somagenics

RealSeq®-AC miRNA Library Kit for Illumina® sequencing

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500-00048

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RealSeq®-AC

I. Overview

8 hours

Step 1. Adapter ligation

Combine: RNA, Buffer 1, RealSeq® Adapter → **Incubate:** 2 min at 70°C, 2 min ice → **Add:** RNase Inhibitor, Buffer 2, Ligase → **Incubate:** 60 min at 25°C

Step 2. Adapter blocking

Add: Blocking Agent → **Incubate:** 10 min at 65°C, Step down to 37°C → **Add:** Blocking Enzyme, Buffer 3 → **Incubate:** 60 min at 37°C, 20 min at 65°C

Step 3. Circularization

Add: RealSeq® Enzyme, Buffer 4 → **Incubate:** 60 min at 37°C

Step 4. Dimer removal

Add: Dimer Removal Agent → **Incubate:** 10 min at 37°C → **Add:** RealSeq® Beads → **Incubate:** 10 min at 37°C

Step 5. Reverse transcription

Add: RT Primer, dNTPs → **Incubate:** 5 min at 65°C → **Add:** RT Buffer, RNase free water, RT Enzyme, RNase Inhibitor → **Incubate:** 60 min at 42°C, 20 min at 65°C

Step 6. PCR amplification

Add: PCR Buffer, dNTPs, FP and RP, PCR Polymerase, RNase free water → **PCR:** 30 sec at 94°C, 10-22 Cycles (15 sec at 94°C, 30 sec at 62°C, 15 sec at 70°C), 5 min at 70°C

Step 7. Size selection

AMPure XP Beads*

*User supplied

Illumina Sequencing

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II. RealSeq®-AC Kit Contents

Core kit box (Store at -20°C) (Box 1 of 2)

Tube	Component	Tube	Component
1	Buffer 1	11	Dimer Removal Agent
2	RealSeq® Adapter	12	RT Primer
3	RNase Inhibitor	13	dNTPs
4	Buffer 2	14	RT Buffer
5	Ligase	15	RT Enzyme
6	Blocking Agent	16	PCR Buffer
7	Blocking Enzyme	17	PCR Polymerase
8	Buffer 3	18	RNase-Free Water
9	RealSeq® Enzyme	+	miRNA Control
10	Buffer 4	AD	Adapter Dilution Buffer

Primer box (Store at -20°C) (Box 1 of 2)

Cat. 500-00012 (12 reactions)

Tube	Component
FP	Forward Primer (FP)
RP1 - 12	Reverse Primers, Index 1 - 12*

Cat. 500-00048 (48 reactions)

Tube	Component
FP	Forward Primer (FP)
RP1 - 24	Reverse Primers, Index 1 - 24*

* For sequences see Appendix C, page 18.

Beads (Store at +4°C)

Tube	Component
B	RealSeq® Beads

III. Warnings and Recommendations

- Do not use the kit past the expiration date
- Do not remove **enzymes** from -20°C until immediately before use and return to -20°C immediately after use.
- Vortex and centrifuge each component before use.
- Use a 96-well aluminum block on ice when handling PCR tubes.
- Do not freeze RealSeq® Beads

IV. User-supplied Reagents, Consumables, and Laboratory Equipment (not included)

- Sterile nuclease-free PCR tubes
- Sterile nuclease-free 1.5 ml tubes
- Magnetic stand for PCR tubes (e.g. Diagenode #B0400001)
- 96-well aluminum block
- Agencourt® AMPure® XP (Beckman #A63880)
- 96-100% Ethanol (molecular biology grade)
- Bioanalyzer® DNA 1000 kit (Agilent #5067-1504) or Tape Station D1000 DNA kit (Agilent #5067-5582 & 5037-5583)
- Qubit® Fluorometer (ThermoFisher Scientific) and Qubit® dsDNA HS Assay Kit, 100 assays (Thermofisher Scientific #Q32851)

V. Input Requirements

- This kit was optimized using 100 ng of Human Brain Total RNA (ThermoFisher #AM7962).
- High quality total RNA with RNA Integrity Number (RIN) > 7 is recommended as input material.
- Using partially degraded RNA will result in a higher proportion of short sequencing reads (< 15 nt) that correspond to degraded rRNAs.
- Not all RNA extraction and purification kits isolate small RNAs, users should confirm that the method used also isolates small RNAs.
- When preparing libraries for the first time we highly recommend using the included miRNA Control to prepare a control library.
- To prepare a control library, use 1 µl of the control miRNA instead of the RNA sample. See Appendix A (Figure 2) for an example library profile with the miRNA Control.

