diagenode

Innovating Epigenetics Solutions

D-Plex Small RNA DNBSEQ™ Kit

Small RNA library preparation kit for MGI® sequencers

Cat. No. C05030051 (Core module – 24 rxns)

USER GUIDE

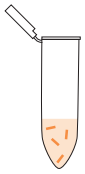
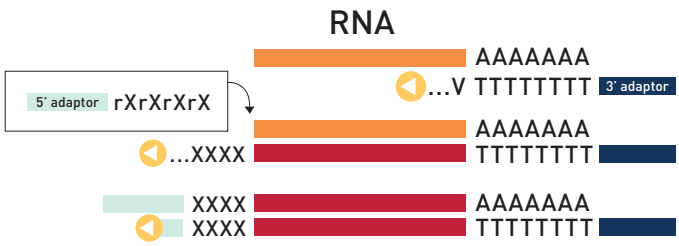
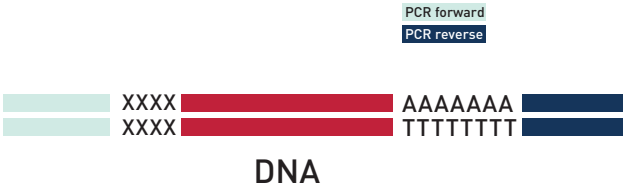
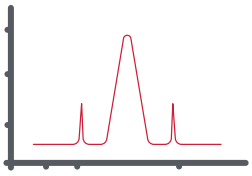
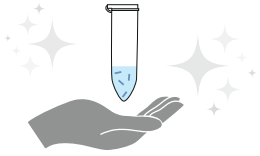
V2 04_2021

Summary

Please read this manual carefully before starting your experiment

Barcodes are not included in this kit and are available separately. Please check "Required Materials Not Provided" section.

Kit Method Overview

			Time	Pause
STEP 1		RNA sample preparation	20 min	❄
STEP 2		RNA dephosphorylation and tailing	1h30	
STEP 3		Reverse transcription	>2h	❄
STEP 4		PCR amplification	60 min	❄
STEP 5		Library purification		
STEP 6		Library clean-up and shaping		
STEP 7		Library control and quantification		

Introduction

The Diagenode D-Plex Small RNA DNBSEQ™ Library Preparation Kit is a tool designed for the study of the **small non-coding transcriptome**. The present kit incorporates the unique **D-Plex technology** to generate double-stranded DNA libraries from RNA samples ready to be used for the DNA single-strand circularization step required for **DNBSEQ sequencing** on MGI sequencers.

The D-Plex technology utilizes the innovative **capture and amplification by tailing and switching, a ligation-free method** for library preparation and offers key advantages such as:

- **Ultra-low input capability**
- Ease of use in a **one day, one tube protocol**
- **High library complexity**

The library preparation protocol works on either total intact RNA (RIN≥8) extracted and purified from a given sample or a small RNA fraction (<200nt), that might very well represent the circulating content of a **liquid biopsy-type of sample** (blood serum and plasma). The input requirements of the method are flexible and allow the user to perform the method within a wide range of RNA quantities going **from 10 pg** of circulating RNA **up to 100 ng** for total RNA.

The core of the technology relies on **ligation-free reactions** to attach the MGI adaptors to both ends of the library construct. Therefore, the results generated with the D-Plex Small RNA DNBSEQ kit will vastly differ from those produced with a ligase-based approach. For instance, the results generated with a D-Plex kit will encompass a **vast spectrum of small non-coding RNAs** (miRNAs, snoRNAs, snRNAs, piRNAs) whereas a ligase-based approach will enrich the sequencing library in 5'-P – 3'-OH RNAs, mainly mature miRNAs.

Diagenode therefore recommends having a **clear understanding of the scientific question** being asked in a given experiment before proceeding to a small RNA-seq library preparation as the choice of technology will strongly impact the end result.

Kit Materials

Table 1. Components of D-Plex Small RNA DNBSEQ Kit

Description	Cap color	Qty (24 rxns)	Storage (°C/°F)
Dephosphorylation Buffer (DB)	Yellow	48 µl	-20°C/-4°F
Dephosphorylation Reagent (DR)	Yellow	12 µl	-20°C/-4°F
Tailing Reagent (TR)	Red	12 µl	-20°C/-4°F
Small Tailing Buffer (STB)	Red	24 µl	-20°C/-4°F
RT Primer H DNBSEQ (RTPH)	Purple	24 µl	-20°C/-4°F
RT Primer M DNBSEQ (RTPM)	Purple	24 µl	-20°C/-4°F
Reverse Transcription Reagent (RTR)	Purple	24 µl	-20°C/-4°F
Reverse Transcription Buffer (RTB)	Purple	120 µl	-20°C/-4°F
Template Switching Oligo DNBSEQ (TSO)	Purple	48 µl	-20°C/-4°F
PCR Master Mix (PCRMM)	Green	1200 µl	-20°C/-4°F
Positive Control miRNA (CTL+)	Black	12 µl (1 ng/ µl)	-20°C/-4°F For long term storage: -80°C/-112°F
Nuclease-free Water	Clear	2000	Room Temperature

Required Materials Not Provided

Barcodes

Specific D-Plex DNBSEQ Barcodes were designed and validated to fit this technology and are **not included in this core module**, providing you with total flexibility. They can be bought separately according to your needs. Please choose the format that suits you best among the compatible references to:

Table 2. DNBSEQ barcode sets and references

Barcodes Modules	Reference
D-Plex 24 DNBSEQ Barcodes - Set A	C05030060
D-Plex 24 DNBSEQ Barcodes - Set B	C05030061

Multiplexing

The validated PCR reverse primers bear the MGIEasy DNA Barcodes that can be used for library multiplexing up to 48. In case of a multiplexing scenario, it is recommended to follow MGI's library pooling guidelines that are explained in the D-Plex DNBSEQ Barcodes manual.

