epicentre®
an Illumina® company

ScriptSeq™ Complete Kit*

(Human/Mouse/Rat)–Low Input

Cat. No. SCL6H – 6 Reactions
(Contains 1 box of Cat. No. LIH1206,
1 box of Cat. No. LIMC126 and 1 box of SSV21106)

Cat. No. SCL24H – 24 Reactions
(Contains 1 box of Cat. No. LIH1224,
1 box of Cat. No. LIMC1204 and 1 box of SSV21124)

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

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

Connect with Epicentre on our blog **(epicentral.blogspot.com)**,
Facebook **(facebook.com/EpicentreBio)**, and Twitter **(@EpicentreBio)**.

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, including:

Clinical research samples

Human/Mouse/Rat

Blood

Bacteria

Plants

Other

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, including:

Clinical research samples

Human/Mouse/Rat

Blood

Bacteria

Plants

Other

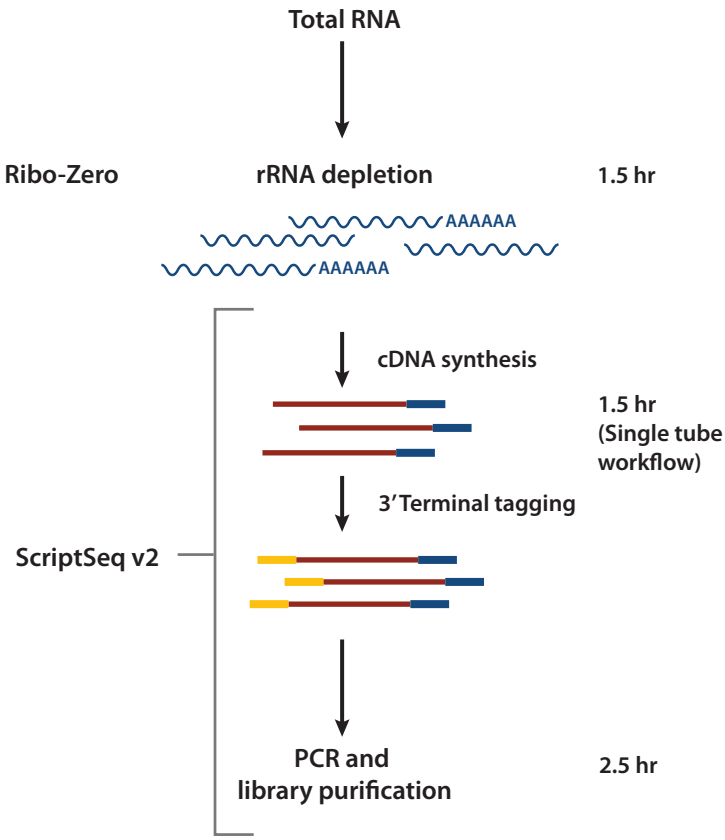


Figure 1. An overview of the ScriptSeq Complete Kit (Human/Mouse/Rat)–Low Input procedure. rRNA is first removed from the sample using the Ribo-Zero Magnetic Kit (Human/Mouse/Rat)–Low Input. The ScriptSeq v2 RNA-Seq Library Preparation Kit is then used to make the RNA-Seq library from the Ribo-Zero treated RNA.

The ScriptSeq Complete Kit (Human/Mouse/Rat)–Low Input is composed of three boxes:

– Ribo-Zero™ rRNA Removal Reagents (Human/Mouse/Rat)–Low Input

– Magnetic Core Kit–Low Input

– ScriptSeq™ v2 RNA-Seq Library Preparation Kit

1. RNA Sample Considerations

DNA-Free RNA

Treat the RNA sample with DNase I to remove all traces of DNA. Then, remove the DNase I prior to the Ribo-Zero reaction. DNA contamination will interfere with rRNA removal and is the main cause of loss of directionality when sequencing the ScriptSeq Complete library. The RNA sample should be free of salts (e.g., Mg^{2+} or guanidinium salts) or organics (e.g., phenol and ethanol).

Amount of RNA

The Ribo-Zero process uses 100 ng - 1 µg of DNA-free RNA. The sample should be dissolved in RNase-Free Water or TE Buffer. It is important to quantify the amount of total RNA in the sample as accurately as possible in order to use the appropriate amount of Ribo-Zero rRNA Removal Solution in Part 3.B. Use Table 1 (Part 3.B) to determine the maximum volume in which the total RNA sample can be dissolved prior to performing the Ribo-Zero procedure.

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

RNA extracted from FFPE tissue can be used to prepare rRNA-depleted ScriptSeq Complete 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.

Ribo-Zero treatment of FFPE RNA samples does not require any modifications to the procedure presented here. However, the use of FFPE RNA does require procedural modifications to the ScriptSeq library preparation procedure. Be sure to read Part 5.A, 5.D, and Appendix 1 before proceeding.

If possible, determine the size distribution of an FFPE RNA sample (e.g., Agilent 2100 Bioanalyzer) before beginning the Ribo-Zero procedure. The size of the FFPE RNA will determine the best method to purify the ScriptSeq Complete library in Part 5.F.

2. Kit Contents and Specifications

Ribo-Zero™ Kit (Human/Mouse/Rat)–Low Input–Used in Section 1

The kit components are supplied in tubes with colored caps for easier identification.
Each kit contains one box of Cat. No. LIH1206/LIH1224 and one box of Cat. No. LIMC126/LIMC1204.

