epicentre®
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ScriptSeq™ v2 RNA-Seq Library Preparation Kit*

SSV21106 – 6 Reactions

SSV21124 – 24 Reactions

Important! *Epicentre's FailSafe™ PCR Enzyme (available separately) is required for use with this kit.*

Epicentre (an Illumina company) warrants that its products will retain full activity for 1 year from the date of receipt by the user if used and stored properly. For full warranty details, see Terms and Conditions available on the Epicentre website at www.epibio.com.

** Covered by issued and/or pending patents.*

1. Kit Contents and Quality Control

Component Name	Tube Label	Volume		Cap Color
		6-rxn	24-rxn	
ScriptSeq v2 cDNA Synthesis Primer	cDNA Primer	18 µl	55 µl	Green
RNA Fragmentation Solution	Fragmentation Solution	10 µl	30 µl	
ScriptSeq v2 cDNA Synthesis PreMix	cDNA Synthesis PreMix	25 µl	80 µl	Red
100 mM DTT	100 mM DTT	100 µl	100 µl	
StarScript AMV Reverse Transcriptase	StarScript AMV Reverse Transcriptase	8 µl	15 µl	
ScriptSeq Finishing Solution	Finishing Solution	10 µl	30 µl	
ScriptSeq v2 Terminal Tagging PreMix	Terminal Tagging PreMix	60 µl	200 µl	Blue
DNA Polymerase	DNA Polymerase	8 µl	15 µl	
Exonuclease I	Exo I	10 µl	30 µl	Yellow
FailSafe PCR PreMix E	FailSafe PCR PreMix E	200 µl	650 µl	
Forward PCR Primer	Forward PCR Primer	10 µl	30 µl	
Reverse PCR Primer	Reverse PCR Primer	10 µl	30 µl	
Nuclease-Free Water	Nuclease-Free Water	500 µl	500 µl	Clear

Storage: Store the kit at –15°C to –25°C in a freezer without a defrost cycle.

Additional Required Reagents and Equipment:

FailSafe™ PCR Enzyme Mix (Epicentre; cat. nos. FSE51100, FSE5101K)

Recommended: Wide bore pipet tip for use in Step 3.C (e.g., Pure™ 200G sterile tip; catalog number #3531, Molecular Bioproducts)

Recommended: 2100 Bioanalyzer (Agilent Technologies)

Optional: MinElute PCR Purification columns (Qiagen)

Optional: Agencourt AMPure XP System (Beckman Coulter) and magnetic plate, rack, or stand for 1.5-ml tubes

Optional: ScriptSeq Index PCR Primers (Epicentre; Cat. Nos. RSBC10948, SSIP1202, SSIP1203, SSIP1204)

Quality Control:

The ScriptSeq v2 RNA-Seq Library Preparation Kit is function-tested in a control reaction using 5 ng of rat liver poly(A) RNA. At least 400 ng of a di-tagged library must be produced in a final volume of 20 µl with a peak between 150-300 bp as assayed using an Agilent Bioanalyzer.

2. Preparation

rRNA Removal

The ScriptSeq v2 Kit uses a random-primed cDNA synthesis reaction. Therefore, the best sequencing results are obtained using an RNA preparation depleted of rRNA. The ScriptSeq v2 Kit can be used with poly(A)$^+$ RNA or with rRNA-depleted RNA. To maximize removal of rRNA, we recommend using one of Epicentre's Ribo-Zero™ Kits.

DNA-Free RNA

Treat the RNA sample with DNase I (for example using Baseline-ZERO DNase; Epicentre Cat. No. DB0711K, DB0715K) to remove all traces of DNA. Then, remove the DNase I prior to Ribo-Zero treatment or poly(A) enrichment. DNA contamination will interfere with rRNA removal and is the main cause of loss of directionality when sequencing the ScriptSeq v2 library. The RNA sample should be free of salts (e.g., Mg^{2+} or guanidinium salts) or organics (e.g., phenol and ethanol).

Automated ScriptSeq Kit Reactions

Epicentre does not provide support for any liquid handling robotic platform or protocol. Please contact the manufacturer of the liquid handling instrument for hardware and software support.

