Protocol Taken from Illumia's ScriptSeq v2 RNA-seq LibraRY PREPARATION KIT(SSV21106). See it for full explanation.

IMPORTANT- Failsafe PCR Enzyme is required for this kit and is sold separately.

3.A. Fragment the RNA and Anneal the cDNA Synthesis Primer

Important!

1. If using severely fragmented RNA, such as that obtained from FFPE samples, use the procedure described in **Appendix 1**.

2. The RNA can be fragmented by methods other than those described in Part 3.A. If fragmenting the RNA by other methods, the fragmented RNA must be purified and dissolved in a maximum of 9 µl of Nuclease-Free Water. Then, use the procedure described in **Appendix 1** to anneal the cDNA synthesis primer and perform cDNA synthesis.

Required in Part 3.A.

3.A.1. In a 0.2-ml PCR tube, assemble the following reaction mixture. If a "no template" control reaction is performed, substitute Nuclease-Free Water for the RNA sample.

ScriptSeqTM v2 RNA-Seq Library Preparation Kit

Component Name	Tube Label	Cap Color
cDNA Synthesis Primer	cDNA Primer	Green
RNA Fragmentation Solution	Fragmentation Solution	
Nuclease-Free Water	Nuclease-Free Water	Clear

Use 500 pg to 50 ng of rRNA-depleted or poly(A)$^+$ RNA per reaction.

. x µl Nuclease-Free Water

. y µl rRNA-depleted or poly(A)$^+$ RNA

. 1 µl Fragmentation Solution

. 2 µl cDNA Primer

. 12 ul Total Volume

. 3.A.2. Fragment RNA: Incubate at 85°C for 5 minutes in a thermocycler with heated lid.

. 3.A.3. Stop the fragmentation reaction by placing the tube on ice.

3.B. Synthesize cDNA

Required in Part 3.B.

Thermocycler settings for Part 3.B

25°C for 5 minutes (cDNA synthesis)

42°C for 20 minutes (cDNA synthesis)

37°C Pause/Hold

37°C for 10 minutes (Finishing Solution)

95°C for 3 minutes (Inactivate Finishing Solution)

25°C Pause/Hold

3.B.1. On ice, prepare the cDNA Synthesis Master Mix: For each reaction, combine on ice:

3.0 µl cDNA Synthesis PreMix

0.5 µl 100 mM DTT

0.5 µl StarScript AMV Reverse Transcriptase

4.0 µl Total volume per reaction

Component Name	**Tube Label**	**Cap Color**
ScriptSeq v2 cDNA Synthesis PreMix	cDNA Synthesis PreMix	Red
100 mM DTT	100 mM DTT	
StarScript AMV Reverse Transcriptase	StarScript AMV Reverse Transcriptase	

ScriptSeq Finishing Solution	Finishing Solution	

3.B.1 Gently but thoroughly mix the cDNA Synthesis Master Mix by pipetting.

. 3.B.2. Add 4 µl of the cDNA Synthesis Master Mix to each reaction on ice from Part 3.A, Step 3, and mix by pipetting.

. 3.B.3. Incubate at 25°C for 5 minutes followed by 42°C for 20 minutes.

. 3.B.4. Cool the reactions to 37°C and Pause/Hold the thermocycler.

. 3.B.5. Remove one reaction or one strip of tubes at a time from the thermocycler. Add 1.0 µl of Finishing Solution, and mix gently but thoroughly by pipetting. Return the reaction to the thermocycler before proceeding with the next.

. 3.B.6. Incubate at 37°C for 10 minutes.

. 3.B.7. Incubate each reaction at 95°C for 3 minutes. Then, cool the reactions to 25°C and Pause/Hold the thermocycler. Prepare the Terminal Tagging Master Mix as described in Part 3.C, Step 1.

3.C. Synthesize 3′-Tagged DNA required in Part 3.C.

Important! The Terminal-Tagging PreMix is a viscous solution. Mix it thoroughly before use.Recommended: *Wide bore pipet tip (e.g., PureTM 200G sterile tip; catalog number #3531, Molecular Bioproducts) when pipetting the Terminal*

Tagging PreMix and the Terminal Tagging Master Mix.