General equipment and reagents

- Gloves to wear at all steps
- Single channel pipettes and corresponding RNase-free filter tips: 10 µl, 20 µl, 200 µl, 1,000 µl
- RNase AWAY™ decontamination reagent (ThermoFisher Scientific, 10328011)
- RNase-free tubes: 0.2 ml, 0.5 ml, 1.5 ml
- Table top centrifuge with strip rotor
- Vortex agitator
- Tube holder for 0.2 ml, 0.5 ml, 1.5 ml tubes
- DiaMag 0.2 ml tube magnetic rack (Diagenode, B04000001)
- DiaMag 1.5 ml tube magnetic rack (Diagenode, B04000003)
- Thermal cycler

Post-PCR libraries purification

- MicroChIP DiaPure columns (Diagenode, C03040001)
- Absolute ethanol (VWR, 20821.310)
- DNase-free, RNase-free ultrapure water

Post-PCR libraries shaping

- Agencourt® AMPure® XP Beads (Beckman Coulter, A63881)
- Absolute ethanol (VWR, 20821.310)
- DNase-free, RNase-free ultrapure water

Precise library size-selection with agarose/polyacrylamide gel-cut

Agarose (4-6%) or polyacrylamide (4-6%) self-made/pre-casted gels with the necessary electrophoresis equipment (TBE 0.5X buffer, electrophoresis tank, generator...) including the material required for the gel cut (scalpel blade, tweezers and pestle) and the supplementary MinElute® Gel Extraction Kit (Qiagen, 28604).

DNA library size and yield estimation

- Agilent 2100 Bioanalyzer® (Agilent) and Agilent High Sensitivity DNA Kit (Agilent, 5067-4626)
- Qubit® Fluorometer (Thermo Fisher Scientific) and Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851)

Sequencing

- MGIEasy Circularization Kit (MGI, 1000005259)
- DNBSEQ-G400RS High-throughput Sequencing Set (Small RNA FCL SE50) (MGI, 1000016998)
- CoolMPS High-throughput Sequencing Set (DNBSEQ-G400RS Small RNA FCL SE50) (MGI, 1000019478)

Remarks Before Starting

General recommendations

- Read the complete manual before first time use
- Decontaminate the working area as well as all the tools used to perform the library preparation with RNase AWAY™ reagent
- Wear gloves at all steps to protect the RNA sample from degradation by contaminants and nucleases
- All containers and storage areas must be free of contaminants and nucleases
- Add enzymes to reaction solutions last and thoroughly incorporate them by pipetting up and down the solution several times
- When mixing reagents, do not vortex but pipet up and down
- When using the protocol for the first time for a definite type of sample, we strongly recommend using the positive miRNA control included in the kit

Important notes

The D-Plex Small RNA DNBSEQ protocol has been developed for inputs ranging from 100 ng to 100 pg of total RNA (RIN≥8) and inputs ranging from 10 ng to 10 pg of small RNA (<200 nt) either isolated from cells or biological fluids, extracellular vesicles, or others.

A starting volume of 13 µl is used in the library preparation process. RNA has to be dissolved in nuclease-free water in this volume before starting the protocol.

It is advised, when performing the protocol for the first time with a particular template, to include a reaction with the positive control provided in the kit and a reaction without template as negative control. This negative control is useful to check for the absence of contaminants in the working environment.

The D-Plex Small RNA DNBSEQ library preparation incorporates the different classes of small non-coding RNAs in the final library for sequencing. If coding RNA or long non-coding RNA degradation products are of particular interest to the researcher, it is possible to perform an optional step (described in the detailed protocol, step 2.1) that will help enrich for those products in the library.

The following protocol describes the library preparation of one single sample. If the interest is to prepare more than one sample in parallel, please scale up accordingly.

> ***CAUTION**: The incubations during the D-Plex Small RNA DNBSEQ library preparation take place in a thermal cycler with a heated lid at 105°C.*

Starting material

The input used for the D-Plex library preparation needs to be free of contaminants and the RNA must be solubilized in nuclease-free water.

Table 3. Input recommendations

Template	Starting amount	RIN
Total RNA	100 pg - 100 ng	≥ 8
Small RNA (<200nt)	10 pg - 10 ng	N/A
Circulating RNA	10 pg - 10 ng	N/A

Template

Both total RNA and small RNA (<200nt) can be used for input in the D-Plex Small RNA DNBSEQ library preparation.

Small RNAs can be isolated either from biological fluids (e.g. blood plasma or serum), exosomes or from cells using a column-based method (e.g. miRNeasy Mini Kit (Qiagen, 217004)) capable of retaining the small RNA fraction (<200nt). Please be aware that not all the products on the market are able to efficiently isolate RNAs under 200nt.

For total RNA samples, we strongly recommend to first perform a quality check of the RNA using the Bioanalyzer® 2100 instrument with the associated Agilent RNA 6000 Pico Kit (Agilent, 5067-1531). Please be aware that sample degradation may result in the underrepresentation of the small non-coding RNAs in the final library.

The D-Plex method offers a protocol in which the RNA sample is treated with a dephosphorylation reagent (DR) that will dephosphorylate the 3'end of an RNA molecule (if it is phosphorylated) and monophosphorylate the 5'end (if it is hydroxylated). This protocol might constitute a special interest for researchers studying the degradation products of coding RNAs and long non-coding RNAs.

Positive control: cel-miR-39-3p

A positive control is supplied in the kit for 12 reactions at a concentration of 1 ng/µl. This synthetic miRNA is meant to be used in a library preparation set to monitor the library preparation efficiency or as a spike-in control in a given RNA sample to account for eventual matrix effect. Typically, 1 ng of synthetic miR39 prepared with the D-Plex protocol and amplified for 12 PCR cycles will yield a minimum of 400 ng of dsDNA after DiaPure purification.

The synthetic miRNA mimics the mature sequence of cel-miR-39-3p from Caenorhabditis elegans and is 22nt long.