Amount of RNA

The standard kit reaction uses 500 pg to 50 ng of rRNA-depleted RNA or poly(A)$^+$ RNA. Typically, 10-15 cycles of PCR are sufficient to generate the sequencing library.

RNA from Formalin-Fixed Paraffin-Embedded (FFPE) Tissue

RNA extracted from FFPE tissue can be used to prepare ScriptSeq v2 libraries. However, the quality of FFPE RNA can be highly variable due to the tissue-fixation procedure, age of the sample, storage conditions, fixation reversal process, etc. Therefore, we cannot guarantee success with every FFPE RNA sample.

The use of FFPE RNA requires procedural modifications to the ScriptSeq v2 library preparation procedure. Be sure to read Part 3.A, 3.D, 3.F.2., and Appendix 1 before proceeding.

StarScript AMV Reverse Transcriptase

The StarScript AMV Reverse Transcriptase is isolated from Avian Myeloblastosis Virions. The enzyme is carefully purified to ensure ≤20-fold AMV genome coverage per 50 M sequencing reads. This corresponds to approximately 3,020 reads in a 51-base sequencing run and approximately 1,540 reads in a 100-base sequencing run.

Sequencing a ScriptSeq RNA-Seq Library

ScriptSeq libraries are compatible with single read, paired-end and multiplex sequencing on any Illumina® sequencer. See Appendix 2 for more details.

