RealSeq®-AC miRNA Library Kit for Illumina® sequencing

Cat. No.
500-00012
500-00048

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RealSeq®-AC

I. Overview

8 hours

Step 1. Adapter ligation

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

Step 2. Adapter blocking

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

Step 3. Circularization

Add:	Incubate:
RealSeq® Enzyme RealSeq® Buffer	60 min at 37°C

Step 4. Dimer removal

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

Step 5. Reverse transcription

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

Step 6. PCR amplification

Add:	PCR:
PCR Buffer dNTPs FP and RP PCR Polymerase RNase free water	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

SPRI Beads

Illumina Sequencing

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II. RealSeq®-AC Kit Contents

Core kit box (Store at -20°C) (Box 1)

Tube	Component	Tube	Component
RB	RNA Buffer	RTP	RT Primer
A	RealSeq® Adapter	dNTP	dNTPs
RI	RNase Inhibitor	RTB	RT Buffer
LB	Ligation Buffer	RTE	RT Enzyme
L	Ligase	PB	PCR Buffer
BA	Blocking Agent	PP	PCR Polymerase
BE	Blocking Enzyme	DRA	Dimer Removal Agent
BB	Blocking Buffer	AD	Adapter Dilution Buffer
RSE	RealSeq® Enzyme	+	miRNA Control
RSB	RealSeq® Buffer	W	RNase-Free Water

Primer box 1 (Store at -20°C) (Box 2)

Cat. No.	Tube	Component
500-00012 / 48	FP	Forward Primer (FP)
500-00012	RP1 - 12	Reverse Primers, Index 1 - 12*
500-00048	RP1 - 24	Reverse Primers, Index 1 - 24*

Primer box 2 (Store at -20°C) (Box 3) Only for 500-00048

Cat. No.	Tube	Component
500-00048	RP25 - 48	Reverse Primers, Index 25 - 48*

* For sequences see Appendix C, page 18.

Beads (Store at +4°C)

Tube	Component
B	RealSeq® Beads (+4°C)
SPRI	SPRI Beads (+4°C)

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.
- Ensure the **RealSeq® Adapter** and **miRNA Control** are always on ice to minimize degredation.
- Vortex and centrifuge each component before use.
- Always have PCR tubes on **ice** when handling.
- **Do not** freeze RealSeq® Beads or SPRI Beads.
- For ease, thermocyclers can be pre-programmed with all the reactions for a continuous workflow. Go to Appendex A for a list of temperatures.

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
- 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, as well as decreased overall percentage of miRNA reads.
- 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 B (Figure 2) for an example library profile with the miRNA control.

Guidelines for different input amounts:

Input Amount	RealSeq®-AC Adapter dilution (per library)**	PCR Cycles
1 µg total RNA	none	10-13
100 ng total RNA*	1µl **Tube A** + 1µl **Tube AD**	13-16
10 ng total RNA	1µl **Tube A** + 1µl **Tube AD**	16-19
1 ng total RNA	1µl **Tube A** + 3µl **Tube AD**	19-22
1 µl miRNA Control	1µl **Tube A** + 1µl **Tube AD**	13

* 100ng of total RNA is recommended. Higher amounts of RNA may enrich for other classes of small RNAs (i.e. piRNA, snRNA, and snoRNA)

** For optimal libraries, prepare a new dilution for every start of the protocol. Re-using the dilutions may cause degradation of the adapter.

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 to 1 ng)	up to 3 µl
• RNA Buffer (RB)	3 µl*
• RealSeq® Adapter (A)	1µl**
RNase Free Water (W)	Variable
Total Volume	**7 µl**

***RNA Buffer (RB) is very viscous. Pipet slowly.**

****See Table in Input Requirements (Section V) 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.
- While the samples are still on ice, add the following reagents to the sample tube. Mix by pipetting and spin down.

Reagent	Volume to add
• RNase Inhibitor (RI)	1 µl
• Ligation Buffer (LB)	1 µl
• Ligase (L)	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 (BA)**.
- Add **2.5 µl** of ● **Blocking Agent (BA)** 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 (BE)	1.1 µl
● Blocking Buffer (BB)	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 (RSE)	1 µl
RealSeq® Buffer (RSB)	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 (DRA)** to each sample tube in the thermocycler. Mix by pipetting, and incubate in the thermocycler with the following profile:

Step Type	Temperature	Time
Hold	37°C	10 min

- Prepare **RealSeq® Beads** (Stored at +4°C)

  ***WARNING*: Do NOT use SPRI 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.

- Quicky spin down the tubes in a microcentrifuge, then place on a magnetic rack for 1 minute or until all beads settle against the side of the tube. Transfer 20 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 (RTP)	2 µl
dNTPs (dNTP)	2 µl
Total Volume	**24 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 (RTB)	4 µl
RNase free Water (W)	9 µl
RT Enzyme (RTE)	2 µl
RNase Inhibitor (RI)	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 (PB)	20 µl
dNTPs (dNTP)	3 µl
Forward Primer (FP)	7 µl
PCR Polymerase (PP)	4 µl
RNase Free Water (W)	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 SPRI Beads, **DO NOT** use RealSeq®beads for size selection.

- **Take out the SPRI 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 SPRI Beads

- Prepare 70% ethanol (500 µl per sample).
- Ensure SPRI Beads is at room temperature, and resuspend before use.
- Vortex and spin down each PCR reaction. Transfer 50 µl of sample to new PCR tubes.
- Add 70 µl of SPRI Beads to each sample. 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 until all the beads separate from solution (wait for the solution to clear before proceeding to the next step). (~3-6 minutes)
- Carefully remove the cleared solution from the tube and discard. Take care to not disturb the beads in the process.
- 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 until the pellet appears dry and is no longer shiny. (~3-6 minutes)

- Once the bead pellet has dried, remove the tubes from magnet and add 12.5 µl of RNase free water (W). 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: Thermocycler Programming

Thermocyclers can be programmed in advance for all reactions.