Ribo-Zero rRNA Removal Reagents (Human/Mouse/Rat)–Low Input (Cat. No. LIH1204/LIH1224)

Component Name	Tube Label	Volume		Cap Color
		6-rxn	24-rxn	
RiboGuard RNase Inhibitor (100 U/µl)	RiboGuard™ RNase Inhibitor	10 µl	15 µl	Blue
Ribo-Zero rRNA Removal Solution (Human/Mouse/Rat)–Low Input	rRNA Removal Solution–(H/M/R)	30 µl	110 µl	
Ribo-Zero Reaction Buffer	Ribo-Zero™ Reaction Buffer	20 µl	60 µl	
Glycogen (10 mg/ml)	Glycogen	20 µl	60 µl	Clear
Sodium Acetate (3 M)	Sodium Acetate	120 µl	500 µl	
RNase-Free Water	RNase-Free Water	1 ml	3 x 1.5 ml	

Storage: Store this kit box and its contents at –70°C to –80°C.

***Magnetic Core Kit (Cat. No. LIMC126/LIMC1204)*–Used in Section 1**

Component Name	Tube Label	Volume		Cap Color
		6-rxn	24-rxn	
Magnetic Beads	Magnetic Beads	600 µl	4 x 6 µl	Clear
Magnetic Bead Resuspension Solution	Magnetic Bead Resuspension Solution	250 µl	1 ml	Yellow/Clear
RNase-Free Water	RNase-Free Water	1.5 ml	3 x 1.5 ml	Clear

Storage: Store this kit box and its contents at 4°C (Do Not Freeze!).

Additional Required Reagents and Equipment for the Ribo-Zero procedure:

Magnetic rack or stand for 1.5 ml tubes
0.2-ml or 0.5-ml and 1.5 ml microcentrifuge tubes (RNase-free)
Thermocycler or other temperature control device for 0.2-ml or 0.5-ml tubes
Water bath, heating block, or other temperature control device for 1.5-ml tubes
RNA purification kits such as Agencourt RNAClean™ XP Kit (Beckman Coulter), or Qiagen RNeasy™ MinElute® Cleanup Kit, or ice-cold 100% and 70% ethanol for ethanol precipitation.
Vortex mixer

ScriptSeq™ v2 RNA-Seq Library Preparation Kit–Catalog No. SSV21106/SSV21124–Used in Section 2

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 Reverse Transcriptase	StarScript 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 –20°C in a freezer without a defrost cycle.

Additional Required Reagents and Equipment for the ScriptSeq v2 Kit procedure:

FailSafe™ PCR Enzyme Mix (Epicentre)
Optional: MinElute PCR Purification columns (Qiagen)
Optional: Agencourt AMPure XP System (Beckman Coulter) and magnetic plate, rack, or stand for 1.5-ml tubes

Performance Specifications and Quality Control

A Ribo-Zero Magnetic Kit (Human/Mouse/Rat)–Low Input reaction removes >99% of 28S, 18S, and 5.8S and >95% of 5S rRNAs from intact Human Reference RNA as assessed by qRT-PCR before and after a Ribo-Zero Magnetic Kit reaction.

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 size distribution of 100-1,000 bp, and a peak range of 150-300 bp as assayed using an Agilent Bioanalyzer.

3. Kit Procedure

Section 1: The Ribo-Zero™ rRNA removal procedure

Quick Protocol for Ribo-Zero™ Kit (Human/Mouse/Rat)–Low Input

For experienced users only! The detailed procedure begins at Step 3.A

Step	Procedure	Pages
Prepare Magnetic Beads	Add 90 µl Magnetic Beads to RNase-free tube. Place in magnetic stand for 1 minute at RT. Wash with 90 µl RNase-Free Water. Place in magnetic stand, repeat wash step. Resuspend in 35 µl Resuspension Solution Optional: Add 0.5 µl RiboGuard RNase Inhibitor	5
Treat sample with rRNA Removal Solution	Mix in 40 µl total volume: 100 ng - 1 µg total RNA 2 -4 µl rRNA Removal Solution 2 µl Reaction Buffer Incubate 10 min @ 68˚C, then 5 minutes @ RT	6
Remove rRNA	Mix previously prepared Magnetic Beads Add RNA mixture, mix well by pipetting, vortex briefly Incubate 5 minutes @ RT, vortex Incubate 5 minutes @ 50˚C Place in magnetic stand, transfer supernatant (rRNA-depleted sample) to RNase-free tube	7
Purify rRNA-depleted sample	Ethanol precipitation or alternative method	7

3.A. Preparation of the Magnetic Beads

Required in Part 3.A

Component Name	Tube Label	Cap Color
Magnetic Beads	Magnetic Beads	Clear
RNase-Free Water	RNase-Free Water	
Magnetic Bead Resuspension Solution	Magnetic Bead Resuspension Solution	Yellow
RiboGuard RNase Inhibitor (100 U/µl)	RiboGuard RNase Inhibitor	Blue

Important! *Allow the Magnetic Core Kit components to equilibrate to room temperature before proceeding!*

Remove the Ribo-Zero rRNA Removal Reagents from –70°C to –80°C storage, thaw the tubes, and place them on ice. Wash the beads by using either the batch washing or individual washing procedure.

3.A.1. Batch Washing Procedure

1. For each Ribo-Zero reaction, 90 µl of the Magnetic Beads is required.

   Note: *Mix the Magnetic Beads well by pipetting or gentle vortexing.*
2. Determine the amount of Magnetic Beads required for the total number of reactions and dispense a maximum of 1,080 µl into each 1.5-ml RNase-free microcentrifuge tube (sufficient for 12 reactions). Pipet the Magnetic Bead suspension slowly to avoid air bubbles and to ensure pipetting of the correct volume. Store unused Magnetic Beads at 4°C.

