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ScriptMiner™ Small RNA-Seq Library Preparation Kit

(SinglePlex; Illumina®-Compatible)

SMSP10908 – 8 Reactions

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1. Introduction

ScriptMiner Small RNA-Seq Library Preparation Kit (SinglePlex; Illumina®-compatible) Process:

1. **3′-Adaptor Oligo Ligation.** A 3′-tagging sequence (the 3′-Adaptor Oligo) is ligated to the 3′ end of the RNA. Small RNA with 3′ ends containing either a 2′,3′-OH or 2′-*O*-methyl, 3′-OH are efficiently tagged in the reaction.
2. **Modification of the 5′ End of the RNA (Optional).** If desired, small 5′-capped and 5′-triphosphorylated RNAs can be converted to 5′-monophosphorylated RNA for subsequent ligation of the 5′ Adaptor Oligo and inclusion in the sequencing library.
3. **Removal of Excess 3′-Adaptor Oligo.** Excess 3′-Adaptor Oligo, which can cause high background in the sequencing reaction, is significantly reduced using a proprietary Degradase Enzyme.
4. **5′-Adaptor Oligo Ligation.** A 5′-tagging sequence (the 5′-Adaptor Oligo) is ligated to the 5′ end of 5′-monophosphorylated RNA.
5. **Di-tagged RNA Purification.** The 5′- and 3′-tagged RNA (di-tagged RNA) is purified by a spin-column method (provided by the user).
6. **cDNA Synthesis.** The purified, di-tagged RNA is reverse-transcribed into cDNA using the cDNA Synthesis Primer and MMLV Reverse Transcriptase. The RNA is then removed by addition of RNase.
7. **PCR Amplification:** The di-tagged cDNA is amplified by limited-cycle PCR using the Forward and Reverse PCR Primers provided in the kit and a proofreading thermostable DNA polymerase (provided by the user).
8. **Purification of PCR Products** (provided by the user).

Additional Required Reagents and Equipment:

RNA purification columns, such as RNA Clean & Concentrator™-5 Kit (Zymo Research; Cat. No. R1015 or R1016), or RNA Clean & Concentrator™-25 Kit (Zymo Research; Cat. No. R1017 or R1018)

MinElute PCR Purification columns (Qiagen; Cat. No. 28004 or 28006)

Thermostable DNA Polymerase with proofreading activity such as Epicentre's FailSafe™ Enzyme Mix.

Performance Specifications and Quality Control

The ScriptMiner mRNA-Seq Library Preparation Kit (Illumina®-compatible) is function-tested in a control reaction using 100 fmol of 22-nucleotide (nt) Control RNA Oligo. The 92-bp PCR product (di-tagged cDNA) must be present, after 15 cycles of PCR, when 15%-20% by volume of the PCR samples are resolved on 8% native PAGE in 1X TBE buffer.

