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Seq-Star™ Stranded RNA-seq Kit (Illumina)

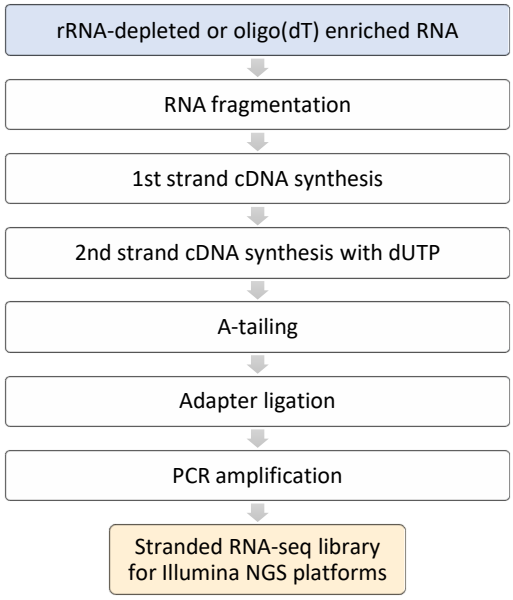
Cat#: AS-MB-11 (Set A); (Set B)

Instruction Manual version 1.0

Product summary

Product description

Seq-Star™ Stranded RNA-seq Kit (Illumina) includes the enzyme and reagent components for constructing stranded RNA-seq libraries for mRNA and long coding RNA transcriptome. The kit uses dUTP to ensure extremely high RNA strand specificity, which is particularly important for long non-coding RNAs often transcribed in sense and antisense strand directions. The Kit procedures generate low bias, high fidelity, high quality libraries with uniform read coverage along the RNA transcript lengths, starting from 10 ~ 400 ng rRNA-depleted or oligo(dT)-enriched input RNA. The libraries are fully compatible with Illumina sequencing platforms.



Starting materials

- 10 ~ 400 ng rRNA-depleted or oligo(dT)-enriched RNA

Kit components

Components	Size 24 reactions	Storage
Fragmentation Buffer	120 µL	-20°C
1st Strand Synthesis Buffer	120 µL	-20°C
Seq-Star™ RTase	12 µL	-20°C
2nd Strand Synthesis Buffer	360 µL	-20°C
2nd Strand Enzyme Mix	24 µL	-20°C
A-Tailing Buffer	36 µL	-20°C
A-Tailing Enzyme	36 µL	-20°C
Ligase Buffer	168 µL	-20°C
Seq-Star™ DNA Ligase	60 µL	-20°C
Beads Suspension	2.5 ml	-20°C
Primer Mix	60 µL	-20°C
2×PCR Mix	300 µL	-20°C
Adapter Set (Set A)	10 µL	-20°C
Adapter Set (Set B)	10 µL	-20°C

Additional required materials

- Seq-Star™ DNAClean Beads (Cat# AS-MB-007, Arraystar) or Agencourt AmPure XP beads (Beckman Coulter)
- Magnetic stand (tube compatible)
- Pipettors and tips
- Thermal cycler
- Agilent 2100 Bioanalyzer (*optional*)
- Fresh 80% ethanol
- Nuclease-free water

Protocol

Part I: RNA Fragmentation

1. Prepare the following mix in a 200 µL PCR-tube for each sample:

10 ~ 400 ng poly(A+) or rRNA-depleted RNA	5.0 µL
Fragmentation Buffer	5.0 µL
Total volume	**10.0 µL**

2. Mix thoroughly by gently pipetting up and down several times.
3. Perform fragmentation using the parameters:

Input RNA	Desired size	Fragmentation
Intact	100-200 bp	8 min@94°C
	200-300 bp	6 min@94°C
	300-400 bp	6 min@85°C
Partially degraded	100-300 bp	1-6 min@85°C
Degraded	100-200 bp	30 sec@65°C

4. Place the tube on ice for 2min and proceed immediately to next step.

Part II: 1st Strand cDNA Synthesis

5. Add the following Mix into the fragmented RNA from Step 4.

1s Strand Synthesis Buffer	5.0 µL
Seq-Star™ RTase	0.5 µL
Total volume	**5.5 µL**

6. Mix thoroughly by gently pipetting up and down 10 times.
7. Incubate the tube in a thermal cycler for [25°C, 20 min; 42°C, 15 min; 42°C, 15 min; 4°C, hold].
8. Transfer the tube on ice. Proceed immediately to the 2nd strand synthesis step.