- **Guidelines for different input amounts:**

Table 1

Input Amount	RealSeq®-AC Adapter dilution*	PCR Cycles
1 µg total RNA	none	10-13
100 ng total RNA	1/2	13-16
10 ng total RNA	1/2	16-19
1 ng total RNA	1/4	19-22
1 µl miRNA Control	1/2	13

* To prepare dilutions of the adapter use the Adapter Dilution Buffer (Tube AD) provided, and only dilute the amount required for the number of samples being processed.

VI. Experimental Protocol

1. Adapter Ligation

- Heat thermal cycler to 70°C.
- Prepare separate PCR microtubes for each RNA sample.
- RNA samples can be added up to a volume of 3 µl.

Reagent	Volume to add
RNA (1 µg - 1 ng)	up to 3 µl
Buffer 1 (Tube 1)	3 µl
RealSeq® Adapter (Tube 2)*	1* µl
RNase Free Water (Tube 18)	Variable
Total Volume	**7 µl**

***See Table 1 in Input Requirements for Adapter dilutions.**

- Place all sample tubes into a thermal cycler at 70°C.
- Heat sample tubes for 2 minutes at 70°C and transfer to ice for at least two minutes.
- Add the following reagents to the sample tube. Mix by pipetting and spin down.

Reagent	Volume to add
RNase Inhibitor (Tube 3)	1 µl
Buffer 2 (Tube 4)	1 µl
Ligase (Tube 5)	1 µl
Total Volume	**10 µl**

- Run the ligation reaction in a thermal cycler with the following profile:

Step Type	Temperature	Time
Hold	25°C	60 min
Hold	65°C	5 min

- **Proceed immediately to next step (Adapter Blocking).**

2. Adapter Blocking

- Thaw, vortex and spin Blocking Agent (Tube 6).
- Add 2.5 µl of Blocking Agent (Tube 6) to each sample tube. Mix by pipetting and spin down.
- Incubate with the following profile:

Step Type	Temperature	Time
Hold	65°C	5 min
Step down*	65 to 37°C	Approx. 5 min

***Step down from 65°C to 37°C at a rate of 0.1°C per second (approximately 5 mins).**

- Add the following reagents to each sample tube. Mix by pipetting and spin down.

Reagent	Volume to add
Blocking Enzyme (Tube 7)	1.1 µl
Buffer 3 (Tube 8)	6.4 µl
Total Volume	**20 µl**

- Run Blocking reaction in a thermal cycler with the following profile:

Step Type	Temperature	Time
Hold	37°C	60 min
Hold	65°C	20 min

- **Proceed immediately to next step (Circularization).**

or

***Stopping Point*:** Alternatively libraries can be stored overnight at -20°C. To restart, thaw samples on ice before proceeding to next step.

3. Circularization

- Perform circularization by adding the following reagents to each sample tube. Mix by pipetting and spin down.

Reagent	Volume to add
RealSeq® Enzyme (Tube 9)	1 µl
Buffer 4 (Tube 10)	1 µl
Total Volume	**22 µl**

- Place samples into a thermal cycler with the following profile:

Step Type	Temperature	Time
Hold	37°C	60 min

- **Proceed immediately to next step (Dimer Removal).**

4. Dimer Removal

- When Circularization is complete, add 1 µl of Dimer Removal Agent (Tube 11) to each sample tube, mix by pipetting, and incubate at 37°C for 10 min.
- Prepare RealSeq® Beads (Do NOT use AMPure beads!):
  - Thoroughly vortex the beads for at least 30 seconds.
  - Pipet 20 µl of the bead suspension into a new PCR tube.
  - Place the tube on the magnetic rack for 1 minute or until all the beads settle against the side of the tube.
  - Remove and discard the supernatant.
- Immediately resuspend beads with all 23 µl from sample tube and incubate for 10 min at 37°C.
- Place beads on a magnetic rack for 1 minute or until all beads settle against the side of the tube, transfer 22 µl of supernatant into a clean PCR tube.
  - **Proceed immediately to next step (Reverse Transcription).**

5. Reverse Transcription

- Add the following reagents to each sample tube.