   Note: *When setting up more than 12 Ribo-Zero reactions, either multiples of 1,080-µl aliquots can be washed in a RNase-free 1.5 ml microcentrifuge tubes, or a larger volume can be washed in RNase-free 15-ml tubes (e.g., using a 15-ml magnetic stand).*
3. Place the 1.5-ml microcentrifuge tube containing the Magnetic Beads on the magnetic stand for at least 1 minute (until the solution appears clear).
4. With the 1.5-ml microcentrifuge tube still on the stand, remove and discard the supernatant.

   ⚠ ***Caution:*** *The supernatant contains 0.1% sodium azide.*
5. Remove the 1.5-ml microcentrifuge tube from the stand and add an equal volume of RNase-Free Water. Mix well by repeated pipetting or by vortexing at medium speed.
6. Repeat steps 3, 4 and 5 (i.e. wash the beads a total of 2 times with RNase-Free water).
7. Remove the 1.5-ml microcentrifuge tube from the magnetic stand. Add a volume of Magnetic Bead Resuspension Solution equal to the number of reactions x 35 µl (e.g., for 12 reactions, add 12 x 35 µl = 420 µl Magnetic Bead Resuspension Solution). Mix well by repeated pipetting or by vortexing at medium speed.
8. Aliquot 35 µl of the washed Magnetic Beads into each new 1.5-ml RNase-free microcentrifuge tube (corresponding to the number of Ribo-Zero reactions).
9. Optional: Add 0.5 µl of RiboGuard RNase Inhibitor to each tube of resuspended Magnetic Beads, and mix briefly by vortexing.
10. Store the microcentrifuge tubes at room temperature until required in Part 3.C.

3.A.2. Individual Washing Procedure

Note: *Mix the Magnetic Beads well by pipetting or gentle vortexing.*

1. For each reaction, pipet 90 µl of Magnetic Beads into a 1.5-ml RNase-free microcentrifuge tube. Pipet the Magnetic Bead suspension slowly to avoid air bubbles and to ensure pipetting of the correct volume. Store unused Magnetic Beads at 4°C.
2. Place each 1.5-ml microcentrifuge tube on the magnetic stand for at least 1 minute (until the solution appears clear).
3. With the 1.5-ml microcentrifuge tube still on the stand, remove and discard the supernatant.

   ⚠ ***Caution:*** *The supernatant contains 0.1% sodium azide.*
4. Remove the 1.5-ml microcentrifuge tube from the magnetic stand and add 90 µl of RNase-Free Water to each tube. Mix well by repeated pipetting or vortexing at medium speed.
5. Repeat steps 2, 3 and 4 (i.e. wash the beads a total of 2 times with RNase-Free water).
6. Remove the 1.5-ml microcentrifuge tube from the magnetic stand. Add 35 µl of Magnetic Bead Resuspension Solution to each tube. Mix well by repeated pipetting or vortexing at medium speed.
7. Optional: Add 0.5 µl of RiboGuard RNase Inhibitor to each tube of resuspended Magnetic Beads, and mix briefly by vortexing.
8. Store the microcentrifuge tubes at room temperature until required in Part 3.C.

3.B. Treatment of the Total RNA Sample with Ribo-Zero rRNA Removal Solution

Required in Part 3.B

Component Name	Tube Label	Cap Color
Ribo-Zero Reaction Buffer	Ribo-Zero Reaction Buffer	Blue
Ribo-Zero rRNA Removal Solution (Human/Mouse/Rat)–Low Input	rRNA Removal Solution (H/M/R)	
RNase-Free Water	RNase-Free Water	Clear

Additionally required for each reaction (provided by user):

Magnetic stand or rack
0.2-ml or 0.5-ml microcentrifuge tube (RNase-free)
Vortex mixer

⚠ ***Important!*** *The maximum volume of the RNA sample and the volume of the Ribo-Zero rRNA Removal Solution used per reaction is dependent on the amount of total RNA in the sample (see Table 1).*

Table 1. Volumes of Ribo-Zero™ rRNA Removal Solution.

Amount of Input Total RNA	Maximum Volume of Total RNA That Can Be Added to Each Reaction	Volume of Ribo-Zero rRNA Removal Solution Used per Reaction
100 ng - 250 ng	16 µl	2 µl
>250 ng - 1 µg	14 µl	4 µl

1. In a 0.2-ml or 0.5-ml RNase-free microcentrifuge tube, combine in the order given:

x	µl	RNase-Free Water
2	µl	Ribo-Zero Reaction Buffer
100 ng - 1	µg	Total RNA sample (see Table 1)
y	µl	Ribo-Zero rRNA Removal Solution (see Table 1)
20	µl	Total volume

2. Gently mix the reaction(s) by pipetting and incubate at 68°C for 10 minutes. Store the remaining Ribo-Zero rRNA Removal Solution and Ribo-Zero Reaction Buffer at –70°C to –80°C.
3. Remove the reaction tube(s) and incubate each at room temperature for 5 minutes.

3.C. Magnetic Bead Reaction and rRNA Removal

Required in Part 3.C: 50°C water bath or heating block for 2.0-ml tubes.

1. Using a pipette, add the treated RNA from Part 3.B to the 1.5-ml microcentrifuge tube containing the washed Magnetic Beads and, without changing the pipet tip, ***immediately and thoroughly mix the contents of the tube by pipetting at least 10 times. Then, vortex the tube immediately at medium setting for 10 seconds and place at room temperature.*** Repeat this process for each sample.