5'-UCACCGGGUGUAAAUCAGCUUG-3'

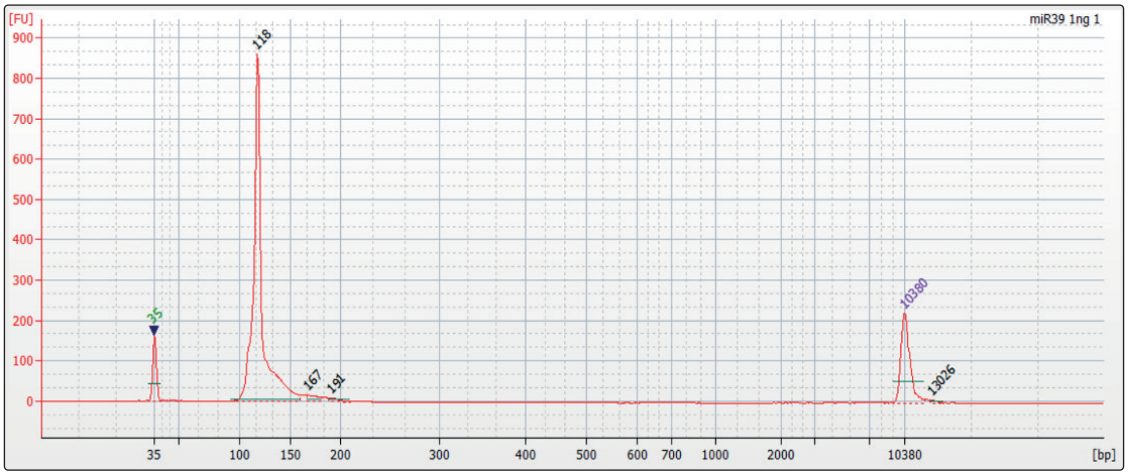
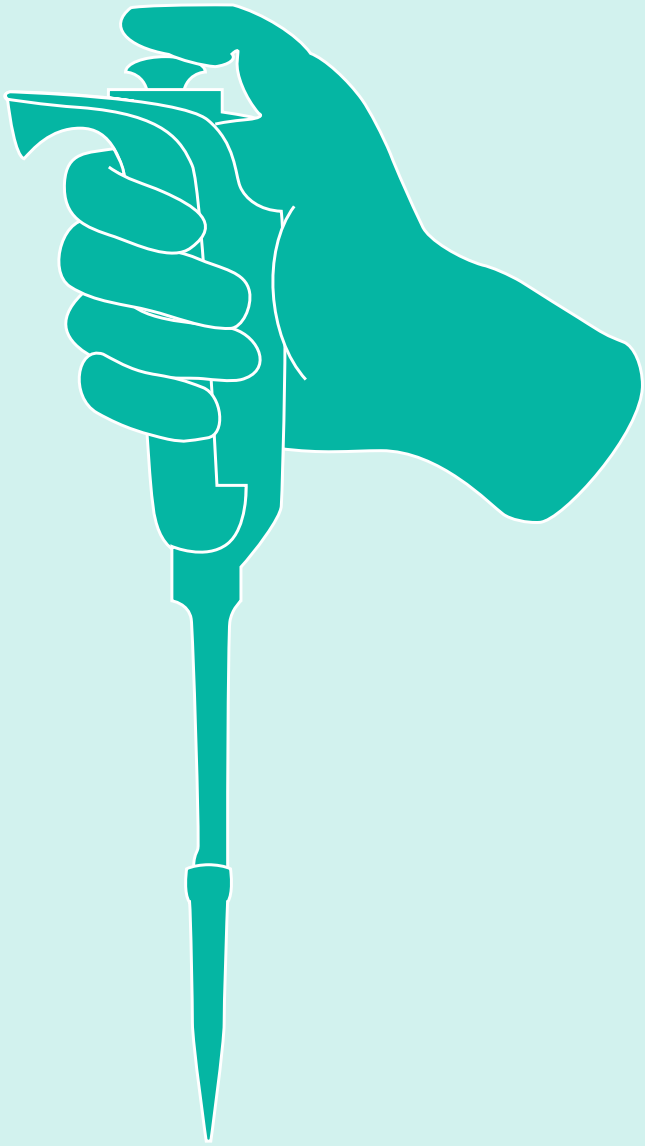


Figure 1: DNA electropherogram of a D-Plex Small RNA DNBSEQ library prepared from 1ng of miR39 (12X PCR cycles) after DiaPure purification. 1 ng of the library was loaded on the Bioanalyzer.

SHORT PROTOCOL



FOR EXPERIENCED USERS

Short Protocol for experienced users

Notice before starting

D-Plex Small RNA DNBSEQ library preparation is incorporating the different classes of small non-coding RNAs in the final library for sequencing. If coding RNA or long non-coding RNA degradation products are of particular interest to the researcher, it is possible to perform an optional (step 3) that will help enrich for those products in the library.

1. Prepare the RNA sample in nuclease-free water to get a total volume of **13 µl**.

2. Add **2 µl of Dephosphorylation Buffer (DB)** and incubate for 10 minutes at 70°C. Then immediately put on ice for 2 minutes.

3. **OPTIONAL [**To proceed to RNA 3'-end dephosphorylation, add **0.5 µl of Dephosphorylation Reagent (DR)** to the reaction tube. Incubate for 15 minutes at 37°C. Then put on ice**]**.

4. Prepare the **Tailing Master Mix (TMM)** by mixing **0.5 µl of Tailing Reagent (TR)** and **1 µl of Small Tailing Buffer (STB)**.

5. Add **1.5 µl of TMM** and incubate for 40 minutes at 37°C + 20 minutes at 65°C. Then put on ice to cool down.

6. Add **1 µl of Reverse Transcription Primer DNBSEQ (RTP)**:

   - For 100 ng-10 ng total RNA or 10 ng-1 ng small RNA fraction, use **RT Primer H DNBSEQ (RTPH)**.
   - For 10 ng-100 pg total RNA or 1 ng-10 pg small RNA fraction, use **RT Primer M DNBSEQ (RTPM)**.

7. Incubate for 10 minutes at 70°C. Then slowly decrease to 25°C for 2 minutes by ramping down at 0.5°C/sec.

8. Take the **Template Switch Oligo DNBSEQ (TSO)** tube out of the freezer and let it thaw on ice.

9. Prepare the **Reverse Transcription Master Mix (RTMM)** by mixing **5µl of Reverse Transcription Buffer (RTB)** and **1µl of Reverse Transcription Reagent (RTR)** in a nuclease-free PCR tube.

10. Add **6µl of Reverse Transcription Master Mix (RTMM)** and incubate for 15 minutes at 25°C.

11. Add **2 µl of Template Switch Oligo DNBSEQ (TSO)**.

12. Incubate for 120 minutes at 42°C + 10 minutes at 70°C.

13. Add **10 µl of D-Plex Forward Primer DNBSEQ (FP), 10 µl of D-Plex barcoded Reverse Prime DNBSEQ (RP)**, and **50 µl of PCR Master Mix (PCRMM)**.