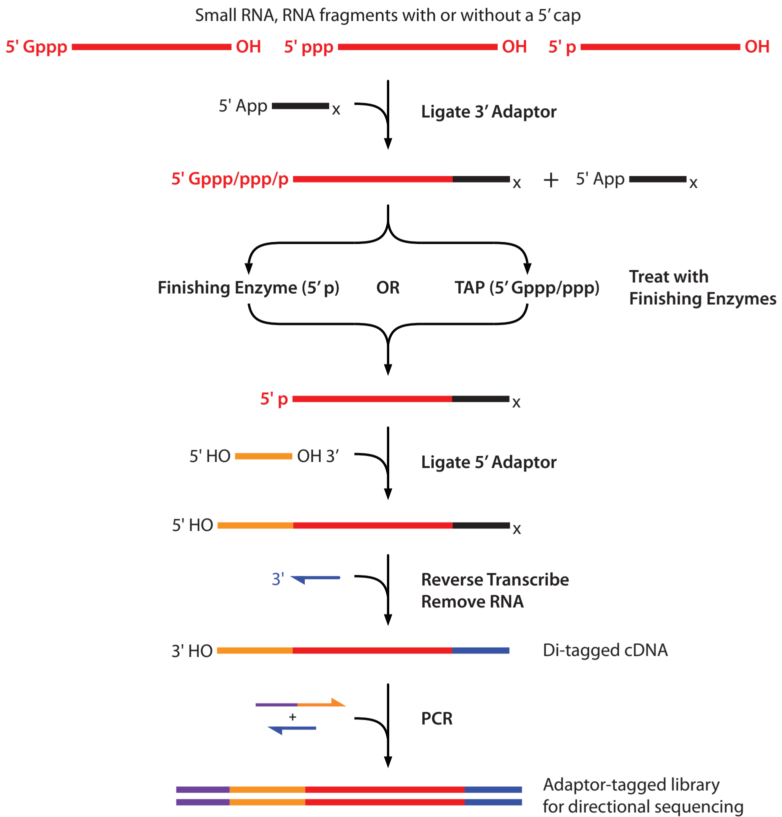

Figure 1. **An overview of the procedure for the ScriptMiner™ Small RNA-Seq Library Preparation Kit (SinglePlex, Illumina®-compatible).**

2. Kit Contents

Component Name	Tube Label	Volume	Cap Color
ScriptMiner™ 3′-RNA Ligation Buffer	3′ Ligation Buffer	10 µl	Green
PEG Solution	PEG Solution	80 µl	
DTT	DTT	25 µl	
RiboGuard™ RNase Inhibitor	RiboGuard™ RNase Inhibitor	10 µl	
ScriptMiner™ SinglePlex 3′-Adaptor Oligo	3′ Adaptor Oligo	10 µl	
ScriptMiner™ 3′-RNA Ligase	3′ RNA Ligase	10 µl	
ScriptMiner™ Finishing Enzyme	Finishing Enzyme	10 µl	
Tobacco Acid Pyrophosphatase	TAP	10 µl	
Ligation STOP Solution	Ligation STOP Solution	40 µl	
ScriptMiner™ Degradase Buffer	Degradase Buffer	20 µl	Clear
$MgCl_2$	$MgCl_2$	70 µl	
ScriptMiner™ Degradase Enzyme	Degradase Enzyme	10 µl	
ScriptMiner™ Control RNA Oligo (1 µM)	Control RNA Oligo (1 µM)	6 µl	
Nuclease-Free Water	Nuclease-Free Water	1.6 ml	
ScriptMiner™ 5′-RNA Ligation Buffer	5′ Ligation Buffer	10 µl	Blue
ScriptMiner™ SinglePlex 5′-Adaptor Oligo	5′ Adaptor Oligo	20 µl	
ATP	ATP	10 µl	
ScriptMiner™ 5′-RNA Ligase	5′ RNA Ligase	10 µl	
MMLV Reverse Transcription Buffer	MMLV RT Buffer	40 µl	Red
ScriptMiner™ SinglePlex cDNA Primer	cDNA Primer	10 µl	
dNTP PreMix	dNTP PreMix	20 µl	
MMLV Reverse Transcriptase	MMLV Reverse Transcriptase	10 µl	
RNase	RNase	10 µl	
FailSafe™ PCR PreMix E	FailSafe™ PCR PreMix E	1.3 ml	Yellow
ScriptMiner™ SinglePlex Forward PCR Primer	Forward PCR Primer	55 µl	
ScriptMiner™ SinglePlex Reverse PCR Primer	Reverse PCR Primer	55 µl	

Important! *The components of this kit should only be used to prepare ScriptMiner SinglePlex (nonbarcoded) Illumina®-compatible small RNA-Seq libraries. Do not use the components of this kit with other ScriptMiner Kits. Do not use components from other ScriptMiner kits with this kit.*

Storage: Store the kit at –20°C in a freezer without a defrost cycle.

3. Preparation

Input RNA:

The RNA purification method should be designed such that it includes small RNA (18-40 nt). Any of the following can be used as input RNA in the standard kit reaction:

- *Good:* From 1 to 5 µg of total RNA, isolated by a method that includes small RNA (18-40 nt).
- *Better:* Size-enriched small RNA of <200 nt prepared using commercially available column methods (provided by the user).
- *Best:* At least 100 pg of polyacrylamide gel–selected small RNA of about 18-40 nt, e.g., isolated with the flashPAGE™ Fractionator (Ambion).

The RNA sample should be dissolved in 8 µl of Nuclease-Free Water.