   Important! *Always add the treated RNA sample to the washed Magnetic Beads and immediately mix by pipetting. Never add the Magnetic Beads to the treated RNA sample. Immediate and thorough mixing prevents the beads from forming clumps that can significantly impact the efficiency of the rRNA removal.*
2. Incubate the 1.5 ml microcentrifuge tube at room temperature for 5 minutes.
3. Following incubation, mix the reactions by vortexing at medium speed for 10 seconds and then place at 50°C for 5 minutes in an appropriate water bath or heating block. Avoid any significant condensation during this incubation step.
4. After the 5-minute incubation at 50°C, remove the microcentrifuge tubes and immediately place them on a magnetic stand for at least 1 minute (until the solution appears clear).
5. Carefully remove each supernatant (50-52 µl) containing the RNA and transfer to a labeled 1.5-ml RNase-free microcentrifuge tube.

   ⚠ ***Important!*** *The supernatant contains rRNA-depleted RNA.*

   Optional: *If a small amount of Magnetic Beads are still visible in the supernatant, place the collected supernatant onto the magnetic stand for 1 minute. Remove the supernatant containing the rRNA-depleted RNA and transfer to a new 1.5-ml RNase-free microcentrifuge tube.*
6. Place the supernatant (RNA solution) on ice and immediately proceed to Part 3.D. Alternatively, the supernatant may be stored at –70°C to –80°C before completing Part 3.D.

3.D. Purification of the rRNA-Depleted Sample

The rRNA-depleted samples can be purified by three methods: ethanol precipitation, AMPure beads, or spin columns. Ethanol precipitation and the modified RNeasy MinElute procedure provide optimal recovery of small RNAs that may be lost with other purification methods; however, ethanol precipitation can be challenging for inexperienced users. We also provide an alternative protocol using AMPure beads (provided by the user) for ease of automation but this will not quantitatively recover small RNAs.

3.D.1. Ethanol Precipitation of the rRNA-Depleted Sample

Component Name	Tube Label	Cap Color
RNase-Free Water	RNase-Free Water	Clear
Sodium Acetate (3 M)	Sodium Acetate	
Glycogen (10 mg/ml)	Glycogen	

Additionally required for each reaction (provided by user):

1.5-ml microcentrifuge tube (RNase-free)
Ice-cold 70% and 100% ethanol

1. Adjust the volume of each sample to 180 µl using RNase-Free Water.
2. Add 18 µl of 3 M Sodium Acetate to each tube.
3. Add 2 µl of Glycogen (10 mg/ml) to each tube and mix by gentle vortexing.
4. Add three volumes (600 µl) of ice-cold 100% ethanol to each tube and mix thoroughly by gentle vortexing.
5. Place the tubes at –20°C for at least 1 hour.
6. Centrifuge the tubes at >10,000 x g in a microcentrifuge for 30 minutes. Carefully remove and discard the supernatant.
7. Wash the pellet with ice-cold 70% ethanol and centrifuge at >10,000 x g for 5 minutes. Carefully remove and discard the supernatant.
8. Repeat Step 7 (above) one more time.
9. Centrifuge briefly to collect any residual supernatant. Carefully remove and discard the supernatant and allow the pellet to air dry at room temperature for 5 minutes.
10. Dissolve the pellet in 10 µl of RNase-Free Water or buffer. The Ribo-Zero-treated RNA can be used immediately or stored at –70°C to –80°C.

3.D.2. Agencourt RNAClean XP Kit (Cat. No. A63987, Beckman Coulter)

Note: *A fresh 80% ethanol solution is required for Steps 5 and 7 below.*

1. Vortex the AMPure RNAClean XP Beads until they are well dispersed, then add as follows:

   Add 160 µl of the mixed AMPure XP Beads to each 1.5-ml microcentrifuge tube containing 85-90 µl of rRNA-depleted RNA from Part 3.C, Step 6. Mix thoroughly by gently pipetting the entire volume 10 times.
2. Incubate the tube(s) at room temperature for 15 minutes. During incubation, prepare 80% ethanol solution required for Steps 5 and 7.
3. Place the tube(s) on the magnetic stand at room temperature for at least 5 minutes (until the liquid appears clear).
4. Remove and discard the supernatant from each tube. Do not disturb the beads.
5. With the tube(s) still on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to each tube, without disturbing the beads.
6. Incubate at room temperature for at least 30 seconds while still on the magnetic stand. Then remove and discard all of the supernatant from each tube. Do not disturb the beads.
7. Repeat Steps 5 and 6 (total of two 80% ethanol washes).
8. Allow the tubes to air dry on the magnetic stand at room temperature for 15 minutes.
9. Add 11 µl of RNase-Free water to each tube.
10. Thoroughly resuspend the beads by gently pipetting 10 times.
11. Incubate the tubes at room temperature for 2 minutes.
12. Place the tubes back onto the magnetic stand at room temperature for at least 5 minutes (until the liquid appears clear).
13. Transfer the clear supernatant from each tube to an appropriate collection tube, always leaving at least 1 µl of the supernatant behind to avoid carryover of magnetic particles. Store on ice for immediate use or store at –70°C or –80°C until required.

3.D.3. RNeasy MinElute Cleanup Kit (Cat. No. 74204, Qiagen)

Note: *RNA purification kits from other suppliers may also be used; however, performance may vary.*

1. Adjust the sample to a volume of 100 µl with RNase-Free Water. Add 350 µl of Buffer RLT, and mix well.
2. Add 550 µl of 96%-100% ethanol to the RNA, and mix well by pipetting. Do not centrifuge.
3. Transfer half of the sample (~500 µl) to an RNeasy MinElute spin column placed in a 2-ml collection tube (supplied in the Qiagen kit). Close the lid gently, and centrifuge for 15 seconds at 8,000 x g (~10,000 rpm). Discard the flow-through. Reuse the collection tube for Step 5.
4. Transfer the remaining sample and repeat the centrifugation. Discard the flow-through and collection tube.
5. Place the RNeasy MinElute spin column in a new 2-ml collection tube (supplied in the Qiagen kit). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 seconds at 8,000 x g (~10,000 rpm) to wash the spin-column membrane. Discard the flow-through.