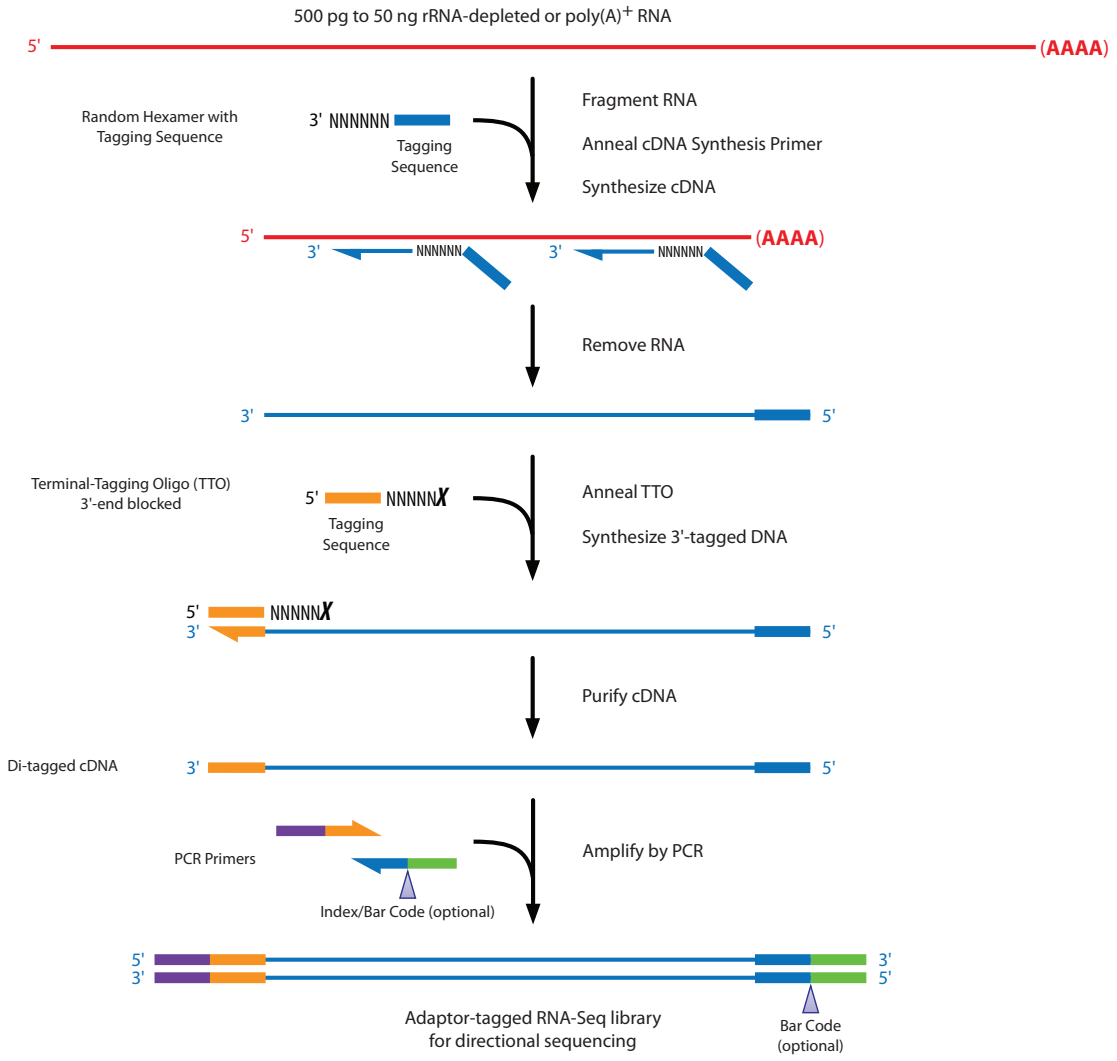


Figure 1. An overview of the procedure for the ScriptSeq™ v2 RNA-Seq Library Preparation Kit.

3. Kit Procedure

Remove all components except enzymes and Finishing Solution, allow to thaw, and store on ice. Centrifuge each tube briefly to collect liquid to the bottom of the tube. It is highly recommended that enzyme and Finishing Solution be stored in a benchtop cooler (–20˚C) to avoid repeated freeze-thaws.

Quick Protocol for ScriptSeq™ v2 RNA-Seq Library Preparation Kit

For experienced users only! The detailed protocol begins at Step 3.A on the next page.

Step	Procedure	Pages
Fragment RNA	If using RNA from FFPE samples, go to Appendix 1 (RNA fragmentation is not required). 1. Mix the following: x µl Nuclease-Free Water y µl Ribo-Zero-treated RNA (500 pg to 50 ng) 1 µl RNA Fragmentation Solution 2 µl cDNA Primer 12 µl Total volume 2. Incubate at 85°C for 5 minutes in thermocycler then place on ice.	5
Synthesize cDNA	1. Mix the following per reaction: 3.0 µl cDNA Synthesis PreMix 0.5 µl 100 mM DTT 0.5 µl StarScript AMV Reverse Transcriptase 4.0 µl Total Volume of cDNA Synthesis Master Mix 2. Add 4 µl of cDNA Synthesis Master Mix to each reaction. Mix by pipetting. 3. Incubate at 25°C for 5 min followed by 42°C for 20 min. 4. Cool reaction to 37°C. 5. Add 1.0 µl of Finishing Solution to each reaction. Mix by pipetting. 6. Incubate at 37°C for 10 min. Incubate at 95°C for 3 min, cool to 25°C and Pause the thermocycler.	5
Synthesize 3′-Tagged DNA	1. Mix the following per reaction: 7.5 µl Terminal Tagging Premix 0.5 µl DNA Polymerase 8.0 µl Total volume of Terminal Tagging Master Mix **Solution is viscous!** Mix thoroughly by pipetting using a wide-bore pipette tip. 2. Add 8.0 µl of Terminal Tagging Master Mix to each reaction. Mix by pipetting using a wide-bore pipette tip. 3. Incubate reaction at 25°C for 15 minutes. Incubate reaction at 95°C for 3 minutes. Cool to 4°C. The cDNA is now di-tagged.	6
Purify cDNA	Choose Qiagen MinElute or Agencourt AMPure purification. Elute in 22.5 µl.	6
PCR Amplify	If adding an optional Index, go to Part 3.E of protocol and skip this Quick Reference Protocol. If **not** adding an Index: Mix in a 0.2-ml PCR tube: 25 µl FailSafe PCR PreMix E 1 µl Forward PCR Primer 1 µl Reverse PCR Primer 22.5 µl di-tagged cDNA 0.5 µl FailSafe PCR Enzyme (supplied by the user) 50 µl Total volume **PCR cycle conditions:** Denature the DNA at 95°C for 1 minute followed by cycles of: 95°C for 30 seconds 55°C for 30 seconds 68°C for 3 minutes 10 - 15 cycles using high quality RNA 12 - 15 cycles using FFPE RNA Incubate at 68°C for 7 minutes after the final cycle	7
Purify Library	AMPure purification. Qiagen purification only suggested for very short FFPE RNA samples (<200 nt) and will result in adaptor-dimer contamination.	8
QC Library	Quantify by Qubit™ or PicoGreen and visualize on Agilent BioAnalyzer	9

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.