Types of Small RNAs Included in the Library:

RNA 3′-end modifications: The standard kit reaction produces a library from RNAs with 3′ ends containing either a 2′,3′-OH group (such as mammalian miRNA) or a 2′-*O*-methyl, 3′-OH group (such as piwi-interacting RNA [piRNA] and plant miRNA).

RNA 5′-end modifications: The standard kit reaction produces a library from RNAs with a 5′-monophosphorylated end (5′ pNNNN…), such as miRNA. However, an optional treatment of the RNA with Tobacco Acid Pyrophosphatase (TAP; provided in the kit) enables the user to prepare libraries from 5′-monophosphorylated RNA, small 5′-capped (5′ GpppNNNN…), and small 5′-triphosphorylated (5′ pppNNNN…) RNAs.

Size of the Adaptor-Tagged Library:

The process for the ScriptMiner Small RNA-Seq Library Preparation Kit (SinglePlex; Illumina-compatible) adds 70 nt to the RNA. Forty-nine nucleotides are added to the 5′ end of the RNA and 21 nt are added to the 3′ end of the RNA.

Sequencing the ScriptMiner Small RNA-Seq (SinglePlex; Illumina®-compatible) Library:

The ScriptMiner SinglePlex library can be sequenced using the standard Illumina Small-RNA Sequencing Primer. See Appendix 1 for more details.

4. ScriptMiner Procedure

ScriptMiner Control Reaction: To help ensure success, we recommend using the 22-nt Control RNA Oligo when first becoming familiar with the ScriptMiner Kit procedure. To run a Control Reaction, see Appendix 2.

3′-Adaptor Oligo Ligation

Remove the ScriptMiner Kit from storage at -20°C. Warm the PEG Solution to room temperature. The room-temperature PEG Solution will be used in both Part A, Step 1 and Part C, Step 1. Place the remaining kit components on ice.

Required in Part A

<table>
<tr><th>Component Name</th><th>Tube Label</th><th>Cap Color</th></tr>
<tr><td>PEG Solution</td><td>PEG Solution</td><td rowspan="9">Green</td></tr>
<tr><td>ScriptMiner™ 3′-RNA Ligation Buffer</td><td>3′ Ligation Buffer</td></tr>
<tr><td>DTT</td><td>DTT</td></tr>
<tr><td>RiboGuard™ RNase Inhibitor</td><td>RiboGuard™ RNase Inhibitor</td></tr>
<tr><td>ScriptMiner™ SinglePlex 3′-Adaptor Oligo</td><td>3′ Adaptor Oligo</td></tr>
<tr><td>ScriptMiner™ 3′-RNA Ligase</td><td>3′ RNA Ligase</td></tr>
<tr><td>ScriptMiner™ Finishing Enzyme</td><td>Finishing Enzyme</td></tr>
<tr><td>Ligation STOP Solution</td><td>Ligation STOP Solution</td></tr>
<tr><td>Tobacco Acid Pyrophosphatase (optional)</td><td>TAP</td></tr>
<tr><td>Nuclease-Free Water</td><td>Nuclease-Free Water</td><td>Clear</td></tr>
</table>

1. Prepare a 3′-Adaptor Ligation Master Mix. For each sample, combine in order:

0.75 µl	ScriptMiner 3′-RNA Ligation Buffer
0.75 µl	DTT
0.5 µl	RiboGuard RNase Inhibitor
3.0 µl	PEG Solution
1.0 µl	ScriptMiner SinglePlex 3′-Adaptor Oligo
6.0 µl	Total volume

2. Add 6 µl of the 3′-Ligation Master Mix to each small-RNA sample. Thoroughly mix each viscous reaction by vortexing to ensure it is homogeneous.

   Important! *Be sure to use the ScriptMiner 3′-RNA Ligase in the Green-cap tube in this step.*

3. Add 1 µl of ScriptMiner 3′-RNA Ligase to each reaction and thoroughly mix the contents by pipetting several times.
4. Incubate the reactions at room temperature (~25°C) for 2 hours.