   Note: *Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.*

6. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 minutes at 8,000 x g (~10,000 rpm) to wash the spin-column membrane. Discard the flow-through and collection tube.

   Note: *After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.*

7. Place the RNeasy MinElute spin column in a new 2-ml collection tube (supplied in the Qiagen kit).
8. Open the lid of the spin column, and centrifuge at full speed for 5 minutes. Discard the flow-through and collection tube.
9. To avoid damage to the spin-column lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (i.e., if the rotor rotates clockwise, orient the lids counterclockwise).
10. It is important to dry the spin-column membrane since residual ethanol may interfere with downstream applications. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.
11. Place the RNeasy MinElute spin column in a new 1.5-ml collection tube (supplied in the Qiagen kit). Add 12 µl of RNase-Free Water directly to the center of the spin-column membrane. Close the lid gently, and centrifuge for 1 minute at full speed to elute the RNA. Recovery is usually 10 µl.

    The eluted RNA can be used immediately or stored at –70°C to –80°C.

4. Before Proceeding to ScriptSeq v2 Library Preparation

It is not necessary, but if desired the quality of the Ribo-Zero treated RNA can be assessed before proceeding to the ScriptSeq v2 Library prep procedure in Section 2. If assessing the quality of the Ribo-Zero treated RNA using an Agilent 2100 Bioanalyzer, use the Agilent RNA6000 Pico Chip and load 2-4 ng of the Ribo-Zero treated RNA. The Agilent RNA Nano Chip does not provide sufficient sensitivity. Note that if the Ribo-Zero treated RNA was purified by ethanol precipitation in Step 3.D.1, small RNAs such as tRNA are recovered along with the mRNAs and large noncoding RNAs. Therefore, the presence of a high proportion of small RNA in the Ribo-Zero treated sample should not be interpreted as degradation of the RNA.

For the Ribo-Zero-treated RNA, use Table 2 as a guide to the maximum volume of the purified RNA to use in ScriptSeq v2 library preparation. The indicated volume corresponds to to the amount of Ribo-Zero treated RNA typically recovered from 1 ug of total RNA.

Note: *Ribo-Zero-treated RNA purified by any of the three methods described in Part 3.D will be recovered in approximately 10 µl of RNase-Free Water.*

Table 2. Volume of Ribo-Zero™-treated RNA to use in ScriptSeq™ v2 library preparation.

RNA Sample	Amount of Total RNA Used in the Ribo-Zero Procedure	Maximum Volume of Purified Ribo-Zero-Treated RNA for ScriptSeq v2 Reaction
Human/Mouse/Rat	1 µg	5 µl
	500 ng	9 µl
	100 ng	9 µl