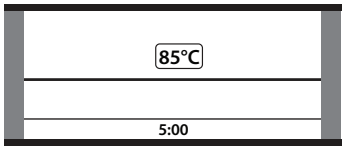
Required in Part 3.A.

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

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.

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	µl	Total volume per reaction



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.

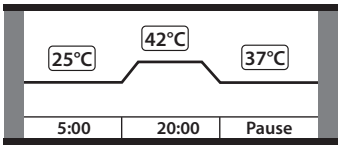
3.B. Synthesize cDNA

Required in Part 3.B.

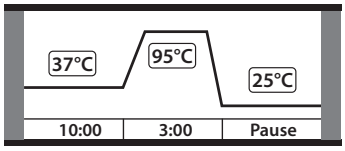
Component Name	Tube Label	Cap Color
ScriptSeq v2 cDNA Synthesis PreMix	cDNA Synthesis PreMix	Red
100 mM DTT	100 mM DTT	
StarScript AMV Reverse Transcriptase	StarScript AMV Reverse Transcriptase	
ScriptSeq Finishing Solution	Finishing Solution	

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

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.

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

⚠ ***Important! The Terminal-Tagging PreMix is a viscous solution. Mix it thoroughly before use.***

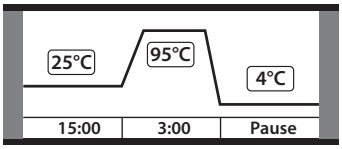
Recommended: *Wide bore pipet tip (e.g., Pure™ 200G sterile tip; catalog number #3531, Molecular Bioproducts) when pipetting the Terminal Tagging PreMix and the Terminal Tagging Master Mix.*

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:

7.5	µl	Terminal Tagging Premix
0.5	µl	DNA Polymerase
8	µl	Total 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.***

- 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.

10. 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.
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 24.5 µl of Nuclease-Free Water to each well/tube and remove from the 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 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.
18. 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 amplifies 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 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.

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

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.*

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

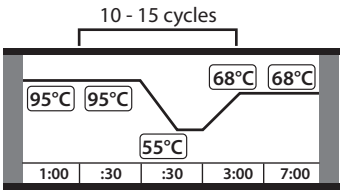
22.5	µl	of di-tagged cDNA from Part 3.D
1	µl	Forward PCR Primer
1	µl	Reverse PCR Primer (or ScriptSeq Index PCR Primer, or user-defined barcode Reverse PCR Primer)
25	µl	FailSafe PCR PreMix E
0.5	µl	FailSafe PCR Enzyme (1.25 U)
50	µl	Total volume per reaction

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	10 - 15 cycles using high quality RNA 12 - 15 cycles using FFPE RNA



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

See Part 3.G for examples of amplified ScriptSeq RNA-Seq libraries. During the PCR, read Part 3.F to determine which post-PCR purification procedure is best suited to your sample. After the PCR procedure is complete, proceed immediately to Part 3.F.

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.

3.F.2. MinElute PCR Purification Kit for Highly Fragmented RNA Samples

Use the MinElute PCR Purification Kit to purify ScriptSeq v2 libraries made from FFPE RNA of average size <200 nt. This procedure uses the Exonuclease I enzyme provided in the ScriptSeq v2 Kit.

1. Remove excess PCR primers by adding 1 µl of Exonuclease I to each reaction and incubate the reactions at 37°C for 15 minutes.
2. Purify the library using the MinElute Kit procedure described by the manufacturer.

3.G. Assess Library Quantity and Quality

The library should be quantified by your laboratory's standard methods. The size distribution can be assessed using the 2100 Bioanalyzer (Agilent) and a High Sensitivity DNA Chip. Fig. 3 shows representative 2100 Bioanalyzer (Agilent) profiles of RNA-Seq libraries produced by the ScriptSeq v2 Kit.

