RNA-Seq Strand Specific Library Preparation Protocol

PolyA enrichment

Sample Name								
Concentration (ng/µl)								
Volume of RNA (µl)								
Volume of Water (µl)								

Up to 40ug can be used with 100uL of dynal beads. We use Invitrogen Dynal Oligo(dT) beads (Cat # 610-06) for PolyA enrichment (2 rounds). (Optional) Measure yield with Nanodrop.

0. Turn on the heat blocks – one at 65C, the other 80C.
1. Dilute 5 µg (up to 40 ug) of total RNA with nuclease-free water to 50 µl in a 1.5 ml RNase-free non-sticky Eppendorf tube—Lobind—(USA Scientific, Cat #1415-2600).
2. Heat the samples at 65 °C for 3 minutes to disrupt the secondary structures, and place the tube on ice.
3. Aliquot 100 µl of Dynal Oligo(dT) beads into a 1.5 ml RNase-free non-sticky Eppendorf tube.
4. Wash beads twice with 100 µl Binding Buffer. Discard supernatant.
5. Resuspend beads with 50 µl Binding Buffer, and add the 50 µl of denatured total RNA from Step 2. Rotate the tube at RT for 5 minutes and discard supernatant.
6. Wash beads twice with 100 µl Washing Buffer B. Discard supernatant.
7. Aliquot 80 µl Binding Buffer for each sample in a fresh 1.5 ml RNase-free non-sticky Eppendorf tube.
8. Remove supernatant from the beads of Step 6, add 20 µl of 10 mM Tris-HCl and heat the beads at 80 °C for 2 minutes to elute mRNA. Immediately put the beads on the magnet stand and transfer the supernatant (mRNA) to the tube from Step 7. Add 100 µl of Washing Buffer B to the remaining beads and wait until Step 10.
9. Heat the mRNA samples at 65 °C for 3 minutes to disrupt the secondary structures, and place the tube on ice.
10. Discard the supernatant from the beads in Step 8, and wash the beads with 100 µl Binding Buffer.
11. Remove supernatant from the beads and add 100 µl of denatured mRNA from Step 9. Rotate the tube at RT for 5 minutes.
12. Discard supernatant and wash the beads twice with 100 µl Washing Buffer B.
13. Discard supernatant. Add 10 µl of 10 mM Tris-HCl and heat the beads at 80 °C for 2 minutes to elute mRNA. Immediately put the beads on the magnet stand and transfer the supernatant (mRNA) to a fresh 200 µl thin wall PCR tube. There should be ~9 µl mRNA.

RNA Fragmentation

1. On ice, mix:

Enriched mRNA (approx. 200ng)	9 µL
Random (hexamer) primers (3ug/ul, Invitrogen, Cat #48190-011)	1 µL
5X First-strand buffer (Invitrogen ds cDNA synthesis kit)	4 µL

2. Mix well, centrifuge briefly and heat at 85 °C for 7-8 min in thermocycler with 200 µl thin wall PCR tube. When finished, immediately place on ice.

First-Strand cDNA synthesis

1. Add directly to sheared mixture:

2 µL	0.1 M DTT
1 µL	10 mM RNase-free dNTP mix (Invitrogen, Cat# 18427-013)
1 µL	RNaseOUT (Invitrogen Cat# 10777-019)
2 µL	Superscript III Reverse Transcriptase (Invitrogen, Cat# 18080-04) (Superscript II is also okay)

2. Total volume is 20 µL. Heat mixture to 50 ^{o}C for 1 hour followed by 85 ^{o}C for 15 minutes to inactivate enzymes, then place on ice. Next you will perform your second strand synthesis directly in the same mixture.

Second Strand Synthesis with dUTP mix

1. To each first-strand reaction tube on ice, add:

92 µL	ddH20
30 µL	5X Second-Strand Buffer (Invitrogen, Cat# 10812-014)
3 µL	**10 mM dNTP—use dUTP mix (add U, no T) if strandedness desired**
4 µL	E. coli DNA Polymerase I (NEB, Cat# M0209L)
1 µL	E. coli RNase H (Epicentre, Cat# R0601K)

Total volume is 150 µL. Mix well, centrifuge briefly, incubate at 16 ^{o}C for 2 hours.

2. Clean-up the reaction using QIAquick PCR Purification kit. Elute in 35 µl EB buffer. At this point, double stranded cDNA can be stored at -20°C or continue to the next step below.

End Repair using 'End-It DNA End Repair Kit'

Epicentre (Cat# ER0720)

1. Combine and mix the following components in a Eppendorf tube
   34 µl double-stranded cDNA to be end-repaired
   5 µl 10X End-Repair Buffer
   5 µl 2.5mM dNTP Mix
   5 µl 10mM ATP
   1 µl End-Repair Enzyme Mix
   50 µl Total reaction volume

2. Incubate at **room temperature** on bench for 45 minutes, and inactivate the enzymes at 70 °C for 10 min.

3. Purify on one QIAquick column using the QIAquick PCR Purification Kit (Cat #28106), eluting in 33 µl of Buffer EB.

Addition of 'A' base to 3' Ends

Use Klenow (3'→5' exo-) from NEB (Cat# M0212S)

**Before starting, make up stocks of 1 mM dATP using NEB 100 mM dATP, e.g. add 5 µL of 100mM dATP to 495 µL Qiagen Buffer EB; then make 50 µL aliquots and freeze at -20°C. Defrost aliquots only once.