14. Amplify the library according to the following program:

<table>
<tr><th>Temperature</th><th colspan="2">Time & Cycles</th></tr>
<tr><td>98°C</td><td colspan="2">30 seconds</td></tr>
<tr><td>98°C</td><td>15 seconds</td><td rowspan="3">n cycles*</td></tr>
<tr><td>62°C</td><td>30 seconds</td></tr>
<tr><td>72°C</td><td>30 seconds</td></tr>
<tr><td>72°C</td><td colspan="2">10 minutes</td></tr>
<tr><td colspan="3">Hold at 4°C or freeze until further processing</td></tr>
</table>

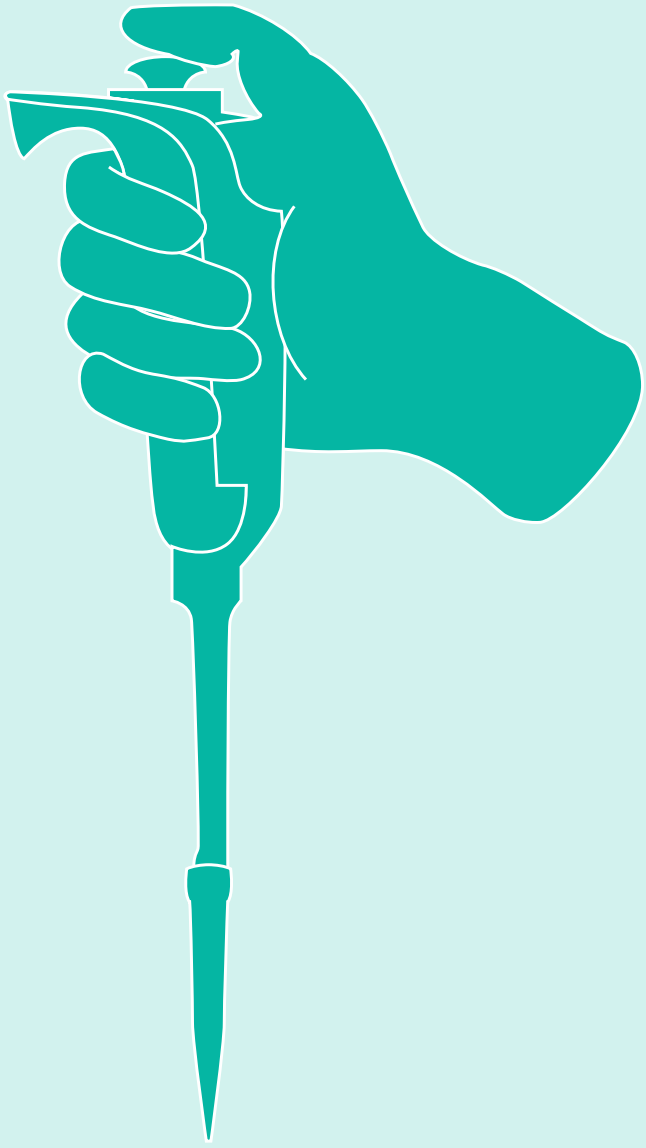
**Perform* n *cycles depending on the initial RNA input:*

- *from **10 cycles**: for 100 ng total RNA or 10 ng small RNA/circulating RNA*
- *up to **20 cycles**: for 100 pg total RNA or 10 pg small RNA/circulating RNA*

15. Purify the PCR product using the MicroChIP DiaPure Kit (Diagenode, C03040001), according to the kit instructions. To perform further clean-up or size-selection of the library, refer to sections 6-A-B-C of the detailed protocol.

16. Perform library quantification using QuBit® dsDNA HS Assay Kit and quality check using Bioanalyzer® DNA High Sensitivity Assay Kit according to the manufacturer's instructions.

DETAILED PROTOCOL



STEP 1

RNA sample preparation -  *20 minutes*

1.1 Dilute the RNA sample in **nuclease-free water** to get a total volume of **13 µl**.

1.2 To the **13 µl RNA solution**, add **2 µl of Dephosphorylation Buffer (DB)**. Mix by pipetting up and down thoroughly but gently until solution is homogenous.

1.3 Incubate for 10 minutes at 70°C and then immediately put the tube on ice for 2 minutes. Spin down the reaction tube to collect the sample at the bottom.

STEP 2

RNA dephosphorylation and tailing

1 hour 30 minutes

2.1 **OPTIONAL [**To proceed to the RNA 3'-end dephosphorylation, add **0.5 µl of Dephosphorylation Reagent (DR)** to the reaction tube. Mix by pipetting up and down until solution is homogeneous. Incubate for 15 minutes at 37°C and then put the tube on ice.**]**

2.2 Prepare the **Tailing Master Mix (TMM)** by mixing **0.5 µl of Tailing Reagent (TR)** and **1 µl of Small Tailing Buffer (STB)** in a tube.

2.3 Add **1.5 µl of TMM** to the reaction tube and mix by pipetting up and down until solution is homogeneous.

2.4 Incubate the sample for 40 minutes at 37°C + 20 minutes at 65°C and then put the tube on ice to let it cool down. Spin down the reaction tube to collect the sample at the bottom.

STEP 3

Reverse transcription -  *> 2 hours*

3.1 Add **1 µl of Reverse Transcription Primer DNBSEQ (RTP)** to the reaction tube. Choose the right RTP according to your RNA input:

Table 4: RT primer concentration according to RNA input

Template	Small RNA fraction / biofluid-isolated RNA	
Total RNA	100 pg – 10 ng	RTPH
	10 ng - 100 ng	RTPM
Small RNA (<200nt)	10 pg - 1 ng	RTPH
	1 ng - 10 ng	RTPM
Circulating RNA	10 pg - 1 ng	RTPH
	1 ng - 10 ng	RTPM

3.2 Mix by pipetting up and down until solution is homogeneous. Incubate for 10 minutes at 70°C and then slowly decrease to 25°C by ramping down at 0.5°C/sec (maintain to 25°C for 2 minutes). Spin down the reaction tube to collect the sample at the bottom and then put the tube on ice until further processing.