Reagent	Volume to add
RT Primer (Tube 12)	2 µl
dNTPs (Tube 13)	2 µl
Total Volume	**26 µl**

- Incubate the samples at 65°C for 5 minutes. Chill on ice for at least two minutes and spin down.
- Add the following reagents to each sample tube:

Reagent	Volume to add
RT Buffer (Tube 14)	4 µl
RNase free water (Tube 18)	7 µl
RT Enzyme (Tube 15)	2 µl
Rnase Inhibitor (Tube 3)	1 µl
Total Volume	**40 µl**

- Mix by pipetting and spin down.
- Place samples into a thermal cycler with the following profile:

Step Type	Temperature	Time
Hold	42°C	60 min
Hold	65°C	20 min

- **Proceed immediately to next step (PCR Amplification).**

or

***Stopping Point*:** Alternatively libraries can be stored overnight at -20°C. To restart, thaw samples on ice before proceeding to next step.

6. PCR Amplification

- Prepare PCR reaction mix for each sample. Mix gently by inversion and spin down.

Reagent	Volume to add
PCR Buffer (Tube 16)	20 µl
dNTPs (Tube 13)	3 µl
Forward Primer (Primer Box FP)	7 µl
PCR Polymerase (Tube 17)	4 µl
RNase Free Water (Tube 18)	19 µl
Total Volume PCR Master Mix	**53 µl**

- Add 53 µl of PCR reaction mix to each sample.
- Add 7 µl of Reverse Primer Index (Primer Box) to each sample. Mix by pipetting and spin down.
- Run samples in a thermal cycler with the following profile:

Step Type	Temperature	Time
HOLD	94°C	30 sec
CYCLE (10-22 cycles) (See Section V)	94°C	15 sec
	62°C	30 sec
	70°C	15 sec
HOLD	70°C	5 min

- **Proceed immediately to next step (Size Selection).**

or

***Stopping Point*:** Alternatively libraries can be stored overnight at -20°C. To restart, thaw samples on ice before proceeding to next step.

7. Size Selection

***WARNING*:** For size selection use Agencourt AMPure XP beads (user supplied), **DO NOT** use RealSeq beads for size selection.

- **Take out the AMPure XP beads to the bench top at least 30 minutes before proceeding. This will ensure that the beads warm to room temperature before use.**

Size selection with Agencourt® AMPure® XP Beads

- Prepare 70% ethanol (500 µl per sample) Ensure beads are at room temperature, and resuspend before use.
- Transfer 50 µl of sample from PCR step to new PCR tube.
- Add 70 µl of AMPure® XP beads.
- Mix reagent and PCR thoroughly by pipette mixing 10 times. Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.
- Place the samples on magnet for 3 minutes to separate beads from solution (wait for the solution to clear before proceeding to the next step).
- Remove the cleared solution from the tube and discard.
- Without removing tube from magnet, add 200 µl of freshly prepared 70% ethanol to each sample and incubate for 30 seconds at room temperature. Remove the ethanol and discard, repeat for a total of two washes.
- Briefly spin the tubes (~2,000 g) to collect the remaining liquid at the bottom of each tube. Place the tubes on the magnetic separation device for 30 seconds, then remove all remaining liquid with a pipette.
- Let the sample tubes rest open on the magnet at room temperature for 3 minutes until the pellet appears dry and is no longer shiny.
- Once the bead pellet has dried, remove the tubes from magnet and add 12.5 µl of RNase free water (Tube 18). Mix thoroughly by pipetting up and down to ensure complete bead dispersion.

- Incubate at room temperature for at least 5 minutes.
- Place the samples on a magnet for 3 minutes or longer, until the solution is completely clear.
- Transfer 10 µl of the clear supernatant containing purified PCR products from each tube to a new tube. Ensure that no beads follow the library during this step.
- Quantify library with Agilent Bioanalyzer®/TapeStation® and Qubit® Fluorometer.