1. Combine and mix the following components in a Eppendorf tube:

Eluted DNA	32 µl
Klenow buffer (= NEB Buffer #2)	5 µl
1mM dATP	10 µl
Klenow fragment (3' → 5' exo minus, 5 U/µl)	3 µl
50 µL Total reaction volume	

2. Incubate for 30 minutes at 37°C.

3. Purify on QIAGEN MinElute column (Cat #28006). Elute in 20 µl Buffer EB.

Adapter ligation

Use LigaFast from Promega (Cat# M8221) or Quick Ligation Kit from NEB (M2200L) and the PE Adapter Oligo Mix (part# 1001782) from Illumina.

1. Combine and mix the following components in a Eppendorf tube:

Eluted DNA from Step 8	19 µl
2X DNA ligase buffer	25 µl
Adapter oligo mix	1 µl
DNA ligase (3 U/µl)	5 µl

50 µL Total reaction volume

2. Incubate for 15 minutes at room temperature on bench.

3. Purify on one QIAGEN MinElute column (Cat #28006) using the MinElute PCR Purification Kit and protocol. Elute in 15 µl Buffer EB.

4. Band-isolate the DNA using a 2.5-3.0% NuSieve low melt agarose gel. Run gel at 100V for about 1 hour to 1.5 hour. Cut out the band from 300-600bp for 200-500bp fragments (to take into account the length of adaptors, which is about 80 bp) using clean, SINGLE USE scalpels. If making multiple libraries, be very careful to avoid cross-contamination and load samples in every other lane.

5. Purify the DNA from the agarose slice using a QIAquick Gel Extraction Kit (Cat #28706). Incubate gel slice in QG buffer at 37°C, vortex every 5 minutes. Follow protocol in handbook, including recommended 0.5 ml QG wash. Wash twice with Buffer PE. Elute in 45 µl Buffer EB.

Uridine digestion of second strand cDNA

Do not perform this step if strandedness is not being used.

1. Combine and mix the following components in a Eppendorf tube:

Gel extracted DNA	44 µl
10X buffer	5 µl
UNG or UDG (NEB, Cat# M0280L)	1 µl

50 µL Total reaction volume

2. Mix by pipetting and incubate at 37°C for 15 minutes.

3. Purify using AMPure XP Beads (Agencourt Bioscience, A63880):

   a. Add 40 µL (0.8X) well-mixed AMPure beads, mix well by pipetting up and down 10 times, let sit for 5 minutes at room temperature.

b. Place the tube in a magnetic plate and keep for >3 min, then discard the supernatant.

c. Add 200ul 70% Ethanol to each tube without disturbing the beads, incubate for 30 seconds at room temperature, discard the supernatant.

d. Repeat step 3 (be sure to remove all of the ethanol)

e. Air dry the beads for 3-5 minutes at room temperature.

f. Off the magnet rack, add 45 µL EB to the beads, pipette the beads up and down 10 times, let sit at room temperature for 2 minutes.

g. Place the tube to the magnet rack and keep for 2 minutes, transfer the supernatant (your DNA) to a new LoBind tube.

Real time PCR to Amplify Library

Use Phusion DNA polymerase, NEB (Cat# F-531) and Illumina primers

- "PCR Primer PE 1.0" which is part of the Illumina kit
- "PCR Primer PE 2.0" which is part of the Illumina kit

Primers and adapters can be purchased separately (Cat # PE-102-1003, 100 reactions).

1. Set up PCR Master Mix, make 10% extra reagent for multiple samples, and aliquot

Purified, digested, adaptor ligated DNA	44 µl
2X Phusion HF DNA polymerase Master Mix	50 µl
10x SYBR Green I	2 µl
PCR Primer PE 1.0 (25 µM)	2 µl
PCR Primer PE 2.0 (25 µM)	2 µl
100 µl Total reaction volume	

Amplify using the following PCR protocol:

- 30 sec at 98°C
- [10 sec at 98°C, 30 sec at 65°C, 45 sec at 72°C] x ~15 cycles: watch the real time PCR curves; terminate ("skip") when necessary
- 5 min at 72°C
- Hold at 15°C

2. Take 10 µl of the PCR product and run on an agarose gel to check the size range.

3. Purify on one QIAGEN column using the PCR Purification Kit. Elute in 50 µl Buffer EB. The volume of EB can be adjusted depending on the number of PCR cycles required.

4. Measure the DNA concentration (ng/µl) by Qubit.

Sample Name	Measured Concentration	Actual Concentration

5. Run 1 µl of each library on an Agilent BioAnalyzer. Record the average size and the concentration. The DNA is now ready for sequencing.