3.3 Take the **Template Switch Oligo DNBSEQ (TSO)** tube out of the freezer and let it thaw on ice.

3.4 Prepare the **Reverse Transcription Master Mix (RTMM)** by mixing **5µl of Reverse Transcription Buffer (RTB)** and **1µl of Reverse Transcription Reagent (RTR)** in a nuclease-free PCR tube.

3.5 Add **6µl of Reverse Transcription Master Mix (RTMM)** to the reaction tube and mix by pipetting up and down until the solution is homogenous.

3.6 Incubate for 15 minutes at 25°C and then put the tube on ice.

3.7 Add **2 µl of Template Switch Oligo DNBSEQ (TSO)** to the reaction tube and mix by pipetting up and down until the solution is homogeneous.

3.8 Incubate the sample for 120 minutes at 42°C + 10 minutes at 70°C and then put the tube on ice until further processing.

NOTE: *The reverse transcription (120 minutes at 42°C + 10 minutes at 70°C) may be performed overnight. If so, add an additional hold at +4°C once the first two steps are completed.*

3.9 Spin down the reaction tube to collect the sample at the bottom.

STEP 4

PCR amplification - *1 hour*

4.1 Add **10 µl of D-Plex Forward Primer DNBSEQ (FP)** and **10 µl of D-Plex barcoded Reverse Primer DNBSEQ (RP)** to the reaction tube. Add **50 µl of PCR Master Mix (PCRMM)** and mix directly by pipetting up and down until solution is homogenous.

4.2 Determine the number of PCR cycles (n) you have to perform, according to the RNA input:

Table 5: PCR amplification program for D-Plex Small RNA DNBSEQ library preparation

Input RNA	Amount RNA	*n*
Total RNA	100 pg	20
	1 ng	15
	10 ng	12
	100 ng	10
Small RNA fraction / Fluid-isolated RNA	10 pg	20
	100 pg	15
	1 ng	12
	10 ng	10

4.3 Incubate for PCR amplification according to the following program:

Step	Temperature	Time & Cycles	
1. Initial denaturation	98°C	30 seconds	
2. Denaturation	98°C	15 seconds	*n* cycles
3. Annealing	62°C	30 seconds	
4. Extension	72°C	30 seconds	
5. Final extension	72°C	10 minutes	
Hold at 4°C or freeze until further processing			

***CAUTION**: If you plan to proceed to the size-selection of the library for short inserts (< 50 nt) (see sections 6-B-C), add 2 more cycles to the n cycles displayed above.*

STEP 5

Library purification

NOTE: *It is recommended to purify the amplified library with a spin column-based method. As an alternative method, a 1.8x SPRI beads clean-up can be performed (see section 6A).*

5.1 Purify the amplified library using the MicroChIP DiaPure kit (Diagenode, C03040001) according to the kit instructions. Note that the library volume to start the purification is around **100 µl**.

5.2 Perform the elution in the volume displayed below in a 1.5 ml tube according to the next library processing step:

Table 6: Elution volume after MicroChIP DiaPure purification

Next library processing step	DiaPure elution volume
No further clean-up or size selection	20 µL
SPRI beads clean up (Section 6A)	50 µL
Agarose gel-cut size-selection (Section 6B)	20 µL
Polyacrylamide gel-cut size-selection (Section 6C)	20 µL

STEP 6

Library clean-up and shaping

NOTE: *To purify the libraries, the MicroChip DiaPure purification method should be sufficient and no additional clean-up is required. However, if necessary or preferred, an additional 1.8x SPRI beads clean-up can be performed to remove residual oligos (Section 6.A).*

Choose the appropriate procedure to shape the final form of the library before sequencing:

- **SECTION 6A:** SPRI beads clean-up to retain all the library fragments.
- **SECTION 6B:** Agarose gel-cut size selection for the accurate selection of any length of fragments.
- **SECTION 6C:** Polyacrylamide gel-cut size selection for the accurate selection of any length of fragments

6A – BEADS CLEAN-UP

6A.1 Take the **AMPure XP beads** out of the fridge and resuspend them gently on a rotating wheel or orbital shaker at room temperature before use.

6A.2 Add **90 µl of beads** to the 50 µl of eluted DNA from Step 5 and mix thoroughly by pipetting up and down or vortexing.

6A.3 Incubate at least 5 minutes at room temperature under mild agitation.

6A.4 Spin the tube down and put it in a magnetic rack until the beads are collected to the side of the tube and the solution is completely clear.

6A.5 Carefully remove and discard the supernatant without disturbing the beads.

6A.6 While keeping the tube on the magnetic rack, perform 2 rounds of bead-wash. Add **200 µl of freshly prepared 80% ethanol** for 30 seconds and then remove carefully the supernatant without disturbing the beads.

6A.7 Spin down the tube, place it back in the magnetic rack and remove any remaining ethanol.

6A.8 Leave the tube open to let the beads pellet dry for 2-3 minutes and then remove it from the magnetic rack.

6A.9 Resuspend the beads in **20 µl of nuclease-free water** by slowly pipetting up and down.

6A.10 Incubate the beads in water during 2 minutes at room temperature under mild agitation.

6A.11 Spin the tube down and place it in the magnetic rack until the beads collected to the size of the tube and the solution is completely clear.

6A.12 Carefully collect the supernatant without taking up any beads. Transfer the cleaned-up library in a nuclease-free tube and store it at -20°C for further use.

6B – AGAROSE GEL-CUT SIZE-SELECTION

6B.1 Load your **20 µl eluted DNA library** from step 5 (mixed with appropriate electrophoresis loading buffer) in a well of a **4% agarose gel soaked in 0,5x TBE buffer**. Use a **gel stain** of your best convenience (e.g. 1x SYBR® Gold nucleic acid gel stain or 1x SYBR® Safe DNA gel stain from Thermo Fisher Scientific) and a ladder of your choice (e.g. 25-100bp mixed DNA ladder Bionneer D-1020).

6B.2 Run the electrophoresis until the migration front reaches the end of the gel.

6B.3 View the gel on a UV transilluminator and perform the gel-excision of the band of interest. For example, the miRNA band is around ~120 bp.

6B.4 Put the gel-slice in a 1.5 ml tube and perform the DNA gel extraction using the **MinElute® Gel extraction kit** from Qiagen **BUT do not follow Qiagen instructions**.