Part III: 2nd Strand cDNA Synthesis

9. Add the following mix to the 1st strand cDNA from step 8.

2nd Strand Synthesis Buffer	15 µL
2nd Strand Enzyme Mix	1 µL
Total volume	**16 µL**

10. Mix thoroughly by gently pipetting up and down several times.
11. Incubate the tube at 16°C for 60 min, then chill the tube on ice. Proceed immediately to next step.

Part IV: cDNA purification

12. Add 56 µL Seq-Star™ DNAClean Beads to the 31.5 µL 2nd strand cDNA synthesis reaction.
13. Mix thoroughly by pipetting up and down 10 times.
14. Incubate at room temperature for 5~15 min to allow cDNA binding to the beads.
15. Place the tube at a magnetic stand until the solution becomes completely clear (about 1~2 minutes). Carefully aspirate and discard the supernatant.
16. Keep the tube on the magnetic stand and add 200 µL freshly prepared 80% ethanol. Incubate at room temperature for 30 seconds and aspirate off the supernatant.

    Caution: Do not disturb the separated magnetic beads during operation!

17. Repeat Step 16 once for total two washes. Make sure to remove all residual ethanol from the bottom of the tube.

    Optional: Air drying the beads for less than 5 minutes helps to remove all the residual ethanol. However over drying may result in dramatic loss in yields.

18. Proceed immediately to next step.

Part V: A-Tailing

19. Add the following A-Tailing mix to the cDNA binding beads from Step 17:

Nuclease-free water	12 µL
A-Tailing Buffer	1.5 µL
A-Tailing Enzyme	1.5 µL
Total volume	**15 µL**

20. Resuspend the beads by pipetting up and down 10 times.

21. Incubate the tube in a thermal cycler for [30°C, 20min; 60°C, 15min; 4°C, hold].
22. Proceed immediately to next step.

Part VI: Adapter Ligation and Purification

23. Add the following Adapter Ligation mix to the A-Tailing reaction from step 22:

Nuclease-free water	8.0 µL
Adapter	1.0 µL
Ligase Buffer	7.0 µL
Seq-Star™ DNA Ligase	2.5 µL
Total volume	**18.5 µL**

24. Resuspend the beads by pipetting up and down 10 times.
25. Incubate the tube at 20°C for 15 min.
26. Add 33.5 µL Beads Suspension to the total of 33.5 µL Adapter Ligation reaction.
27. Mix thoroughly by pipetting up and down 10 times.
28. Incubate at room temperature for 5~15 min to allow cDNA binding to the beads.
29. Place the tube at magnetic stand until the solution becomes completely clear (about 1~2 minutes). Carefully aspirate off and discard the supernatant.
30. Keep the tube on magnetic stand and add 200 µL freshly prepared 80% ethanol. Incubate at room temperature for 30 seconds and aspirate off the supernatant.

    Caution: Do not disturb the separated magnetic beads during operation!
31. Repeat Step 30 once for total two washes. Make sure to remove all residual ethanol from the bottom of the tube.

    Optional: Air dry the beads for less than 5 minutes will ensure to remove all the ethanol. However over dry of the beads may result in dramatic yield loss.
32. Remove the tube off the magnetic stand and resuspend the beads in 25 µL nuclease-free water.
33. Incubate at room temperature for 2 min and then perform a **second round purification** in the following steps.
34. Add 25 µL more Beads Suspension to the 25 µL first round purified adapter-ligated cDNA with the beads.
35. Mix thoroughly by pipetting up and down 10 times.
36. Incubate at room temperature for 5~15 min to allow the adapter-ligated cDNA binding to the beads.
37. Place the mix tube at a magnetic stand until the solution becomes completely clear (about 1~2 minutes). Carefully aspirate off and discard the supernatant.
38. Keep the tube on the magnetic stand and add 200 µL freshly prepared 80% ethanol. Incubate at room temperature for 30 seconds and aspirate off the supernatant.
39. Repeat Step 38 once for total two washes. Make sure to remove all residual ethanol from the bottom of the tube.
40. Remove tube off the magnetic stand and resuspend the beads in 11 µL nuclease-free water.
41. Incubate at room temperature for 2 min. Place the tube on the magnetic stand until the supernatant is completely clear from the beads.
42. Transfer 10 µL supernatant containing the library to a new 200 µL PCR tube.

Part VII: Library Size Selection (*optional*)

The purified adapter-ligated cDNA library from Step 42 can be used directly for PCR amplification. Adapter-dimer contamination should be absent.

The library can be further size selected for a desired size range, using Seq-Star™ DNA Size Selection Kit (Cat# AS-MB-008-01/02, Arraystar) in the following steps (Fig. 1). Other similar kits may also be used.