Thermocycler settings for Part 3.C

:25°C for 15 minutes (DNA Polymerase)

95°C for 3 minutes (Inactivate DNA Polymerase)

4°C Hold or ice

3.C.1. On ice, prepare the Terminal Tagging Master Mix. For each reaction, combine on ice:

Component Name	**Tube Label**	**Cap Color**
ScriptSeq v2 Terminal-Tagging Premix	Terminal Tagging PreMix	Blue

DNA Polymerase	DNA Polymerase	

7.5 µl Terminal Tagging Premix
0.5 µl DNA Polymerase
8.0 µl volume per reaction

. 3.C.2. Thoroughly mix the viscous Terminal Tagging Master Mix.

. 3.C.3. Remove one reaction or strip of tubes from the thermocycler (from Part 3.B, Step 7) and add 8.0 µl of the Terminal Tagging Master Mix. Gently but thoroughly mix the reaction by pipetting. Return each reaction to the thermocycler before proceeding with the next.

. 3.C.4. Incubate each reaction at 25°C for 15 minutes.

. 3.C.5. Incubate each reaction at 95°C for 3 minutes. Then, cool the reactions to 4°C on ice or in the thermocycler.

3.D. Purify the cDNA

The di-tagged cDNA must be purified prior to PCR amplification. We recommend using the MinElute PCR Purification Kit (Qiagen) or the Agencourt AMPure XP system (BeckmanCoulter). ***If working with FFPE RNA, you must use the MinElute PCR Purification kit.***

WE USED MinElute PCR Purrification Kit (28004)

- If using the MinElute PCR Purification Kit, follow the manufacturer's directions. Elute the cDNA using 25 µl of the EB Buffer (Elution Buffer) that is provided in the MinElute Kit. The 25-µl volume typically yields a final volume of 22.5 µl. However, if necessary, adjust the eluate to 22.5 µl with EB Buffer. If using a column purification method other than the MinElute PCR Purification Kit, adjust the eluate volume to 22.5 µl.

- If using the AMPure XP System, the purification can be done in a 96-well plate or in the microfuge tubes containing the di-tagged cDNA from Part 3.C, Step 4. The procedure described uses a 1.8X AMPure XP purification scheme.

1. Warm the AMPure XP beads to room temperature. While the beads warm, prepare 400 µl of fresh 80% ethanol at room temperature for each sample.

2. If performing the AMPure XP procedure using a 96-well plate format, transfer each di-tagged cDNA from Part 3.C, Step 4 independently into a well of the plate. If using microfuge tubes, transfer each 70-µl volume to a separate 1.5-ml tube.

3. ***Important! Vortex the AMPure XP beads until they are a homogeneous suspension.***

4. Add 45 µl of the beads to each sample containing di-tagged cDNA from Part 3.C, Step

4.

5. Mix thoroughly by gently pipetting the entire volume of each well/tube 10 times.

6. Incubate the samples at room temperature for 15 minutes.

7. Place the samples in a magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.

8. Remove and discard the supernatant from each well/tube using a pipette. Some liquid may remain in each well/tube. Take care not to disturb the beads.

9. With the samples remaining on the magnetic stand, add 200 µl of 80% ethanol to each well/tube without disturbing the beads.

Incubate the samples at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each. Take care not to disturb the beads.

10. Repeat steps 9 and 10 one more time for a total of two 80% ethanol washes.

11. Allow the samples to air-dry on their magnetic stands for 15 minutes at room temperature.

12. Add 24.5 µl of Nuclease-Free Water to each well/tube and remove from the magnetic stand.

13. Thoroughly resuspend the beads by gently pipetting 10 times.

14. Incubate the samples at room temperature for 2 minutes.

15. Place the samples on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.

16. Transfer 22.5 µl of the clear supernatant, which contains the di-tagged cDNA, from each well/tube to a new 0.2-ml PCR tube.

17. Place the tubes on ice and proceed to Part 3.E or place at –20°C for longer-term storage.

3.E. PCR Amplify the Library and Add an Index (Barcode)

This step generates the second strand of cDNA, completes the addition of the Illumina adaptor sequences, incorporates an Index or a user-defined barcode, if desired, and ampli fies the library by PCR. Typically, 10-15 PCR cycles are performed. At least one PCR cycle must be done. More PCR cycles can be performed if a greater yield of the library is needed.

Adding an Index Read or a user-defined barcode. The standard ScriptSeq v2 reaction

using the Reverse PCR Primer that is included in the kit produces a nonbarcoded library.

- To add an Illumina Index, replace the Reverse PCR Primer that is included in this kit with one of the ScriptSeq Index PCR Primers, available separately from Epicentre (see Related Products). Only Epicentre's ScriptSeq Index PCR Primers are compatible with the ScriptSeq v2 Kit procedure. Carefully read the ScriptSeq Index PCR Primers product literature to ensure proper pooling of Indexed libraries.