   Note: *Steps 5 and 6 will prepare a sequencing library* ***only*** *from RNAs with a 5′ monophosphate (5′ pNNNN…). If you want to prepare a sequencing library from RNAs with a 5′ monophosphate (5′ pNNNN…), a 5′ cap (5′ GpppNNNN…), or a 5′ triphosphate (5′ pppNNNN…) use the* ***alternate protocol*** *provided (Steps 5A and 6A).*

5. After the 2-hour ligation reaction of Step 4, add 1 µl of the ScriptMiner Finishing Enzyme to each reaction. Incubate the reactions at 30°C for 60 minutes. During the incubation, prepare the 3′ Adaptor Degradation Master Mix as described in Part B, Step 1.
6. Add 4 µl of Ligation STOP Solution to each reaction. Mix thoroughly and briefly centrifuge each reaction to collect the contents at the bottom of the tube. Proceed to Part B.

Alternate Protocol:

5A. After the 2-hour ligation reaction of Step 4, add 4 µl of Ligation STOP Solution to each reaction. Mix thoroughly, and briefly centrifuge to collect the contents of each reaction at the bottom of the tubes.

6A. Add 1 µl of TAP to each reaction. Mix thoroughly by pipetting several times. Incubate the reactions at 37°C for 60 minutes. During the incubation, prepare the 3′-Adaptor Degradation Master Mix as described in Part B, Step 1. Proceed to Part B.

Removal of Excess 3′-Adaptor Oligo

This step significantly reduces the amount of excess 3′-Adaptor Oligo for improved sequencing results.

Required in Part B

Component Name	Tube Label	Cap Color
Degradase Buffer	Degradase Buffer	Clear
$MgCl_2$	$MgCl_2$	
Degradase Enzyme	Degradase Enzyme	

1. Prepare the 3′-Adaptor Degradation Master Mix. For each reaction, combine in order:

   2 µl Degradase Buffer
   7 µl $MgCl_2$
   1 µl Degradase Enzyme
   10 µl Total volume

2. Add 10 µl of the 3′-Adaptor Degradation Master Mix to each reaction.
3. Incubate each reaction at 37°C for 30 minutes. During the 30-minute incubation, prepare the 5′-Adaptor Ligation Master Mix (Part C, Step 1).

5′-Adaptor Oligo Ligation

This step adds a tagging sequence, the ScriptMiner SinglePlex 5′-Adaptor Oligo, to the 5′ end of the RNA.

Required in Part C

Component Name	Tube Label	Cap Color
ScriptMiner™ 5′-RNA Ligation Buffer	5′ Ligation Buffer	Blue
ATP	ATP	
ScriptMiner™ 5′-RNA Ligase	5′ RNA Ligase	
ScriptMiner™ SinglePlex 5′-Adaptor Oligo	5′ Adaptor Oligo	
PEG Solution	PEG Solution	Green
Nuclease-Free Water	Nuclease-Free Water	Clear

Important! *Be sure to use the ScriptMiner 5′-RNA Ligation Buffer and ScriptMiner 5′-RNA Ligase in the Blue-cap tubes in this step.*

1. Prepare the 5′-Adaptor Ligation Master Mix. For each reaction, combine in order:

   1 µl ScriptMiner 5′-RNA Ligation Buffer
   5 µl PEG Solution
   1 µl ATP
   2 µl ScriptMiner SinglePlex 5′-Adaptor Oligo
   1 µl ScriptMiner 5′-RNA Ligase
   10 µl Total volume

Thoroughly mix the viscous solution by pipetting several times.

2. Add 10 µl of the 5′-Adaptor Ligation Master Mix to each reaction from Part B, Step 3. Incubate each reaction at room temperature for at least 1 hour. Incubation for an additional 1-2 hours may improve the yield of the library.
3. Add 10 µl of Nuclease-Free Water to each reaction from Step 2. The volume of each reaction is now 50 µl.

The RNA now has tagging sequences at both its 5′ and 3′ ends (di-tagged RNA).

Di-Tagged RNA Purification

This step is required, and is designed to remove buffers and enzymes that will hinder the efficiency of cDNA synthesis in Part E. **Do not** omit this step. We recommend using the Zymo RNA Clean & Concentrator Kits (see page 4). Other purification kits may also work. However, they should be qualified for RNA purification and designed to recover small fragment sizes of >20 nt.