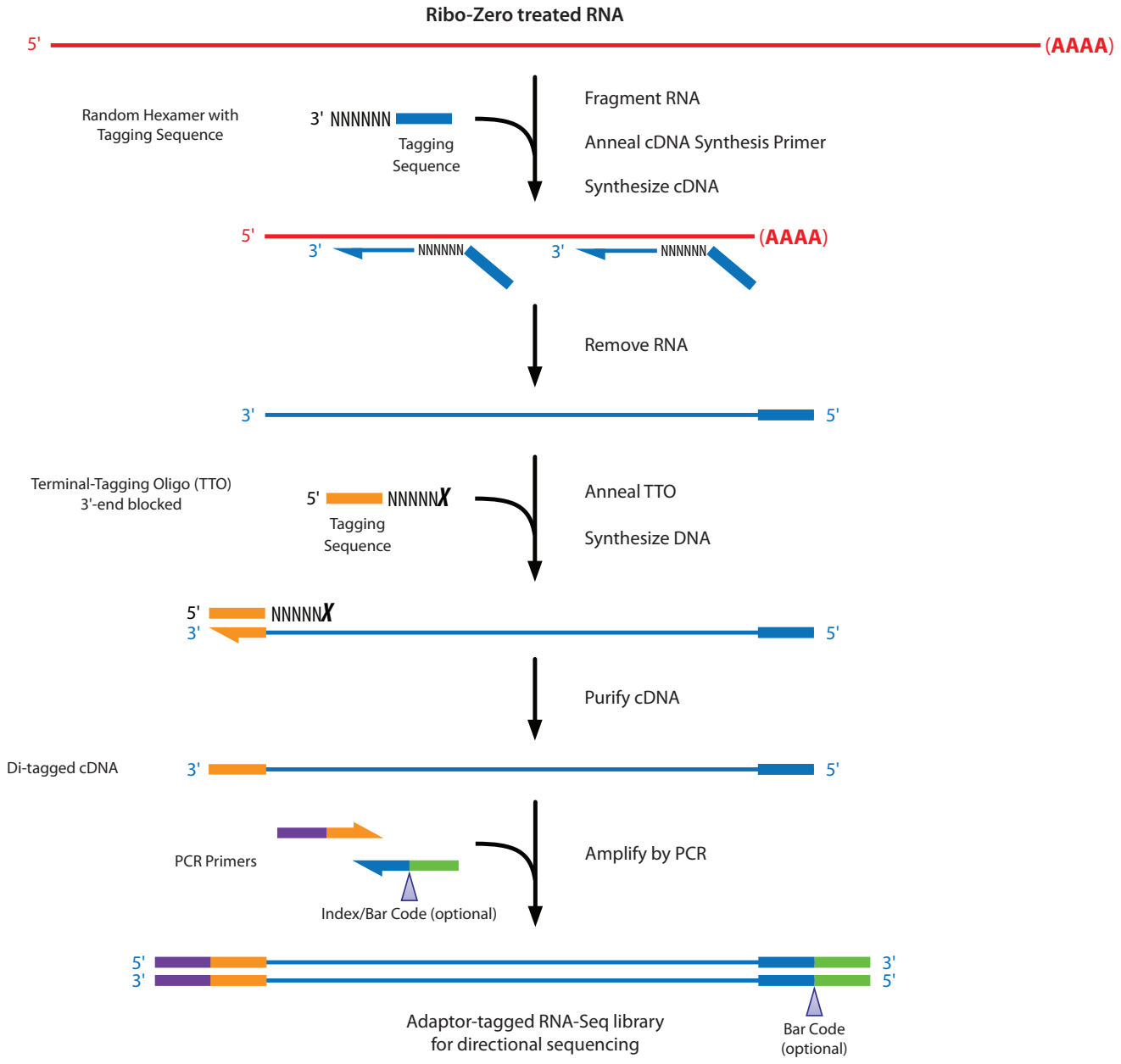


Figure 2. An overview of the procedure for the ScriptSeq™ v2 RNA-Seq Library Preparation Kit.

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

For experienced users only! The detailed procedure begins at Step 5.

Step	Procedure	Pages
Fragment RNA	If using RNA from FFPE or severely degraded samples, go to Appendix 1. 1. Mix the following: x µl Nuclease-Free Water y µl Ribo-Zero treated RNA (See Table 2, pg. 9) 1 µl RNA Fragmentation Solution 2 µl cDNA Synthesis Primer 12 µl Total volume 2. Incubate at 85°C for 5 minutes in thermocycler then place on ice.	12
Synthesize cDNA	1. Mix the following: 3.0 µl cDNA Synthesis PreMix 0.5 µl 100 mM DTT 0.5 µl StarScript Reverse Transcriptase 4 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. 7. Incubate at 95°C for 3 min, cool to 25°C and Pause the thermocycler.	12
Terminal-Tag cDNA	1. Mix the following: 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. 2. Add 8.0 µl of Terminal Tagging Master Mix to each reaction. Mix by pipetting. 3. Incubate reaction at 25°C for 15 minutes. 4. Incubate reaction at 95°C for 3 minutes. Cool to 4°C. The cDNA is now di-tagged.	13
Purify cDNA	Choose Qiagen MinElute or Agencourt AMPure purification. Elute in 22.5 µl.	13
PCR	If adding optional barcodes, go to p. 14 of protocol and skip this Quick Reference Protocol. If **not** adding barcodes: Mix in a 0.2-ml PCR tube: 22.5 µl di-tagged cDNA 25 µl FailSafe PCR PreMix E 1 µl Forward PCR Primer 1 µl Reverse PCR Primer 0.5 µl FailSafe PCR Enzyme 50 µl total volume **PCR cycle conditions:** Denature the dsDNA at 95°C for 1 minute followed by 15 cycles of: 95°C for 30 seconds 55°C for 30 seconds 68°C for 3 minutes Incubate at 68°C for 7 minutes after the final cycle	14
Purify Library	AMPure purification. Qiagen purification only suggested for very short FFPE or severely degraded RNA samples (<200 nt) and will result in adaptor-dimer contamination.	15
QC Library	Quantify by Qubit or PicoGreen and visualize on Agilent BioAnalyzer	16

5. ScriptSeq Kit Procedure

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

5.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 5.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.

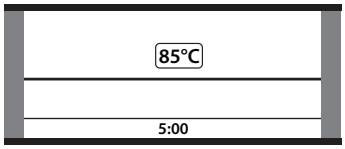
Required in Part 5.A.

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

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.

x	µl	Nuclease-Free Water
y	µl	Ribo-Zero-treated RNA (See Table 2, pg. 9)
1	µl	RNA Fragmentation Solution
2	µl	cDNA Synthesis Primer
12	µl	Total reaction volume

2. Fragment RNA: Incubate at 85°C for 5 minutes in a thermocycler.
3. Stop the fragmentation reaction by placing the tube on ice.



5.B. Synthesize cDNA

Required in Part 5.B.

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

Thermocycler settings for Part 5.B:

25°C for 5 minutes (cDNA synthesis)
42°C for 20 minutes (cDNA synthesis)
37°C Pause

37°C for 10 minutes (Finishing Solution)
95°C for 3 minutes (Inactivate Finishing Solution)
25°C Pause

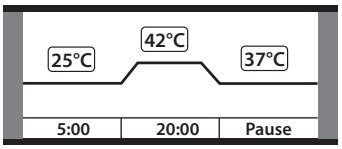
1. On ice, prepare the cDNA Synthesis Master Mix:
   For each reaction, combine on ice:

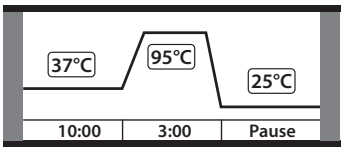
3.0	µl	cDNA Synthesis PreMix
0.5	µl	100 mM DTT
0.5	µl	StarScript Reverse Transcriptase
4.0	µl	Total volume

   Gently but thoroughly mix the cDNA Synthesis Master Mix by pipetting.
2. Add 4 µl of the cDNA Synthesis Master Mix to each reaction on ice from Part 5.A, Step 3, and mix by pipetting.

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 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 each reaction at 95°C for 3 minutes. Then, cool the reactions to 25°C and Pause the thermocycler.