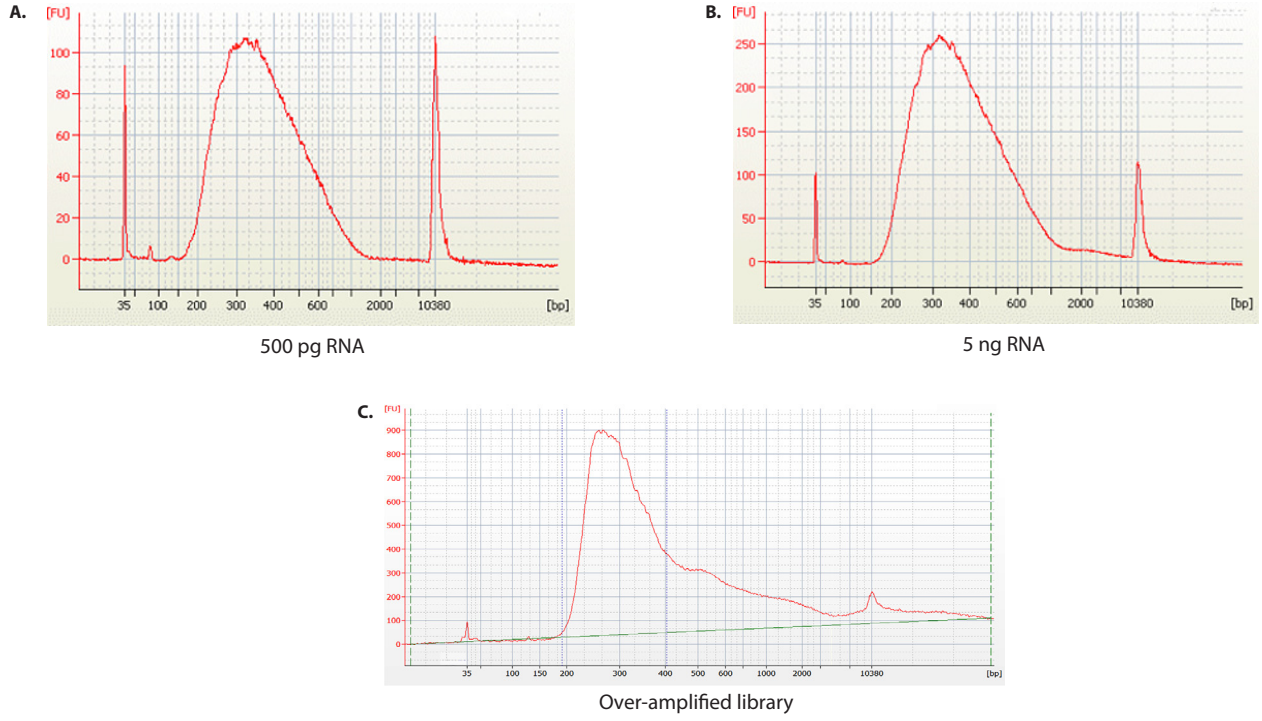


Figure 3. Representative profiles of ScriptSeq™ v2 Kit RNA-Seq libraries. ScriptSeq RNA-Seq libraries were prepared with the indicated amount of human liver poly(A)$^+$ RNA. During the ScriptSeq procedure, the di-tagged cDNAs were purified (Part 3.D) using the MinElute® PCR Purification Kit (Qiagen). In Part 3.E, 15 PCR cycles were performed for both the 500 pg (Fig. 3A) and 5 ng (Fig. 3B) libraries. Figure 3C shows the profile of a library that had been over-amplified (too many PCR cycles) during Step 3.E. If >60% of the over-amplified material is between 200 and 1000 bp, the library can be sequenced. The PCR-amplified libraries were purified (Part 3.F) using the AMPure® XP procedure. Purified libraries were analyzed using the 2100 Bioanalyzer (Agilent) with a High Sensitivity DNA Chip.

4. Appendices

Appendix 1: Preparing a Library from Severely Fragmented RNA and FFPE RNA

Use this procedure when preparing libraries from rRNA-depleted RNA:

- That is highly fragmented, such as sometimes obtained from FFPE samples.
- That has been fragmented using a procedure different than that described in Part 3.A.