Follow the Zymo protocol for total RNA (>17 nt), **except** elute each sample **two** times using 15 µl of Nuclease-Free Water for each elution. Measure the recovered volume as accurately as possible (e.g., using a pipettor). Then, add Nuclease-Free Water to a final volume of 30 µl.

Proceed to Part E, or store the purified di-tagged RNA at –20°C or –70°C.

cDNA Synthesis

This step reverse-transcribes the di-tagged RNA into di-tagged cDNA.

Required in Part E

<table>
<tr><th>Component Name</th><th>Tube Label</th><th>Cap Color</th></tr>
<tr><td>MMLV Reverse Transcriptase Buffer</td><td>MMLV RT Buffer</td><td rowspan="5">Red</td></tr>
<tr><td>dNTP PreMix</td><td>dNTP PreMix</td></tr>
<tr><td>ScriptMiner™ SinglePlex cDNA Synthesis Primer</td><td>cDNA Primer</td></tr>
<tr><td>MMLV Reverse Transcriptase</td><td>MMLV Reverse Transcriptase</td></tr>
<tr><td>RNase</td><td>RNase</td></tr>
<tr><td>DTT</td><td>DTT</td><td>Green</td></tr>
</table>

1. Prepare the **Reverse Transcription Master Mix**. For each reaction, combine in order:

4 µl MMLV Reverse Transcription Buffer
2 µl dNTP PreMix
2 µl DTT
1 µl ScriptMiner SinglePlex cDNA Synthesis Primer
1 µl MMLV Reverse Transcriptase
10 µl Total volume

2. To each 30-µl sample of purified di-tagged RNA from Part D, add 10 µl of the Reverse Transcription Master Mix.
3. Incubate the reactions at 37°C for 15 minutes.
4. Terminate the reactions by heating the tubes at 85°C for 15 minutes.
5. Cool the reactions to 55°C and add 1 µl of RNase to each tube. Incubate the reactions at 55°C for 5 minutes.

The di-tagged cDNA can now be amplified by PCR (Part F) or stored at –20°C.

PCR Amplification

PCR amplification: a) incorporates Illumina-compatible adaptor sequences; and b) amplifies the library for cluster generation. **At least one** PCR cycle must be performed in order to generate a viable sequencing library.

The PCR amplification strategy employed by the ScriptMiner Kit is designed to maximize the yield of the desired library, while minimizing the production of nonspecific or undesired amplification product.

First, perform Analytical PCR as described in Part F, Steps 2-7. The Analytical PCR process identifies the optimal number of PCR cycles that should be performed for each library (di-tagged cDNA).

Once the optimal number of PCR cycles has been identified for each library, perform Preparative PCR as described in Part F, Steps 8-10 to generate sufficient quantities of the library for sequencing.

Required in Part F

<table>
<tr><th>Component Name</th><th>Tube Label</th><th>Cap Color</th></tr>
<tr><td>ScriptMiner™ SinglePlex Forward PCR Primer</td><td>Forward PCR Primer</td><td rowspan="3">Yellow</td></tr>
<tr><td>ScriptMiner™ SinglePlex Reverse PCR Primer</td><td>Reverse PCR Primer</td></tr>
<tr><td>FailSafe™ PCR PreMix E</td><td>FailSafe™ PCR PreMix E</td></tr>
<tr><td>Nuclease-Free Water</td><td>Nuclease-Free Water</td><td>Clear</td></tr>
</table>

Provided by the user: Proofreading PCR enzyme, such as Epicentre's FailSafe PCR Enzyme Mix (see page 15 for ordering information).