- To add a user-defined barcode, see Appendix 3.

- **Choice of PCR enzyme.** This kit is optimized for use with Epicentre's FailSafe PCR Enzyme. We do not recommend using other PCR enzymes, as the yield and quality of the final library may be adversely affected.

- Required in Part 3.E. Provided by the user: FailSafe PCR Enzyme (Epicentre; cat. nos. FSE51100, FSE5101K) ***Important!*** *If you are adding an Index or user-defined barcode to the library, do not use the Reverse PCR Primer that is included in this kit! Instead, use the Index- or barcode-containing oligo as the Reverse PCR Primer in this procedure. Read carefully the ScriptSeq Index PCR Primer product literature to ensure color balancing of the Indexed libraries.*

Component Name	**Tube Label**	**Cap Color**
FailSafe PCR PreMix E	FailSafe PCR PreMix E	Yellow
Forward PCR Primer	Forward PCR Primer	
Reverse PCR Primer	Reverse PCR Primer	
Nuclease-Free Water	Nuclease-Free Water	Clear

1. In a 0.2-ml PCR tube combine on ice:

22.5 µl of di-tagged cDNA from Part 3.D

1 µl Forward PCR Primer

1 µl Reverse PCR Primer (or ScriptSeq Index PCR Primer, or user-defined barcode Reverse PCR Primer)

25 µl FailSafe PCR PreMix E

0.5 µl FailSafe PCR Enzyme (1.25 U)

50 µl Total volume per reaction

2. Perform PCR

Denature the DNA at 95°C for 1 minute.

Followed by 10-15 cycles of:95°C for 30 seconds.

55°C for 30 seconds.

68°C for 3 minutes.

After the appropriate number of PCR cycles, incubate at 68°C for 7 minutes.

3.F. Purify the RNA-Seq Library

Use the AMPure XP system (Beckman Coulter) to purify the ScriptSeq v2 kit libraries, *except for libraries prepared from FFPE RNA with an average size <200 nt*. The AMPure XP System is best at removing the "primer-dimers" that can occur during PCR.

Note: *Use the MinElute PCR Purification system (Qiagen) only for purifying libraries made from FFPE RNA with an average size <200 nt. Libraries purified using the MinElute columns will be contaminated with primer-dimers.*

3.F.1. AMPure XP Purification

This procedure will yield a ScriptSeq library of >200 nts (see also Part 3.G). This procedure uses a 1X AMPure XP purification scheme.

1. Warm the AMPure XP beads to room temperature. While the beads warm, prepare 400 µl of fresh 80% ethanol at room temperature for each sample.

2. If using a 96-well plate format, transfer each amplified library from Part 3.E, Step 2, independently into a well of the plate. If using microfuge tubes, transfer each 50-µl volume to a separate 1.5 ml tube.

3. ***Important!*** *Vortex the AMPure XP beads until they are a homogeneous suspension.*

4. Add 50 µl of the beads to each sample.

5. Mix thoroughly by gently pipetting the entire volume up of each well/tube 10 times.

6. Incubate the samples at room temperature for 15 minutes.

7. Place the samples in a magnetic stand at room temperature for at least 5 minutes, until

the liquid appears clear.

8. Remove and discard the supernatant from each well/tube using a pipette. Some liquid may remain in each well. Take care not to disturb the beads.

9. With the sample remaining on the magnetic stand, add 200 µl of 80% ethanol to each well/tube without disturbing the beads.

10. Incubate the samples at room temperature for at least 30 seconds, then remove and discard all of the supernatant. Take care not to disturb the beads.

11. Repeat steps 9 and 10 one more time for a total of two 80% ethanol washes.

12. Allow the samples to air-dry on their magnetic stands for 15 minutes at room temperature.

13. Add 20 µl of Nuclease-Free Water to each well/tube and remove the plate or 1.5-ml tubes from their magnetic stand.

14. Thoroughly resuspend the beads by gently pipetting 10 times.

15. Incubate the samples at room temperature for 2 minutes.

16. Place the samples on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.

17. Transfer the clear supernatant, which contains the RNA-Seq library, from each well/tube to an appropriate collection tube for assessment of library quantity and quality.

Measure Concentration of each library.

Pool libraries for a final concentration of 10 nM in a volume of 20 ul. Ready for miseq.