4.1.A. Anneal the cDNA Synthesis Primer

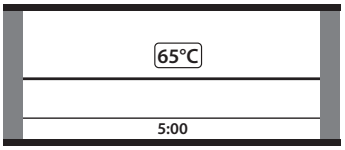
Required in Part 4.1.A.

Component Name	Tube Label	Cap Color
ScriptSeq cDNA Synthesis Primer	cDNA Primer	Green
Nuclease-Free Water	Nuclease-Free Water	Clear

1. If a "no template" control reaction is performed, substitute Nuclease-Free Water for the RNA sample.

x	µl	Nuclease-Free Water
y	µl	rRNA-depleted fragmented or FFPE RNA (500 pg to 50 ng)
2	µl	cDNA Primer
11	µl	Total volume per reaction

2. Incubate at 65°C for 5 minutes in a thermocycler.
3. Stop the reaction by placing the tube on ice.



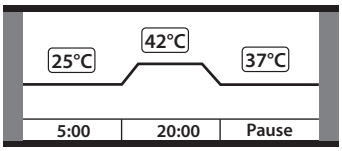
4.1.B. Synthesize cDNA

Required in Part 4.1.B.

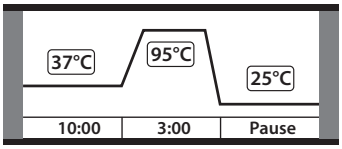
Component Name	Tube Label	Cap Color
RNA Fragmentation Solution	Fragmentation Solution	Green
ScriptSeq v2 cDNA Synthesis PreMix	cDNA Synthesis PreMix	Red
100 mM DTT	100 mM DTT	
StarScript AMV Reverse Transcriptase	StarScript AMV Reverse Transcriptase	
ScriptSeq Finishing Solution	Finishing Solution	
Nuclease-Free Water	Nuclease-Free Water	Clear

Thermocycler settings for Part 4.1.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



Note: *The RNA Fragmentation Solution is added to supplement Mg^{2+} in the cDNA synthesis reaction.*

1. Prepare the cDNA Synthesis Master Mix.

   For each reaction, combine on ice:

1.0	µl	Fragmentation Solution
3.0	µl	cDNA Synthesis Premix
0.5	µl	100 mM DTT
0.5	µl	StarScript AMV Reverse Transcriptase
5	µl	Total volume per reaction

   Gently but thoroughly mix the cDNA Synthesis Master Mix by pipetting 10 times.
2. Add 5 µl of the cDNA Synthesis Master Mix to each reaction on ice from Part 4.1.A, Step 3 and mix gently but thoroughly.
3. Incubate at 25°C for 5 minutes followed by 42°C for 20 minutes.
4. Cool the reactions to 37°C and pause the thermocycler.
5. Remove one reaction or strip of tubes at a time from the thermocycler, add 1.0 µl of Finishing Solution, and mix gently but thoroughly by pipetting. Return each reaction to the thermocycler before proceeding with the next.
6. Incubate at 37°C for 10 minutes.
7. Incubate the reactions at 95°C for 3 minutes. Then, allow the reactions to cool to 25°C and Pause/Hold the thermocycler.
8. Continue with the standard kit procedure beginning at Part 3.C.

Appendix 2: Sequencing the ScriptSeq v2 Library

ScriptSeq RNA-Seq libraries are compatible with TruSeq™ Cluster Kits and can be sequenced on any Illumina® sequencer.

The sequence produced by the Read 1 Sequencing Primer is that of the sense strand of the original fragmented RNA molecule. To map ScriptSeq library sequencing data using TopHat/Cufflinks, use the **fr-secondstrand** command.

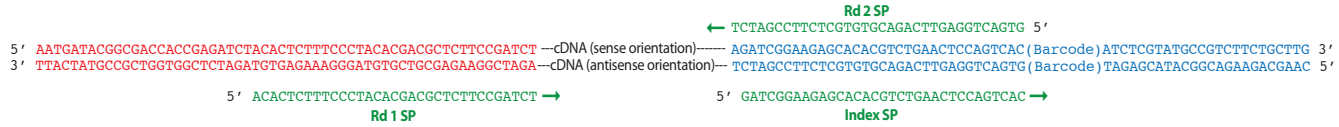


Figure 2. Sequencing a ScriptSeq™ v2 library.