   During the 95°C incubation, prepare the Terminal Tagging Master Mix as described in Part 5.C, Step 1.





5.C. 3′-Terminal-Tag the cDNA

Required in Part 5.C.

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

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

Thermocycler settings for Part 5.C:

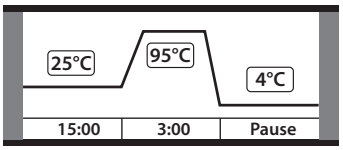
25°C for 15 minutes (DNA Polymerase)
95°C for 3 minutes (Inactivate DNA Polymerase)
4°C Hold or ice

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

   Thoroughly mix the viscous Terminal Tagging Master Mix by pipetting up and down several times or by flicking the tube.
2. Remove one reaction at a time from the thermocycler (from Part 5.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. Incubate each reaction at 25°C for 15 minutes.
4. Incubate each reaction at 95°C for 3 minutes. Then, cool the reactions to 4°C on ice or in the thermocycler.



5.D. Purify the cDNA

The 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 or severely degraded RNA <200 nts, you must use the MinElute PCR Purification kit.*

Option 1: MinElute column purification

Insert this above the "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.

Option 2: AMPure XP System

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 5.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 5.C, Step 4 independently into a well of the plate.
3. ⚠ ***Important!*** *Vortex the AMPure XP beads until they are a homogeneous suspension.*

4. Add 45 µl of the beads to each well of the 96-well plate or to each microfuge tube containing di-tagged cDNA from Part 5.C, Step 4.
5. Mix thoroughly by gently pipetting the entire volume of each well/tube 10 times.
6. If using microfuge tubes, transfer each 70-µl volume to a separate 1.5-ml tube.
7. Incubate the 96-well plate or 1.5-ml microfuge tubes at room temperature for 15 minutes.
8. Place the 96-well plate or the 1.5-ml tubes in a magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
9. 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.
10. With the plate or 1.5-ml tubes remaining on the magnetic stand, add 200 µl of 80% ethanol to each well/tube without disturbing the beads.
11. Incubate the plate or 1.5-ml tubes 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.
12. Repeat steps 10 and 11 one more time for a total of two 80% ethanol washes.
13. Allow the plate or tubes to air-dry on their magnetic stands for 15 minutes at room temperature.
14. Add 24.5 µl of Nuclease-Free Water to each well/tube and remove from the magnetic stand.
15. Thoroughly resuspend the beads by gently pipetting 10 times.
16. Incubate the plate or tubes at room temperature for 2 minutes.
17. Place the plate or tubes on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
18. 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.
19. Place the tubes on ice and proceed to Part 5.E or place at –20°C for longer-term storage.

5.E. 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, 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.
- 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 5.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

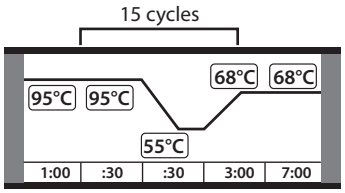
Provided by the user: FailSafe PCR Enzyme Mix (Epicentre).

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.*

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

22.5	µl	di-tagged cDNA from Part 5.D
25	µl	FailSafe PCR PreMix E
1	µl	Forward PCR Primer
1	µl	Reverse PCR Primer (or ScriptSeq Index PCR Primer, or user-defined barcode Reverse PCR Primer)
0.5	µl	FailSafe PCR Enzyme (1.25 U)
50	µl	Total volume

2. Perform PCR:



Denature the ds DNA at 95°C for 1 minute

Followed by 15 cycles* of:

95°C for 30 seconds

55°C for 30 seconds

68°C for 3 minutes

**Fewer PCR cycles can be performed*

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

During the PCR, read Part 5.F to determine which post-PCR purification procedure is best suited to your sample. After the PCR procedure is complete, proceed immediately to Part 5.F.

5.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 or severely degraded 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 or severely degraded RNA with an average size <200 nt. Libraries purified using the MinElute columns will be contaminated with primer-dimers.*

5.F.1. AMPure XP Purification

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 5.E, Step 2, independently into a well of the plate.
3. ⚠ ***Important!*** *Vortex the AMPure XP beads until they are a homogeneous suspension.*
4. Add 50 µl of the beads to each well of the 96-well plate or to each PCR tube.
5. Mix thoroughly by gently pipetting the entire volume up of each well/tube 10 times.
6. If using microfuge tubes, transfer each 100-µl volume to a separate 1.5 ml tube.
7. Incubate the 96-well plate or 1.5-ml microfuge tubes at room temperature for 15 minutes.
8. Place the 96-well plate or the 1.5-ml tubes in a magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
9. 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.
10. With the plate or 1.5-ml tubes remaining on the magnetic stand, add 200 µl of 80% ethanol to each well/tube without disturbing the beads.
11. Incubate the plate or 1.5-ml tubes at room temperature for at least 30 seconds, then remove and discard all of the supernatant. Take care not to disturb the beads.
12. Repeat steps 9 and 10 one more time for a total of two 80% ethanol washes.
13. Allow the plate or tubes to air-dry on their magnetic stands for 15 minutes at room temperature.
14. Add 20 µl of Nuclease-Free Water to each well/tube and remove the plate or 1.5-ml tubes from their magnetic stand.
15. Thoroughly resuspend the beads by gently pipetting 10 times.
16. Incubate the plate or tubes at room temperature for 2 minutes.
17. Place the plate or tubes on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
18. Transfer the clear supernatant, which contains theRNA-Seq library, from each well/tube to an appropriate collection tube for assessment of library quantity and quality.

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

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

5.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).