VII. Appendix A: Example Library Profile

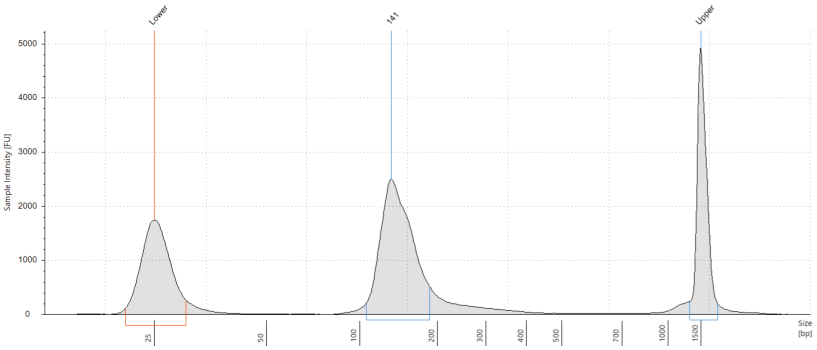

Figure 1. Example TapeStation® profile from a library with an input of 100 ng Brain total RNA amplyfied by 10 cycles of PCR. miRNA sized libraries are approximately 141 bp.

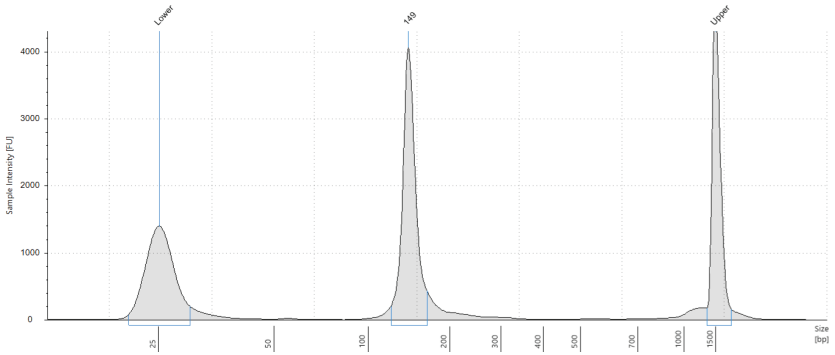

Figure 2. Example TapeStation® profile from a library with an input of 1 µl of miRNA control amplyfied by 13 cycles of PCR. miRNA control libraries are approximately 149 bp.

VIII. Appendix B: Data Analysis

- RealSeq®-AC libraries are completely compatible with bioinformatics tools designed for Illumina's TruSeq Small RNA libraries.
- The final product of a RealSeq®-AC library contains the adapter sequence TGGAATTCTCGGGTGCCAAGG
- This sequence needs to be trimmed from sequenced reads before mapping.
- One of the tools that can be used to perform trimming of adapter sequences is *cutadapt* (Martin et al. 2011).
- The following *cutadapt* command will trim adapter sequences and filter reads with inserts shorter than 15 nt.

```
cutadapt -m 15 -a TGGAATTCTCGGGTGCCAAGG input.fastq > output.fastq
```

- After trimming the alignments can be performed as normal.

IV. Appendix C: Reverse Primer Index Sequence

Tube	Index Sequence
RP1	CGTGAT
RP2	ACATCG
RP3	GCCTAA
RP4	TGGTCA
RP5	CACTGT
RP6	ATTGGC
RP7	GATCTG
RP8	TCAAGT
RP9	CTGATC
RP10	AAGCTA
RP11	GTAGCC
RP12	TACAAG
RP13	TTGACT
RP14	GGAACT
RP15	TGACAT
RP16	GGACGG
RP17	CTCTAC
RP18	GCGGAC
RP19	TTTCAC
RP20	GGCCAC
RP21	CGAAAC
RP22	CGTACG
RP23	CCACTC
RP24	GCTACC

Tube	Index Sequence
RP25	ATCAGT
RP26	GCTCAT
RP27	AGGAAT
RP28	CTTTTG
RP29	TAGTTG
RP30	CCGGTG
RP31	ATCGTG
RP32	TGAGTG
RP33	CGCCTG
RP34	GCCATG
RP35	AAAATG
RP36	TGTTGG
RP37	ATTCCG
RP38	AGCTAG
RP39	GTATAG
RP40	TCTGAG
RP41	GTCGTC
RP42	CGATTA
RP43	GCTGTA
RP44	ATTATA
RP45	GAATGA
RP46	TCGGGA
RP47	CTTCGA
RP48	TGCCGA

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