43. Add 40 µL nuclease-free water to 10 µL of the purified adapter-ligated cDNA for a 50 µL total volume.
44. Perform the size selection procedure according to the kit instructions.

Table 1. Bead amounts for the indicated peak size using Seq-Star™ DNA Size Selection Kit two-round procedure

Peak size (bp)	**300**	**350**	**400**	**500**	**600**	**700**
Beads (µL) 1st round	40	35	30	27.5	25	22.5
Beads (µL) 2nd round	10	10	10	7.5	7.5	7.5

45. Elute the size selected DNA in 11 µL nuclease-free water.

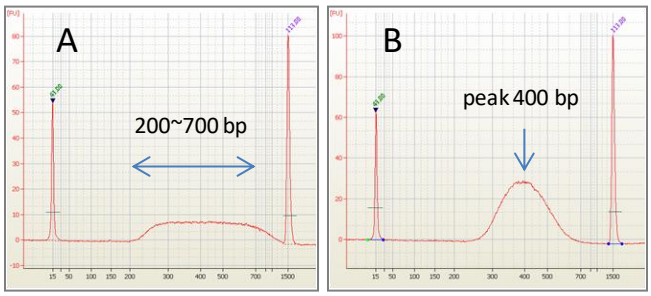


Figure1. Bioanalyzer fragment analysis of a library constructed from the oligo(dT)-enriched mRNA using Seq-Star™ Stranded RNA-seq Kit. The library without size selection has a size distribution of 200~700 bp. Adapter dimers are absent (A). The library sized with Seq-Star™ DNA Size Selection Kit has the peak size of 400 bp (B).

Part VIII: Library Amplification

46. Add 12.5 µL 2×PCR Mix and 2.5 µL Primer Mix to the 10 µL purified adapter-ligated cDNA from step 42 or step 45.
47. Mix thoroughly by pipetting up and down 10 times.
48. Run the PCR program as [98°C, 45 s; 6-14 cycles of (98°C, 15 s; 60°C, 30 s; 72°C, 30 s); 72°C, 5 min; 4°C, hold].

    Note: The number of PCR amplification cycles depends on the input RNA amount. 10~14 cycles for 10~50 ng, 8~12 cycles for 50~200 ng, and 6~10 cycles for 200~400 ng input RNA.
49. Add 25 µL Seq-Star™ DNAClean Beads to the 25 µL PCR product.
50. Mix thoroughly by pipetting up and down 10 times.
51. Incubate at room temperature for 5~15 min to allow cDNA binding to the beads.
52. Place the tube at a magnetic stand until the solution becomes completely clear (about 1~2 minutes). Carefully aspirate off and discard the supernatant.
53. Keep the tube on the magnetic stand and add 200 µL freshly prepared 80% ethanol. Incubate at room temperature for 30 seconds and aspirate off the supernatant.

    Caution: Do not disturb the separated magnetic beads during operation!
54. Repeat Step 53 once for total two washes. Make sure to remove all the remaining ethanol from the bottom of the tube.

    Optional: Air drying the beads for < 5 minutes helps to remove residual ethanol. However over drying may result in dramatic loss in yields.
55. Remove the tube off the magnetic stand and resuspend the beads in 21.5 µL nuclease-free water.
56. Incubate at room temperature for 2 min. Place the tube on the magnetic stand until the supernatant is completely clear from the beads.
57. Transfer 20 µL supernatant containing the final library to a new tube.

Troubleshooting

Problem	Possible causes	Suggestion
Low library yield	Insufficient amount of starting RNA	Use more sensitive method to measure low RNA concentration (*e.g.* Agilent 2100 Bioanalyzer). Use qPCR to evaluate the efficiency of oligo(dT) enrichment or rRNA removal.
	Contamination of enzymatic inhibitors in RNA samples	Make sure no residual RNA-binding inhibitors or organics in your input RNA samples!

Appendix

Index sequences for Illumina multiplexing barcodes

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Set A
#01 CGATGT; #02 TGACCA; #03 ACAGTG; #04 GCCAAT;
#05 CAGATC; #06 CTTGTA; #07 AGTCAA; #08 AGTTCC;
#09 ATGTCA; #10 CCGTCC; #11 GTCCGC; #12 GTGAAA
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Set B
#13 ATCACG; #14 TTAGGC; #15 ACTTGA; #16 GATCAG;
#17 TAGCTT; #18 GGCTAC; #19 GTGGCC; #20 GTTTCG;
#21 CGTACG; #22 GAGTGG; #23 ACTGAT; #24 ATTCCTTT
```