6B.5 Add **600 µl of buffer GQ** and incubate overnight at 37°C under an agitation of 300 rpm.

6B.6 After gel dissolution, spin the tube down, collect the solution to transfer it in a MinElute® column.

6B.7 Centrifuge for 20 seconds at 16.000g and discard the flow-through.

6B.8 Add **0.75 ml of buffer PE** to the MinElute® column, incubate for 1 minute and centrifuge for 1 minute at 16.000g.

6B.9 Discard the flow-through and centrifuge the column for 1 minute at 16.000g.

6B.10 Place the MinElute® column in a clean 1.5ml tube, add **20 µl of Buffer EB** and incubate 2 minutes at room temperature.

6B.11 Centrifuge for 1 minute at 10.000g and collect the eluted DNA library. Store at -20°C for further use.

6C – POLYACRYLAMIDE GEL-CUT SIZE-SELECTION

6C.1 Load your **20 µl eluted DNA library** from step 5 (mixed with appropriate electrophoresis loading buffer) in a well of a **4-6% polyacrylamide gel soaked in 2xTBE buffer**. Use a **gel stain** of your best convenience (e.g. 1x SYBR® Gold nucleic acid gel stain or 1x SYBR® Safe DNA gel stain from Thermo Fisher Scientific) and a ladder of your choice (e.g. 25-100bp mixed DNA ladder Bionneer D-1020).

6C.2 Run the electrophoresis until the migration front reaches the end of the gel.

6C.3 View the gel on a UV transilluminator and perform the gel-excision of the band of interest. For example, the miRNA band is around ~120 bp.

6C.4 Put the gel-slice in a 1.5 ml tube and perform the DNA gel extraction using the **MinElute® Gel extraction kit** from Qiagen **BUT do not follow Qiagen's instructions.**

6C.5 Crush the gel slice in the tube with a DNase-free pestle or cut it into very small pieces and then add 600 µl of buffer GQ and incubate overnight at 37°C under a 300 rpm agitation to let DNA diffuse out of the gel.

6C.6 Spin the tube down at full speed, collect supernatant and transfer it in a MinElute® column;

6C.7 Centrifuge 20 secondes at 16.000g and discard flow-through.

6C.8 Add **0.75 ml of buffer PE** to the MinElute® column, incubate for 1 minute and centrifuge 1 minute at 16.000g.

6C.9 Discard the flow-through and centrifuge the column for 1 minute at 16.000g.

6C.10 Place the MinElute® column in a clean 1.5ml tube, add **20 µl of Buffer EB** and incubate 2 minutes at room temperature.

6C.11 Centrifuge 1 minute at 10.000g and collect the eluted DNA library. Store at -20°C for further use.

STEP 7

Library quality control and quantification

7.1 For quantification, measure the library concentration using **QuBit® dsDNA HS Assay kit** according to the manufacturer's instructions.

7.2 For library size estimation, use the **Bioanalyzer® DNA High Sensitivity assay kit** according to the manufacturer's instructions.

D-Plex Small RNA DNBSEQ Construct

Figure 2: The D-Plex Small RNA DNBSEQ construct bears the MGIEasy DNA Barcodes allowing library multiplexing up to 48.

Sequencing Recommendations

The D-Plex Small RNA DNBSEQ Kit allows the production of dsDNA libraries, which are designed for DNBSEQ sequencing on MGI sequencers. Therefore, a D-Plex Small RNA DNBSEQ library pool needs to undergo a circularization reaction prior to the subsequent DNB-production, flow-cell loading and sequencing procedure. For this circularization step, we refer to the **MGIEasy Circularization Kit** (MGI, 1000005259).

To obtain high quality data, we recommend the MGI DNBSEQ-G400 sequencing system with the usage of the new CoolMPS technology (**CoolMPS High-throughput Sequencing Set** (DNBSEQ-G400RS Small RNA FCL SE50) (MGI, 1000019478))

The D-Plex Small RNA DNBSEQ library construct bears the MGIEasy DNA Barcodes that can be used for library multiplexing up to 48. In case of a multiplexing scenario, it is then recommended to follow MGI's library pooling guidelines that are explained in the D-Plex Small RNA DNBSEQ Barcodes manual.

The complete architecture of the D-Plex Small RNA DNBSEQ construct is provided in Figure 2. The empty library size (adapters + template switch + A tail) is equal to 108 bp.

***IMPORTANT NOTICES**:*

- *Given the high complexity of D-Plex Small RNA DNBSEQ libraries, it is advised to sequence one library with a minimum of 20M (millions) SE (single-end) reads in order to sufficiently cover the library content for downstream analysis.*
- *Since the library sequence starts with the template switch motif, we recommend to initiate the sequencing with 3 dark cycles.*

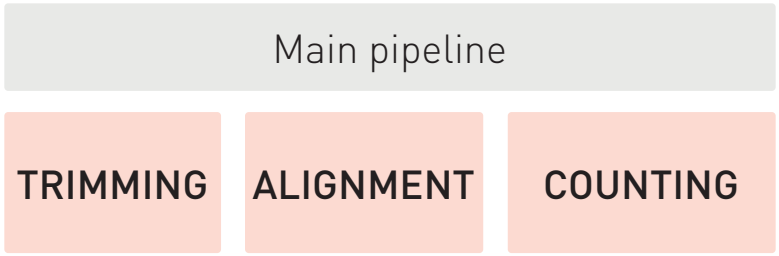
Table 7: Processing and sequencing recommendations

ssDNA circularization	MGIEasy Circularization Kit (MGI, 1000005259)
Sequencing system	MGI DNBSEQ-G400
Sequencing set	DNBSEQ-G400RS High-throughput Sequencing Set (Small RNA FCL SE50) (MGI, 1000016998)
Sequencing chemistry	CoolMPS High-throughput Sequencing Set (DNBSEQ-G400RS Small RNA FCL SE50) (MGI, 1000019478)
Sequencing parameters	SE50, dark cycles 1-3

Data Analysis Recommendations

The following is a guideline to allow you to start analyzing your D-Plex sequenced data. Depending on the aim of your analysis you might need to try different parameters and tools.

The D-Plex libraries contain special sequences that need particular treatment in order to get the best results out of your datasets. These special sequences are the poly-A tail and the template switch motif. This guide will take you through the basic processes of trimming, alignment and counting, using software tools and settings that we validated. Though naturally other tools and methods can also be used, please pay attention to finding the optimal settings for your experiments, e.g. do not run a paired-end pipeline for single reads.