1. Prepare the PCR Master Mix. For each di-tagged cDNA made in Part E, Step 5, prepare a 100-µl PCR by combining:

Volume	Component
2-5 µl	Di-tagged cDNA from Part E, Step 5
40-43 µl	Nuclease-Free Water
50 µl	FailSafe PCR PreMix E
2 µl	ScriptMiner SinglePlex Forward PCR Primer
2 µl	ScriptMiner SinglePlex Reverse PCR Primer
1 µl	Proofreading PCR enzyme (2.5 Units/µl)
100 µl	Total volume

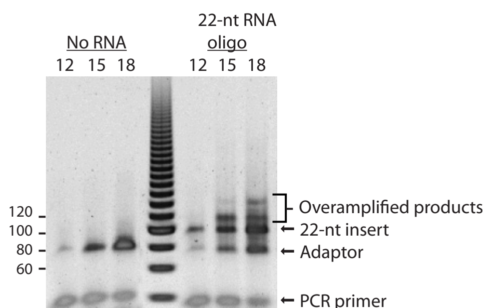

Figure 2. Analysis of PCR-amplified products resulting from ScriptMiner™ (SinglePlex) reactions. Reactions contained nuclease-free water (No RNA; lanes 1-3) or 1 pmol of the 22-nt ScriptMiner Control RNA. Reactions were amplified for 12, 15, or 18 cycles as in Part F, Step 3, and reaction products were examined in a 8% native polyacrylamide gel in 1X TBE buffer. The 92-bp cDNA produced from the ScriptMiner Control RNA Oligo is indicated. Note that increasing the number of PCR cycles can generate nonspecific (overamplified) products. In the above example, 12-15 PCR cycles is considered optimal.

Analytical PCR (Steps 2-7) is first performed in order to identify the optimal number of PCR cycles for each library (di-tagged cDNA).

2. For each 100-µl PCR prepared in Step 1, aliquot 30 µl into each of three PCR tubes. Label the tubes: PCR1-12, PCR1-15, and PCR1-18 to designate the di-tagged cDNA and the number of PCR cycles that are being evaluated. If a second library (di-tagged cDNA) is being evaluated at the same time, label tubes for this library PCR2-12, PCR2-15, and PCR2-18.
3. Perform Analytical PCR:

   95°C for 30 seconds to denature the di-tagged cDNA

   Then 12, 15, or 18 PCR cycles of:
   - 94°C for 15 seconds
   - 55°C for 5 seconds
   - 65°C for 5 seconds
4. After 12 PCR cycles, remove the tubes labeled PCR1-12, PCR2-12, etc. Separately, remove 4 µl from each of these tubes into fresh tubes labeled PCR1-12, PCR 2-12, etc. Add 1 µl of 6X Blue Juice (0.25% bromophenol blue, 0.25% xylene cyanole, and 30% glycerol) to each 4-µl aliquot and mix well. Store the tubes at –20°C.
5. Repeat Step 4 after 15 and 18 PCR cycles.
6. Load the samples from Steps 4 and 5 on a 8% native polyacrylamide gel (see Appendix 3). Run the gel at 15 mA until the bromphenol blue reaches the bottom of gel. Stain the gel with SYBR® Gold. Visualize under UV light and compare the gel results with Fig. 2 to identify the optimal number of PCR cycles for each di-tagged cDNA.

   Note: Do not *use an agarose gel, as it will not provide sufficient resolution of the RNA.*

7. Gel analysis: The purpose of the Analytical PCR is to find the appropriate number of amplification cycles that will generate a sufficient amount of library material with the least amount of undesired PCR product. In general, the **minimum** number of PCR cycles needed to visualize the library on a gel is the recommended condition. Too many PCR cycles will result in a higher risk of producing spurious amplification products or artifacts that are visualized as a general smearing and/or concatamers of products on a native polyacrylamide gel as shown in Fig. 2.

The process for the ScriptMiner Small RNA-Seq Library Preparation Kit (SinglePlex; Illumina®-compatible) adds 70 nt to the RNA.

Preparative PCR. Once the optimal number of PCR cycles has been determined for each di-tagged cDNA (Steps 2-7), prepare **two** 100-µl PCRs as described in Step 1 for **each** di-tagged cDNA.

8. Perform Preparative PCR.

   95°C for 30 seconds to denature the di-tagged cDNA

   Then, PCR cycles of:
   - 94°C for 15 seconds
   - 55°C for 5 seconds
   - 65°C for 5 seconds

9. Based on the results of the Analytical PCR (Steps 2-7), perform the optimal number of PCR cycles for each di-tagged cDNA.
10. Combine the two PCRs for each di-tagged cDNA and proceed to Part G.

