RealSeq®-Dual miRNA Library Kit for Illumina® sequencing

Cat. No.
700-00012
700-00048

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RealSeq®-Dual

I. Overview

8 hours

Step 1. Adapter ligation

Combine: → **Incubate:** → **Add:** → **Incubate:**

RNA
RNA Buffer
RealSeq® Adapter

2 min at 70°C
2 min ice

RNase Inhibitor
Ligation Buffer
Ligase

60 min at 25°C

Step 2. Adapter blocking

Add: → **Incubate:** → **Add:** → **Incubate:**

Blocking Agent

5 min at 65°C
Step down
to 37°C

Blocking Enzyme
Buffer 3

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 Buffer
dNTPs
FP and RP
PCR Polymerase
RNase free water

PCR:
30 sec at 94°C
13-22 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

Column Purification* → **AMPure XP Beads***

*User supplied

Illumina Sequencing

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II. RealSeq®-Dual Kit Contents

Core kit box (Store at -20°C) (Box 1)

Tube	Component	Tube	Component
RB	RNA Buffer	RTP	RT Primer - Dual
A	RealSeq® Dual 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	W	RNase-Free Water
RSE	RealSeq® Enzyme	HY	HY4 Removal Agent
RSB	RealSeq® Buffer	+	miRNA Control
DRA	Dimer Removal Agent - Dual		

Primer box (Store at -20°C) (Box 2)

Cat. No.	Tube	Component
700-00012 / 48	FP1 - 8	Forward Primer (FP) 1 -8
700-00012	RP1 - 12	Reverse Primers, Index 1 - 12*
700-00048	RP1 - 48	Reverse Primers, Index 1 - 48*

Primer box 2 (Store at -20°C) (Box 3) Only for 700-00048

Cat. No.	Tube	Component
700-00048	RP25 - 48	Reverse Primers, Index 25 - 48*

* For sequences see Appendix C, page 18.

Beads

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 degradation.
- Vortex and centrifuge each component before use.
- Always have PCR tubes on **ice** when handling.
- **Do not** freeze RealSeq® Beads or SPRI Beads.
- Master-mixes can be used for each reaction. Maximum overage of 10% is recommended.
- 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)
- 96-100% 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 with 100 ng of brain RNA or RNA extracted from 200 µL of Plasma extracted with Quick-cfRNA (Zymo Research)
- This kit is not compatible with plasma samples preserved with heparin.
- Using partially degraded RNA will result in a higher proportion of short sequencing reads (< 15 nt) that correspond to degraded rRNAs.
- Not all Plasma/Serum RNA extraction and purification kits isolate miRNAs with the same efficiency. Users should confirm that the method used isolates miRNAs efficiently.
- 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	PCR Cycles
1 µg total RNA	10-13
100 ng total RNA	13-16
10 ng total RNA	16-19
1 ng total RNA	19-22*
1 µl miRNA Control	13

* Recommended PCR cycles when profiling RNA extracted from biofluid samples

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 10 µL.

Reagent	Volume to add
RNA Input	up to 10 µL
● RNA Buffer (RB)	5 µL*
● RealSeq® Adapter Dual (A)	1 µL
RNase Free Water (W)	Variable
Total Volume	**16 µL**

*** RNA Buffer (RB) is very viscous, pipet slowly or heat to 37°C to facilitate pipetting.**

- 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)	2 µL
● Ligase (L)	1 µL
Total Volume	**20 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 **1 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 µL
Total Volume	**22 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	**24 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 -Dual (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 25 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 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 - Dual (RTP)	2 µL
dNTPs (dNTP)	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 (RTB)	4 µL
RNase free Water (W)	7 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**

* Select a unique FP/RP combination for each sample

- Add 53 µL of PCR reaction mix to each sample.
- Add **7 µL** of a unique **Reverse Primer Index*** 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 (17-22 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 are 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 SPRISelect® Reagent 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 (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: Thermocycler Programming

Thermocyclers can be programmed in advance for all reactions. Set Lid temperature at 95°C.

Step	Temperature		Time
1. Adapter Ligation	70°C		2 min
	ice or 4°C		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 or 4°C		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 Profile

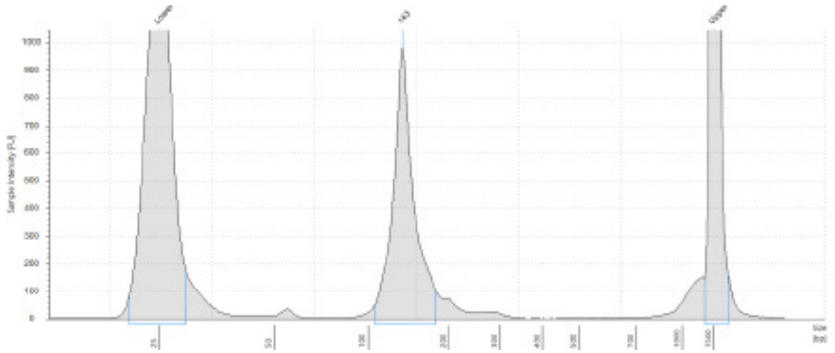

Figure 1. Example TapeStation® profile of a library prepared with RNA extracted from 200 µL of plasma. Library was amplified by 17 cycles of PCR. miRNA sized libraries are approximately 143 bp.

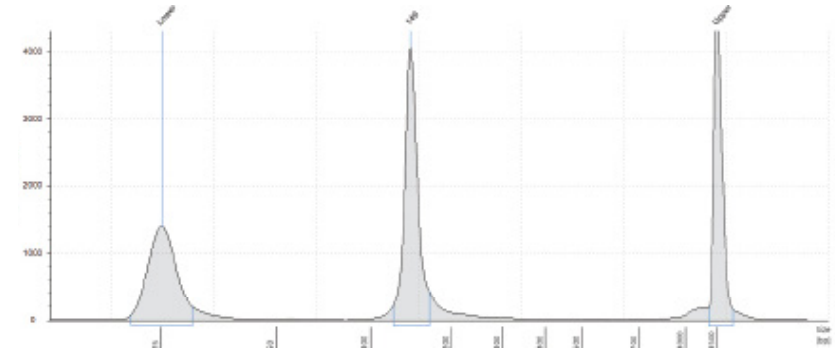

Figure 2. Example TapeStation® profile from a library with an input of 1 µL of miRNA control amplified by 13 cycles of PCR. miRNA control libraries are approximately 149 bp.

IX. Appendix C: Data Analysis

- RealSeq®-Biofluids libraries are completely compatible with bioinformatics tools designed for Illumina's TruSeq Small RNA libraries.
- The final product of a RealSeq®-Biofluids library contains the adapter sequence TGGAATTCTCGGGTGCCAAGG.
- This sequence needs to be trimmed from sequenced reads before mapping.
- RealSeq®-Biofluids libraries also contain an extra base at the 5' end of the inserts that needs to be removed before analysis.
- 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: Primer Index Sequence

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.

Please visit somagenics.com for ordering indexes RP1-RP48.

Tube	Seq	Rev
FP1	TGAACCTT	AAGGTTCA
FP2	TGCTAAGT	ACTTAGCA
FP3	TGTTCTCT	AGAGAACA
FP4	TAAGACAC	GTGTCTTA
FP5	CTAATCGA	TCGATTAG
FP6	CTAGAACA	TGTTCTAG
FP7	TAAGTTCC	GGAACTTA
FP8	TAGACCTA	TAGGTCTA

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