***NOTE**: The links for the tools used in the example pipeline are at the end of this section.*

Trimming

Trimming is mandatory for the reads generated with the D-Plex Small RNA DNBSEQ Kit, because the read length is often longer than the targeted small RNAs, so in many cases the poly-A tail and the 3' adapter are also sequenced. In addition, the 5' end contains the template switch motif that is originally not part of the RNA. The aim of trimming is to remove these artificial sequences that will likely hamper downstream analyses.

In our example below, we use cutadapt to demonstrate how to properly do the trimming for D-Plex reads and get a read set as clean as possible. To minimize the effect of ambiguously read bases, we trim the Ns and allow IUPAC codes in the reads. We then remove the 3' adapter (AAGTCGGAGGCCAAGCGGTCT) and the poly-A tail from the 3' end of the read. Finally, we remove the 5' adapter (GAACGACATGGCTACGATCCGACTTSSS) from the 5' end of the read. We found that this combination of parameters

prevents excessive over-trimming and read loss, while reliably and fully cleans up the read set coming from an average MGI sequencing run. The last step includes discarding reads below 15 nucleotides, as they might be difficult to map uniquely. The example command with cutadapt is below.

```
cutadapt --trim-n -a AAGTCGGAGGCCAAGCGGTCT raw_reads.fastq.gz
| cutadapt --match-read-wildcards -a AAAAA - | cutadapt -O 8
--match-read-wildcards -g GAACGACATGGCTACGATCCGACTTSSS -m 15
-o trimmed_reads.fastq.gz –
```

Alignment

Aligning the trimmed reads needs special treatment as mapping small reads requires the use of specialized aligners that are suitable for mapping small RNA DNBSEQ reads. We strongly recommend aligning to the genome instead of the transcriptome. Indeed, D-Plex tends to generate very high-complexity libraries which often include small RNAs that are not identified yet and therefore would not map to a transcriptome consisting of only known transcripts. Of course, in addition to the genome alignment, the mapped reads can be assigned to known transcripts as well for expression analysis of the known genes.

In our example command below, we use the software STAR which does exactly that: it aligns the reads to the reference genome, then assigns them to known transcripts, using a GTF annotation file. For this example, we assume that the reads were 50 bases long and STAR was run in multithreading mode on 10 cores. NOTE: for mapping any type of unspliced RNA, STAR recommends to index the genome without the GTF file, since STAR only uses splice junctions information for mapping.

```
STAR --runThreadN 10 --genomeDir /genomes/hg19/
--readFilesIn trimmed_reads.fastq.gz --sjdbGTFfile genomes/
hg19/hg19.gtf --alignIntronMax 1 --readFilesCommand zcat
--outFileNamePrefix ./MySample --outSAMtype BAM Unsorted
--outFilterMultimapScoreRange 0 --outFilterMultimapNmax 50
--outFilterMismatchNoverLmax 0.05 --outFilterMatchNmin 15
--outFilterScoreMinOverLread 0 --outFilterMatchNminOverLread
0 --sjdbOverhang 49 --quantMode GeneCounts TranscriptomeSAM
--quantTranscriptomeBAMcompression -1 --outSAMunmapped Within
```

Counting

In our command example below, we use the featureCounts program from the Subread package for counting. The other options specify that we want to keep multimapping reads, since some small RNA biotypes map to multiple loci. Each read will carry a fractioned count (option –fraction). The -O option will allow a read to be assigned to more than one matched meta-feature (this is optional). In the end we will obtain the expression levels of annotated features as unnormalized values (counts).

```
featureCounts -a genomes/hg19/hg19.gtf -o MySample_
featureCounts.txt -T 10 -M -O --fraction MySample_sorted.bam
```

Links for the tools used in the example pipeline

Tool	Link
Website	https://www.diagenode.com/en/
Cutadapt	https://github.com/marcelm/cutadapt
STAR	https://github.com/alexdobin/STAR
Samtools	http://www.htslib.org/download
featureCounts	http://subread.sourceforge.net/

Example of Results

Quality requirements

When the D-Plex Small RNA DNBSEQ protocol is followed accordingly with the appropriate conditions for the RNA input, the following standards of quality can be expected :

Table 8: Minimum yield expected from a D-Plex Small RNA DNBSEQ library after DiaPure purification for different inputs and templates.

Template	Input	DNA quantity after DiaPure purification (ng)
Total RNA (from K562 cells)	100 ng	~100 ng
Total RNA (from K562 cells)	100 pg	~100 ng
Small RNA (<200nt)	1 ng	~50 ng

Electrophoregrams

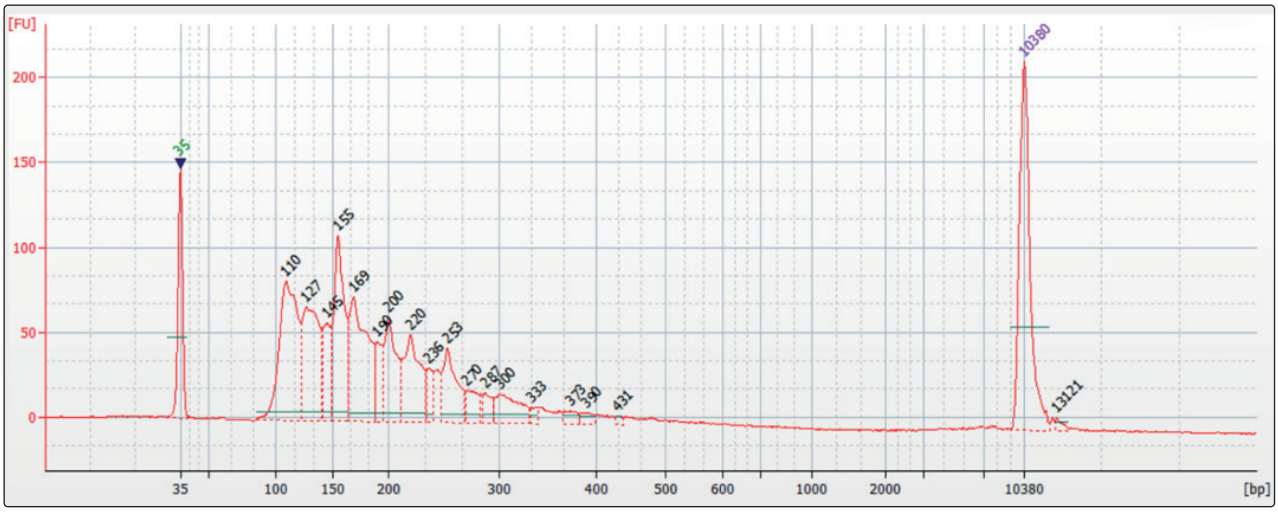


Figure 3: DNA electropherogram of a D-Plex Small RNA DNBSEQ library (DiaPure purification) made from 25 ng of K562 total RNA. 1 ng of the library was loaded on the Bioanalyzer.