Purification of PCR Products

1. Purify the PCR amplification products using a commercially available column-based method, such as the MinElute PCR Purification Kit (Qiagen) or the DNA Clean & Concentrator kit (Zymo Research).
2. Elute the purified DNA with 10 µl of Nuclease-Free Water or Elution Buffer. Add 2 µl of 6X Blue Juice (0.25% bromophenol blue, 0.25% xylene cyanole, and 30% glycerol) and gel-purify the desired products, using the procedure outlined in Appendix 3.

5. Appendices

Appendix 1: Sequencing the ScriptMiner Library

Note: *Nucleotide sequences shown in Appendix 1 are copyrighted to Illumina, Inc. Oligonucleotide sequences © 2006-2010 Illumina, Inc. All rights reserved. The Small-RNA Sequencing Primer used to sequence a ScriptMiner (SinglePlex) library is available from Illumina, Inc.*

When using the Illumina Small RNA Sequencing Primer, the 5′→3′ sense-strand sequence of the original RNA is generated. The first nucleotide read is the exact 5′ nucleotide of the cDNA (Fig. 3).

```
5′ AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACGATC---  cDNA (sense orientation)  --TCGTATGCCGTCTTCTGCTTG 3′
3′ TTACTATGCCGCTGGTGGCTGTCCAAGTCTCAAGATGTCAGGCTGCTAG---cDNA (antisense orientation)--AGCATACGGCAGAAGACGAAC 5′
                 5′ CGACAGGTTCAGAGTTCTACAGTCCGACGATC→
                         Small RNA Sequencing Primer
```

Blue = Sequence added by the 3′-Adaptor Oligo and PCR amplification
Red = Sequence added by the 5′-Adaptor Oligo and PCR amplification
Black = Sequence of the cDNA
Green = Illumina Small-RNA Sequencing Primer

Figure 3. Sequencing the ScriptMiner™ library.

Appendix 2: Control Reactions

Control RNA Oligo (included in the kit): 5′ p-NNCGCUUGCAGAGAGAAAUCNN-OH 3′

To ensure success, we recommend using the RNA Control Oligo when first becoming familiar with the ScriptMiner Kit procedure. This positive control sample consists of a 22-nt, 5′-phosphorylated RNA oligo containing two degenerate nucleotides on both the 5′ and 3′ ends. It is designed to mimic the typical length of a miRNA and provide a robust signal using the ScriptMiner procedure. The RNA Control Oligo is provided as a 1 µM solution. For best results, use at least 1 µl (1 pmol) per ScriptMiner reaction and expect to observe a 92-bp PCR product.

If desired, a No-RNA control reaction can also be run as listed below. If doing a No-RNA control reaction, use 8 µl of Nuclease-Free Water instead of the Control RNA Oligo.

Control Reaction:

Volume	Component
1 µl	Control RNA Oligo RNA (1 pmol)
7 µl	Nuclease-Free Water
8 µl	Total volume

Proceed to Part A, Step 1 of the ScriptMiner procedure on page 7.

Appendix 3: Gel Purification of Library

The process for the ScriptMiner Small RNA-Seq Library Preparation Kit (SinglePlex; Illumina-compatible) adds 70 nt to the RNA. Therefore, the cDNA derived from miRNA or other small RNAs (~18-40 nt) will be ~88-110 bp in length. The provided protocol can be used to size-select the PCR products for subsequent cluster generation and sequence analysis. Other size-selection procedures (provided by the user) may also be used.

To ensure compatibility with subsequent cluster amplification and sequencing quality, a minimum amount of gel-purified library is needed. This requirement is to obtain at least 20 µl of library material at ~10 nM following gel purification. This is equivalent to ~0.2 pmol total yield or ~10 ng (at 0.5 ng/µl) of a 92-bp PCR product produced from the Control RNA Oligo included in the kit (see Appendix 2).