Red	=	sequence incorporated by the Terminal Tagging process and PCR amplification.
Blue	=	sequence incorporated during reverse transcription and PCR amplification.
Black	=	sequence of the cDNA.
Rd 1 SP	=	sequence read is that of the sense strand of the original fragmented RNA molecule.
Rd 2 SP	=	sequence read is that of the antisense strand of the original fragmented RNA molecule.
Index SP	=	first nucleotide read is that of the Index or barcode.

Appendix 3: Adding a User-Defined Barcode to the Library

A barcode is added by the Reverse PCR Primer in Part 3.E of the procedure. A Reverse PCR Primer containing a user-defined barcode sequence must be synthesized by the user and is then used as the Reverse PCR Primer in Part 3.E of the procedure.

The user-defined Reverse PCR Primer(s) *must* be the following sequence:

5′ CAAGCAGAAGACGGCATACGAGAT **desired barcode's reverse complement** GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3′

The primer(s) should be dissolved to a concentration of 10 µM in nuclease-free water.

Important! *The user-defined barcode sequence of the of the custom synthesized Reverse PCR Primer should be the reverse complement of the sequence read. For example, using the Illumina Multiplexing Index Read Sequencing Primer, the user-defined barcode sequence:*

5′...ACGTAC... 3′ will be read as: 5′...GTACGT...3′

Please contact Technical Support if you have questions about adding user-defined barcodes or synthesizing custom reverse PCR primers.

Appendix 4. Troubleshooting and FAQs

Observation/Question	Recommendation
Library Preparation	
Does PCR have to be performed?	Yes. PCR completes the addition of the Illumina adaptor sequences. At least one cycle of PCR must be performed.
There is not enough of the Terminal Tagging Oligo (TTO) Solution	The Terminal Tagging Solution is very viscous. Please spin down the tube in a microcentrifuge at maximum speed for 30 seconds. Make sure your pipettes are calibrated. Pipette the solution slowly. Be sure that TTO solution does not adhere to the outside of the pipette tip when it is withdrawn from the solution. If possible, use a wide-bore pipette tip when pipetting the TTO solution.
There is a bi-modal peak in bioanalyzer trace of my ScriptSeq library	The library was overamplified during the PCR. Re-create the libraries with fewer cycles. Alternatively a double SPRI cleanup can remove large fragments. See also Figure 4C in Step 3.G.
Sequencing	
Which TruSeq Cluster kits are compatible with ScriptSeq Libraries?	TruSeq Cluster kits that use the HP8 and HP10 sequencing primers are compatible with SctiptSeq libraries.
How do I choose which Indexes to use when making Indexed ScriptSeq libraries?	Please see the ScriptSeq Index PCR Primers protocol for more informtation.
On the sequencing sample sheet, what kit setting should I use to sequence the ScriptSeq libraries?	Tru-Seq LT
Can I use the TruSeq LT Index adaptors when making an Indexed ScriptSeq library?	No. Only the ScriptSeq Index PCR Primers can be used to add an Index to a ScriptSeq library. Although the 6-nucleotide Index sequence is the same for each ScriptSeq Index Primer and the corresponding TruSeq Index Adapter, the flanking sequences are different. Therefore, the ScriptSeq Index Primers and TruSeq Index Adapters can not be used interchangably.
Data Anlaysis	
When doing data analysis, which strand do I map to in TopHat/Cufflinks?	Please use the fr-secondstrand command when performing ScriptSeq library analysis.
Are ScriptSeq libraries stranded?	Yes. The sequence generated by the Read 1 Sequencing Primer corresponds to the sense strand of the original fragmented RNA molecule. See Appendix 2.

5. Additional RNA-Seq Sample Prep Products

Ribo-Zero™ rRNA Removal Kits and Globin-Zero™ Gold Kit for globin mRNA/rRNA removal are available separately for many sample types.

ScriptSeq™ Complete Kits, combining a Ribo-Zero™ rRNA removal or Globin-Zero™ globin mRNA/rRNA removal module and a ScriptSeq™ v2 RNA-Seq Library Preparation Kit module, are available for many sample types.

ScriptSeq™ Index PCR Primers for adding an Index to the ScriptSeq libraries.

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