RealSeq®-Dual miRNA Library Kit for Illumina® sequencing

Cat. No.
700-00012
700-00048

20240613_RealSeq®-Dual

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RealSeq®-Dual

I. Overview

Step 1. Adapter ligation

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

Step 2. Adapter blocking

Add: Blocking Agent → **Incubate:** 5 min at 65°C, Step down to 37°C → **Add:** Blocking Enzyme → **Incubate:** 60 min at 37°C, 20 min at 65°C

Step 3. Circularization

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

Step 4. Dimer removal

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

Step 5. Reverse transcription

Add: RT Primer-Dual, 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, 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

SPRIselect® Reagent

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 Appendices D & E, page 19-20.

Beads

Tube	Component
B	RealSeq® Beads (+4°C)
SPRI	SPRIselect® Reagent (+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® Dual 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 SPRIselect® Reagent.
- 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 Appendix 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 B (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® Dual Adapter (A)	1 µl
RNase Free Water (W)	Variable
Total Volume	**16 µl**

***RNA Buffer (RB) is very viscous, pipet slowly.**

- 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 SPRIselect® Reagent!**

  - o Thoroughly vortex the beads for at least 30 seconds.
  - o Pipet 20 µl of the bead suspension into a new PCR tube.
  - o Place the tube on the magnetic rack for 1 minute or until all the beads settle against the side of the tube.
  - o 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 (13-22 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 SPRIselect® Reagent (SPRI), **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 SPRIselect® Reagent

- 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 (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. Set Lid temperature at 95°C.

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

VIII. Appendix B: Example Library Profile

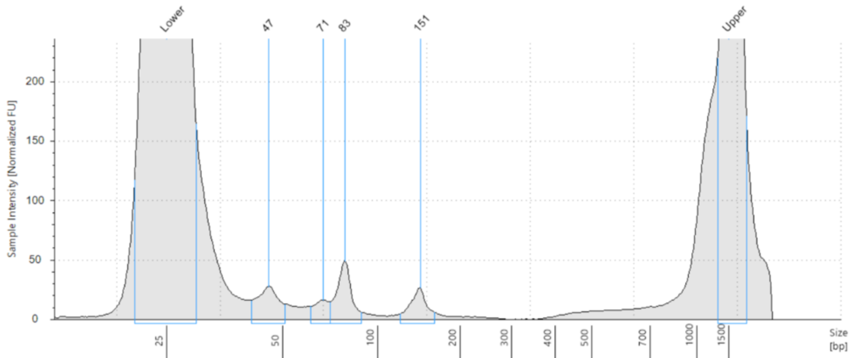

Figure 1. Example TapeStation® profile of a negative control library prepared with water. Library was amplified by 16 cycles of PCR. Adapter dimer peak is ~150 bp.

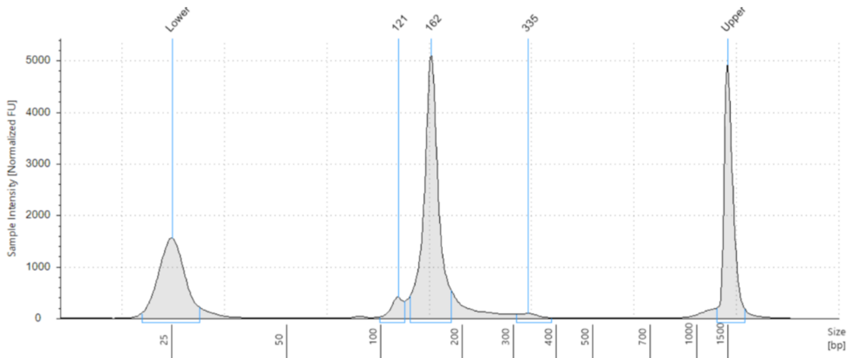

Figure 2. Example TapeStation® profile from a library with an input of 1 µl of miRNA positive control amplified by 13 cycles of PCR. miRNA control libraries are ~165 bp.

IX. Appendix C: Data Analysis

- RealSeq®-Dual libraries are completely compatible with bioinformatics tools designed for Illumina's TruSeq Small RNA libraries.
- The final product of a RealSeq®-Dual library contains the adapter sequence TGGAATTCTCGGGTGCCAAGG.
- This sequence needs to be trimmed from sequenced reads before mapping.
- RealSeq®-Dual 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: Reverse Primer Index Sequence

Tube	Sequence	Reported*	Tube	Sequence	Reported*
RP1	ATTGCCGA	ATCACGAT	RP25	ATGCTACC	ACTGATAT
RP2	ATCTTCGA	CGATGTAT	RP26	ATCCACTC	ATGAGCAT
RP3	ATTCGGGA	TTAGGCAT	RP27	ATCGTACG	ATTCCTAT
RP4	ATGAATGA	TGACCAAT	RP28	ATCGAAAC	CAAAAGAT
RP5	ATATTATA	ACAGTGAT	RP29	ATGGCCAC	CAACTAAT
RP6	ATGCTGTA	GCCAATAT	RP30	ATTTTCAC	CACCGGAT
RP7	ATCGATTA	CAGATCAT	RP31	ATGCGGAC	CACGATAT
RP8	ATGTCGTC	ACTTGAAT	RP32	ATCTCTAC	CACTCAAT
RP9	ATTCTGAG	GATCAGAT	RP33	ATGGACGG	CAGGCGAT
RP10	ATGTATAG	TAGCTTAT	RP34	ATTGACAT	CATGGCAT
RP11	ATAGCTAG	GGCTACAT	RP35	ATGGAACT	CATTTTAT
RP12	ATATTCCG	CTTGTAAT	RP36	ATTTGACT	CCAACAAT
RP13	ATTGTTGG	AGTCAAAT	RP37	ATTACAAG	CGGAATAT
RP14	ATAAAATG	AGTTCCAT	RP38	ATGTAGCC	CTAGCTAT
RP15	ATGCCATG	ATGTCAAT	RP39	ATAAGCTA	CTATACAT
RP16	ATCGCCTG	CCGTCCAT	RP40	ATCTGATC	CTCAGAAT
RP17	ATTGAGTG	GTAGAGAT	RP41	ATTCAAGT	GACGACAT
RP18	ATATCGTG	GTCCGCAT	RP42	ATGATCTG	TAATCGAT
RP19	ATCCGGTG	GTGAAAAT	RP43	ATATTGGC	TACAGCAT
RP20	ATTAGTTG	GTGGCCAT	RP44	ATCACTGT	TATAATAT
RP21	ATCTTTTG	GTTTCGAT	RP45	ATTGGTCA	TCATTCAT
RP22	ATAGGAAT	CGTACGAT	RP46	ATGCCTAA	TCCCGAAT
RP23	ATGCTCAT	GAGTGGAT	RP47	ATACATCG	TCGAAGAT
RP24	ATATCAGT	GGTAGCAT	RP48	ATCGTGAT	TCGGCAAT

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

XI. Appendix E: Custom Sequencing Primer

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

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

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