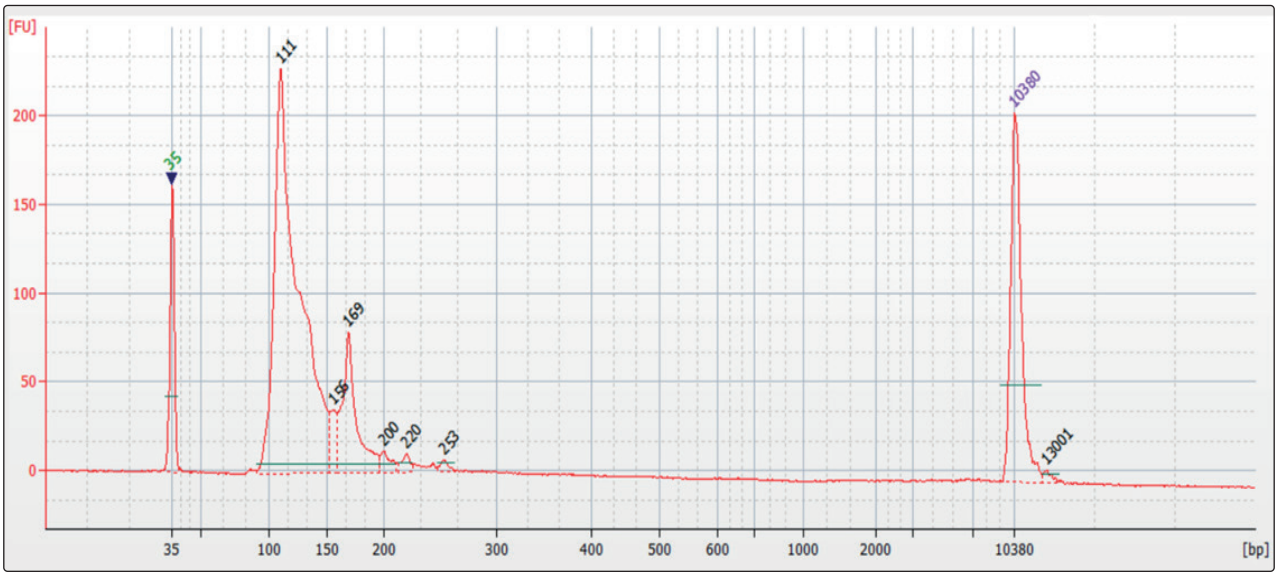


Figure 4: DNA electropherogram of a D-Plex Small RNA DNBSEQ library (DiaPure purification) made from 500 pg of K562 total RNA. 1 ng of the library was loaded on the Bioanalyzer.

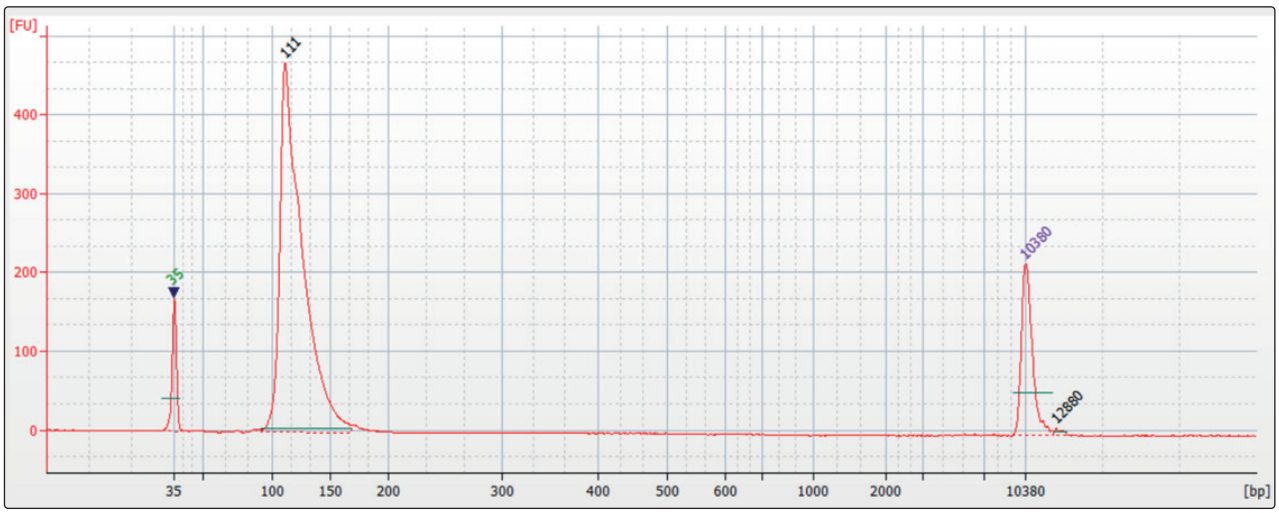


Figure 5: DNA electropherogram of a D-Plex-MGI small RNA library (DiaPure purification) made from 50 pg of circulating RNA, isolated from human plasma. 1 ng of the library was loaded on the Bioanalyzer.

Related Products

Indexes Module Reference	Reference
D-Plex Small RNA DNBSEQ Barcodes - Set A	C05030060
D-Plex Small RNA DNBSEQ Barcodes - Set B	C05030061
DiaMag 0.2 mL tube magnetic rack	B04000001
DiaMag 1.5 mL tube magnetic rack	B04000003
MicroChIP DiaPure columns	C03040001

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