μ l of SRA ladder with 2 μ l of SRA gel loading dye in a sterile, RNase-free, 0.5 μ l microfuge tube.
2. Heat the ligated sample and ladder tubes at 65°C for 5 minutes in a thermal cycler just before loading on the gel.
3. Centrifuge the heated tubes to collect the entire column of the tube.
4. Load both the entire SRA ladder and sample RNA on the same gel with several lanes between them.
5. Run the gel at 200 V for 1 hour.
6. Remove the gel from the apparatus.

Recover the Isolated RNA

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 TBE/ethidium bromide in a clean container for 2 minutes.
4. View the gel on a Dark Reader transilluminator to avoid being exposed to UV light. The SRA ladder is in 20 bp steps up to 100 bp.



NOTE

Prolonged personnel exposure to UV light can damage your DNA.

5. Using a clean scalpel, cut out a band of gel corresponding to the 70–90 nucleotide bands in the marker lane.
6. Place the band 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 300 μ l of SRA 0.3 M NaCl to the gel debris in the 2 ml tube.
9. Elute the DNA by rotating the tube gently at room temperature for 4 hours.

10. Transfer the eluate and the gel debris to the top of a Spin X cellulose acetate filter. Centrifuge the filter in the microfuge for 2 minutes at full speed.
11. Add 3 μ l of glycogen and 750 μ l of room temperature 100% ethanol.
12. Incubate at -80°C for 30 minutes.
13. Immediately centrifuge to 14K for 25 minutes on a 4°C microcentrifuge.
14. Remove the supernatant and discard it.
15. Wash the pellet with 750 μ l of room temperature 75% ethanol.
16. Remove the supernatant and discard it.
17. Allow the RNA pellet to air dry.
18. Resuspend the RNA pellet in 6.4 μ l of ultra pure water.

Reverse Transcribe and Amplify the Small RNA Ligated with Adapters

Reverse transcription followed by PCR is used to create cDNA constructs based on the small RNA ligated with 5' and 3' adapters. This protocol selectively enriches those RNA 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

- ▶ Ultra pure water
- ▶ SRA RT primer
- ▶ RNase OUT
- ▶ 5X Phusion* HF Buffer (Finnzymes Oy)
- ▶ Phusion* DNA Polymerase (Finnzymes Oy)
- ▶ Primer GX1
- ▶ Primer GX2
- ▶ 25 mM dNTP mix

User-Supplied

- ▶ 5' and 3' adapter-ligated RNA (4.5 μ l)
- ▶ SuperScript II Reverse Transcriptase with 100 mM DTT and 5X first strand buffer (Invitrogen, part # 18064-014)

Procedure

Template Preparation

1. Combine the following in a sterile, RNase-free, 0.5 μ l microtube:
 - Purified 5' and 3' ligated RNA (4.5 μ l)
 - SRA RT primer (0.5 μ l)
 The total volume should be 5 μ l.
2. Heat the mixture at 65°C in a thermal cycler for 10 minutes.
3. Place the tube on ice.

Dilute the 25 mM dNTP Mix

1. Premix the following reagents in a separate, sterile, RNase-free, 0.2 μ l microtube. Multiply each volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.
 - Ultra pure water (0.5 μ l)
 - 25 mM dNTP mix (0.5 μ l)
 The total volume should be 1 μ l.
2. Label the tube "12.5 mM dNTP Mix."

Reverse Transcription

1. Add the following reagents in the order listed to the cooled tube:

- 5X first strand buffer (2 μ l)
- 12.5 mM dNTP mix (0.5 μ l)
- 100 mM DTT (1 μ l)
- RNase OUT (0.5 μ l)

The total volume should now be 9 μ l (5 μ l of template preparation and 4 μ l of reverse transcription).

2. Heat the sample to 48°C in a thermal cycler for 3 minutes.
3. Add 1 μ l SuperScript II Reverse Transcriptase.
4. Incubate in a thermal cycler at 44°C for 1 hour.

Prepare the PCR Master Mix

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

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

- Ultra pure water (28 μ l)
- 5X Phusion* HF buffer (10 μ l)
- Primer GX1 (0.5 μ l)
- Primer GX2 (0.5 μ l)
- 25 mM dNTP mix (0.5 μ l)
- Phusion* DNA Polymerase (0.5 μ l)

The total volume should be 40 μ l.

PCR Amplification

1. Add 40 μ l of PCR master mix into a sterile, nuclease-free 0.2 ml PCR tube.
2. Add 10 μ l of single strand reverse-transcribed cDNA.
3. Amplify the PCR in the thermal cycler using the following PCR 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 **Illumina-Supplied**

- ▶ Ultra pure water
- ▶ 25 bp ladder
- ▶ 10X gel elution buffer
- ▶ Spin-X cellulose acetate filter
- ▶ Glycogen
- ▶ 1X resuspension buffer
- ▶ 6X DNA loading dye

User-Supplied

- ▶ Amplified cDNA construct (50 μ l)
- ▶ 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
- ▶ Room temperature 70% ethanol
- ▶ Clean scalpels
- ▶ 21-gauge needles

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. Dilute the 5X TBE buffer to 1X for use in electrophoresis.
2. Assemble the gel electrophoresis apparatus per the manufacturer's instructions.

Run the Gel Electrophoresis

1. Mix 5 μ l of 25 bp ladder with 1 μ l of 6X DNA loading dye.
2. Mix 50 μ l of amplified cDNA construct with 10 μ l of 6X DNA loading dye.
3. Load 5 μ l of 25 bp ladder and loading dye mix in one well on the 6% PAGE gel.
4. Load two wells with 25 μ l each of amplified construct and loading dye mix on 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 Gel Elution Buffer

- ▶ Dilute the 10X gel elution buffer into a fresh 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 (90 μ l)
- 10X gel elution buffer (10 μ l)

The total volume should be 100 μ l.

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 to avoid being exposed to UV light. The 25 bp ladder is in 25 bp steps up to 300 bp.



Prolonged personnel exposure to UV light can damage your DNA.

5. Using a clean scalpel, cut out approximately a 92 bp band in the sample lanes.
6. Place the band 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 μ l of 1X gel elution buffer 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 μ l of glycogen, 10 μ l of 3M NaOAc, and 325 μ l of -20°C 100% ethanol.
13. Immediately centrifuge to 14K for 20 minutes in a benchtop microcentrifuge.
14. Remove and discard the supernatant, leaving the pellet intact.
15. Wash the pellet with 500 μ l 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 μ l resuspension buffer.

Validate the Library

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

Bioanalyzer Method

1. Load 1 μ l 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 should be similar in size to the size-range excised during the gel purification step (~92 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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