Additional required reagents (provided by the user):

Molecular biology grade acrylamide

Gel stain, such as SYBR Gold Dye (Invitrogen)

TBE Running Buffer

20-bp DNA Ladder (Bayou Biolabs)

80% Ethanol (cold)

0.3 M Sodium acetate

Isopropyl alcohol (isopropanol)

TE Buffer

Gel Purification Procedure:

1. Cast a 8% native mini polyacrylamide gel (8 cm x 10 cm) in 1X TBE. Use a preparative comb if possible (width of well 10-15 mm).
2. Load the amplified and purified cDNA sample from Part G, Step 8 next to a 20-bp DNA Ladder (Bayou Biolabs) and run the gel at a constant current of 10-15 mA for about 1 hour or until the bromophenol blue dye is about 2 cm from the bottom of the gel.
3. Stain the gel with SYBR Gold Dye (Invitrogen) according to the manufacturer's instructions and visualize the DNA on a Dark Reader™ Transilluminator (Clare Chemical Research) or similar non-UV light box. The Dark Reader Transilluminator allows the DNA to be visualized without UV light, thus preventing damage to the DNA.
4. Cut out a gel slice containing the DNA band of the desired size.
5. Place the gel plug into a sterile 0.5-ml microcentrifuge tube in which a hole is punched through the bottom using a ~20-gauge needle. Place the 0.5-ml tube inside a 1.5-ml tube and centrifuge at top speed for 30 seconds to shred the gel slice.
6. Remove and discard the 0.5-ml tube, add 300 µl of 0.5 M ammonium acetate to the shredded gel slice, and shake the tube for 2-3 hours in a 37°C incubator.
7. Collect the sample and pipet both the supernatant and gel pieces into a 0.22-micron spin-filter unit. Centrifuge for 30 seconds at 3,000 x g.
8. Collect the flow-through, add 1 µl of glycogen (10 µg/µl) and one volume of isopropyl alcohol (or 3 volumes of 100% ethanol) to the tube containing the aqueous solution. Mix by vortexing vigorously. Store the tube at –70°C for 15 minutes or at –20°C for at least 2 hours.
9. Recover the cDNA by centrifuging at >10,000 x g for 10 minutes at 4°C. Carefully remove and discard the supernatant. Wash the pellet with 100 µl of cold 80% ethanol.
10. Centrifuge at >10,000 x g for 5 minutes at 4°C. Carefully remove and discard the ethanol without disturbing the pellet
11. Air-dry the pellet, but do not over-dry. Resuspend the pellet in ~20-50 µl of Nuclease-Free Water or TE buffer.
12. Quantify the samples using a PicoGreen® assay (Invitrogen), NanoDrop® UV-Vis Spectrophotometer (Thermo Scientific), or other appropriate methods. Analysis of material by PAGE or a Bioanalyzer (Agilent) is also recommended.

Discontinued

6. Related Products

Ribo-Zero™ rRNA Removal Kit (Human/Mouse/Rat)	
RZH1046	6 Reactions
Ribo-Zero™ rRNA Removal Kit (Human/Mouse/Rat) Low Input	
RZH1086	6 Reactions
Ribo-Zero™ rRNA Removal Kit (Gram-Negative Bacteria)	
RZNB1056	6 Reactions
Ribo-Zero™ rRNA Removal Kit (Gram-Positive Bacteria)	
RZPB10106	6 Reactions
ScriptSeq™ mRNA-Seq Library Preparation Kit (Illumina®-compatible)	
SS10906	6 Reactions
SS10924	24 Reactions
FailSafe™ Enzyme Mix Only	
FSE51100	100 Units
FSE5101K	1000 Units
Tobacco Acid Pyrophosphatase (TAP)	
T81050	50 Units at 5 U/µl
T19050	50 Units at 10 U/µl
T19100	100 Units at 10 U/µl
T19250	250 Units at 10 U/µl
T19500	500 Units at 10 U/µl

FailSafe, Ribo-Zero, ScriptMiner, and ScriptSeq are trademarks of Epicentre, Madison, Wisconsin.
Clean & Concentrator is a trademark of Zymo Research, Orange, California

DarkReader is a trademark of Clare Chemical, Dolores, Colorado.

flashPAGE is a trademark of Ambion Inc., Austin, Texas.

Illumina is a registered trademark of Illumina Inc., San Diego, California.

MinElute is a registered trademark of Qiagen Inc., Valencia, California.

NanoDrop is a registered trademark of NanoDrop Technologies Inc., Wilmington, Delaware.

PicoGreen and SYBR are registered trademarks of Molecular Probes, Inc., Eugene, Oregon.

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