Step	Temperature		Time
1. Adapter Ligation	70°C		2 min
	ice		2 min
	25°C		60 min
	65°C		5 min
2. Adapter Blocking	65°C		5 min
	Step down 0.1°C/sec --> 37°C		~5 min
	37°C		60 min
	65°C		20 min
Optional Stopping Point	**-20°C**		**Overnight**
3. Circularization	37°C		60 min
4. Dimer Removal	37°C		~25-30 min
5. Reverse Transcription	65°C		5 min
	ice		2 min
	42°C		60 min
	65°C		20 min
Optional Stopping Point	**-20°C**		**Overnight**
6. PCR Amplification	**Step**	**Temp**	**Time**
	HOLD	94°C	30 sec
	CYCLE (10-22 cycles)	94°C	15 sec
		62°C	30 sec
		70°C	15 sec
	HOLD	70°C	5 min
Optional Stopping Point	**-20°C**		**Overnight**

VIII. Appendix B: Example Library Profiles

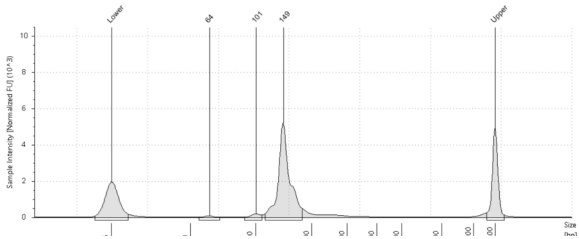

Figure 1. Example D1000 TapeStation® profile from a library with an input of 100 ng Brain total RNA amplified by 10 cycles of PCR. miRNA sized libraries are approximately 141 bp.

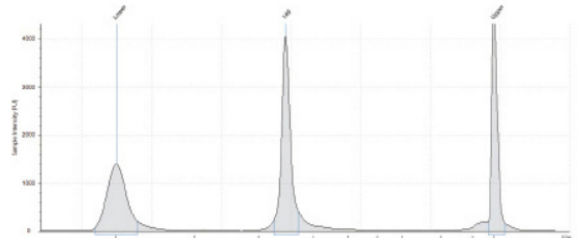

Figure 2. Example D1000 TapeStation® profile from a library with an input of 1 µl of miRNA control amplified by 13 cycles of PCR. miRNA control libraries are approximately 149 bp.

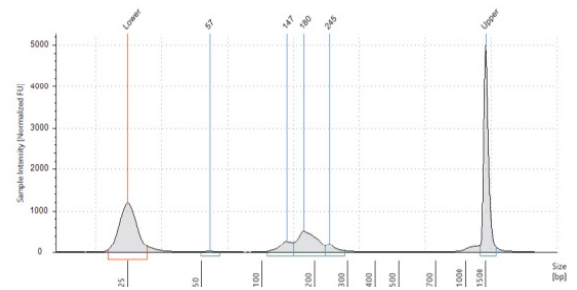

Figure 3. Example D1000 TapeStation® profile from a library with an input of 100 ng of MCF10A cells amplified by 16 cycles of PCR. The libraries are approximately 147-245bp.

IX. Appendix C: 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 -u 1 -m 15 -a TGGAATTCTCGGGTGCCAAGG input.fastq > output.fastq
```

- After trimming the alignments can be performed as normal.

X. Appendix D: Reverse Primer Index Sequences

Tube	Sequence	Reported*	Tube	Sequence	Reported*
RP1	CGTGAT	ATCACG	RP25	ATCAGT	ACTGAT
RP2	ACATCG	CGATGT	RP26	GCTCAT	ATGAGC
RP3	GCCTAA	TTAGGC	RP27	AGGAAT	ATTCCT
RP4	TGGTCA	TGACCA	RP28	CTTTTG	CAAAAG
RP5	CACTGT	ACAGTG	RP29	TAGTTG	CAACTA
RP6	ATTGGC	GCCAAT	RP30	CCGGTG	CACCGG
RP7	GATCTG	CAGATC	RP31	ATCGTG	CACGAT
RP8	TCAAGT	ACTTGA	RP32	TGAGTG	CACTCA
RP9	CTGATC	GATCAG	RP33	CGCCTG	CAGGCG
RP10	AAGCTA	TAGCTT	RP34	GCCATG	CATGGC
RP11	GTAGCC	GGCTAC	RP35	AAAATG	CATTTT
RP12	TACAAG	CTTGTA	RP36	TGTTGG	CCAACA
RP13	TTGACT	AGTCAA	RP37	ATTCCG	CGGAAT
RP14	GGAACT	AGTTCC	RP38	AGCTAG	CTAGCT
RP15	TGACAT	ATGTCA	RP39	GTATAG	CTATAC
RP16	GGACGG	CCGTCC	RP40	TCTGAG	CTCAGA
RP17	CTCTAC	GTAGAG	RP41	GTCGTC	GACGAC
RP18	GCGGAC	GTCCGC	RP42	CGATTA	TAATCG
RP19	TTTCAC	GTGAAA	RP43	GCTGTA	TACAGC
RP20	GGCCAC	GTGGCC	RP44	ATTATA	TATAAT
RP21	CGAAAC	GTTTCG	RP45	GAATGA	TCATTC
RP22	CGTACG	CGTACG	RP46	TCGGGA	TCCCGA
RP23	CCACTC	GAGTGG	RP47	CTTCGA	TCGAAG
RP24	GCTACC	GGTAGC	RP48	TGCCGA	TCGGCA

*Note: **Reported** are the sequences reported by the sequencer.

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