6. Appendices

Appendix 1: Preparing a Library from Severely Fragmented RNA

Use this procedure when preparing libraries of Ribo-Zero treated 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 5.A.

6.1.A. Anneal the cDNA Synthesis Primer

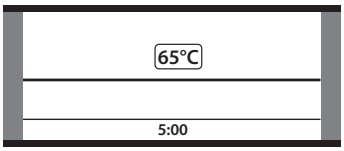
Required in Part 6.1.A.

Component Name	Tube Label	Cap Color
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	Ribo-Zero treated FFPE RNA (See Table 2, pg. 9)
2	µl	cDNA Synthesis Primer
11	µl	Total volume

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



6.1.B. Synthesize cDNA

Required in Part 6.1.B.

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

Thermocycler settings for Part 6.1.B:

25°C for 5 minutes (cDNA synthesis)
42°C for 20 minutes (cDNA synthesis)
37°C Pause

37°C for 10 minutes (Finishing Solution)
95°C for 3 minutes (Inactivate Finishing Solution)
25°C Pause

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

1. Prepare the cDNA Synthesis Master Mix.

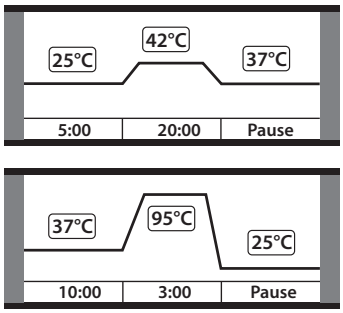
   For each reaction, combine on ice:

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

   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 6.1.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 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 each reaction at 95°C for 3 minutes. Then, allow the reactions to cool to 25°C and Pause the thermocycler.

   During the 95°C incubation, prepare the Terminal Tagging Master Mix as described in Part 5.C, Step 1.
8. Continue with the standard kit procedure beginning at Part 5.C.



Appendix 2: Sequencing a ScriptSeq v2 Library

Note: *Oligonucleotide sequences © 2006-2010 Illumina, Inc. All rights reserved.*

ScriptSeq RNA-Seq libraries are compatible with TruSeq Cluster Kits and can be sequenced on GAII, HiSeq and MiSeq sequencers.

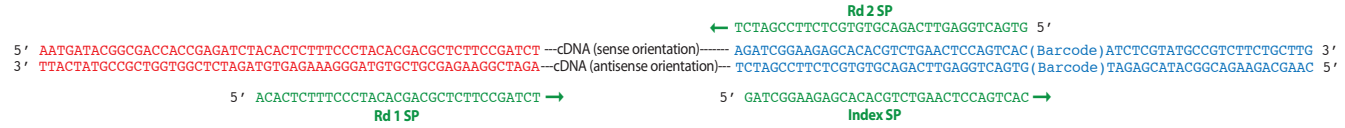


Figure 3. Sequencing a ScriptSeq™ v2 library.

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 generated is that of the sense strand of the original fragmented RNA molecule.
Rd 2 SP	=	sequence generated 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

Note: *Some nucleotide sequences shown in Appendix 3 are copyrighted to Illumina, Inc. Oligonucleotide sequences © 2006-2010 Illumina, Inc. All rights reserved.*

A barcode is added by the Reverse PCR Primer in Part 5.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 5.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 Epicentre's Technical Support if you have questions about adding user-defined barcodes or synthesizing custom reverse PCR primers.

Appendix 4: Profiles of ScriptSeq Complete RNA-Seq Libraries.

Fig. 4 shows representative 2100 Bioanalyzer (Agilent) profiles of RNA-Seq libraries produced by the ScriptSeq Complete Kit (Human/Mouse/Rat)—Low Input.

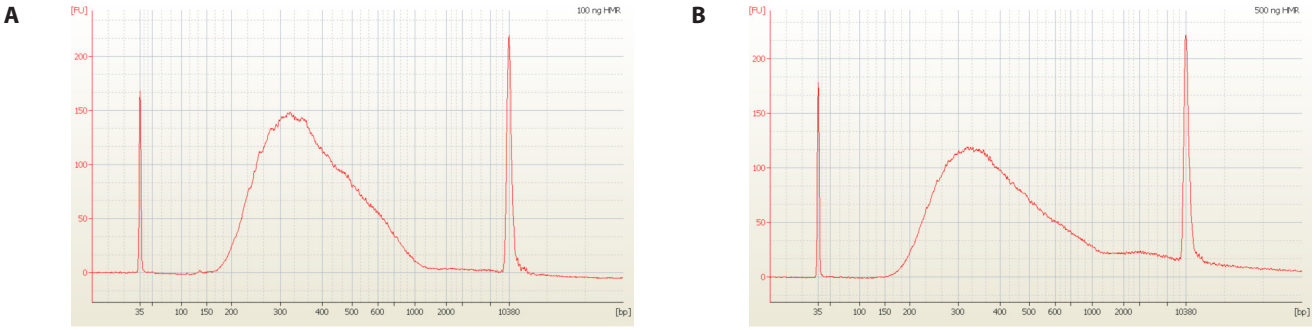


Figure 4. Representative profiles of ScriptSeq™ Complete libraries. Aliquots (0.1-1.0 µg) of Universal Human Reference RNA were treated with the Ribo-Zero Kit as outlined in Table 1. Following rRNA depletion, an aliquot corresponding to either 100 ng (A) or 500 ng (B) total RNA equivalence was used as input for the ScriptSeq v2 process. Average yields based on NanoDrop® quantitation were 21 ng/µl and 50 ng/µl, respectively. Libraries were purified using AMPure XP beads and approximately 2 ng of each library was analyzed on an Agilent 2100 Bioanalyzer using the high-sensitivity DNA assay.

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