diagenode

Innovating Epigenetics Solutions

D-Plex Small RNA-seq Kit

Small RNA library preparation kit for Illumina® sequencing

Cat. No. C05030001 (Core module - 24 rxns)

USER GUIDE

V1 01_2020

Get the electronic version of this user manual on the product page

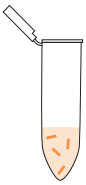
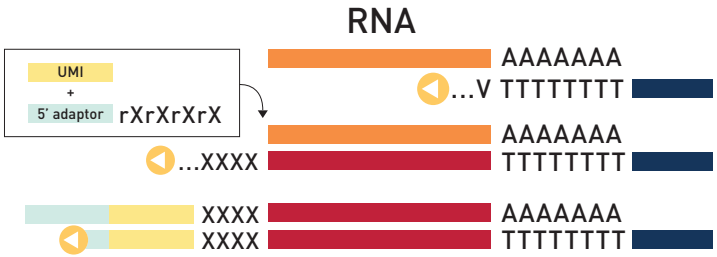
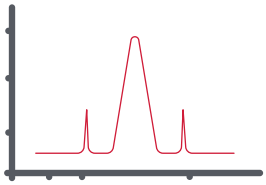
www.diagenode.com/en/p/D-Plex-Small-RNA-seq-Library-Prep-x24

Summary

Please read this manual carefully before starting your experiment

Indexes 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	45 min	❄
STEP 5		Library purification and shaping		
STEP 6		Library quality control and NGS		

Introduction

The Diagenode D-Plex Small RNA-seq Library Preparation Kit is a tool designed for the study of the **small non-coding transcriptome**. The present kit is designed for library preparation for Illumina platforms.

The D-Plex technology is a derivative of the previously established CATS technology and thus retains the same key advantages such as:

- **Low input** capability of the library preparation
- Ease of use in a **one day, one tube protocol**
- **Higher library complexity** than most of other available library preparation kits for small RNA-sequencing

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** of total RNA.

The core of the technology relies on **ligation-free reactions** to attach the Illumina adaptors to both ends of the library construct. Therefore, the results generated with the D-Plex Small RNA-seq kit will vastly differ from those produced with ligase-based approach. For instance, the results generated with the 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.

An important addition to the D-Plex set of features is the use of **unique molecular identifiers (UMI)** to each transcript incorporated in the library. Given this new addition it is now possible to exclude PCR duplicates from a set of reads, thus improving the sncRNA quantification.

Kit materials

Table 1. Components of the D-Plex Small RNA-seq Kit

Component	Cap color	Qty (24 rxns)	Storage
Dephosphorylation Buffer (DB)	Yellow	48 µL	-20°C/-4°F
Dephosphorylation Reagent (DR)	Yellow	12 µL	-20°C/-4°F
Crowding Buffer (CB)	Yellow	120 µL	+4°C/+39,2°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 (RTPH)	Purple	24 µL	-20°C/-4°F
RT Primer M (RTPM)	Purple	24 µL	-20°C/-4°F
Reverse Transcription Reagent (RTR)	Purple	144 µL	-20°C/-4°F
Template Switching Oligo (TSO)	Purple	48 µL	-20°C/-4°F
Removal Reagent (RR)	White	24 µL	-20°C/-4°F
PCR Master Mix (PCRMM)	Green	1200 µL	-20°C/-4°F
Nuclease-free Water	Clear	1848 µL	Room Temp.
Positive Control miRNA (CTL+)	Black	12 µL (1 ng/µL)	-20°C/-4°F
			Long term storage: -80°C/-112°F

***NOTE**: Upon receipt, store the components at the indicated temperature*

Required materials not provided

Indexes

Specific D-Plex indexes 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. Index sets and their corresponding references

Indexes Module	Reference
D-Plex 24 Single Indexes - Set #A	C05030010
D-Plex 24 Single Indexes - Set #B	C05030011

Multiplexing

The validated PCR reverse primers bear the TruSeq (Illumina) small RNA indexes that can be used for library multiplexing up to 48. In case of a multiplexing scenario, it is then recommended to follow Illumina's library pooling guidelines that are explained in the D-Plex Single Indexes 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 (Thermo Fisher Scientific, 10328011)
- RNase-free tubes: 0.2, 0.5, 1.5 mL
- Table top centrifuge with strip rotor
- Vortex agitator
- Tube holder for 0.2, 0.5, 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) and Agencourt® AMPure® XP Beads (Beckman Coulter, A63881)
- Absolute ethanol (VWR, 20821.310)
- DNase-free, RNase-free ultrapure water

Post-PCR libraries size selection

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

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 using the protocol for the first time for a definite type of sample, we strongly recommend using the positive miRNA control included in the D-Plex kit

Important notices

The D-Plex small RNA-Seq 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, etc.

A starting volume of 8 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 library preparation is incorporates the different classes of sncRNAs in the final library for sequencing. If coding RNA or lncRNA 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.

Furthermore, it has also been noted that treating circulating RNA samples with DR tends to increase the abundance of sncRNA in general and decrease the representation of tRNA that may take up a certain percentage of the library (sample and input dependent).

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 #1**: **At first use**, thaw the Template Switch Oligo (TSO) and proceed to a denaturation at 95°C for 2 minutes and then immediately cool down the tube on ice. From then, always keep the **denaturated TSO at -20°C for storage or on ice** while performing the protocol.*
>
> ***CAUTION #2**: The incubations during the D-Plex small RNA-seq library preparation take place in a thermal cycler with a **heated lid at 105°C**.*

Starting material

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 2. Input recommendations

Template	Starting quantity of RNA for library preparation	RIN
Total RNA	100 pg - 100 ng	≥ 8
Small RNA (< 200 nt) / Circulating RNA extracted from biofluids, extracellular vesicles...	10 pg - 10 ng	N/A

Template

Both total RNA and small RNA (<200 nt) can be used for input in the D-Plex 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 (<200 nt). Be aware that not all the products on the market are able to efficiently isolate RNAs under 200 nt.

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). The D-Plex small RNA-seq kit has been developed with high-quality total RNA , (RIN >8) but the kit can also perform well on partially and even highly degraded samples (e.g. FFPE-derived samples). However, 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 an alternative 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 in case it is hydroxylated. This protocol might constitute a special interest for researchers studying the degradation products of coding RNAs and lncRNAs.

It has also been noted that treating circulating RNA samples with DR is increasing the abundance of various sncRNAs and tends to decrease the abundance of tRNAs that may take up a certain percentage of the library (depending on sample type and input).

Positive control: cel-miR-39-3p

A positive control (black cap) 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 to monitor the library preparation efficiency or as a spike-in in a given RNA sample to account for an eventual matrix effect. Typically, 1 ng of synthetic miR39 prepared with the D-Plex protocol and amplified during 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 22 nt long:

5'-UCACCGGGUGUAAAUCAGCUUG-3'

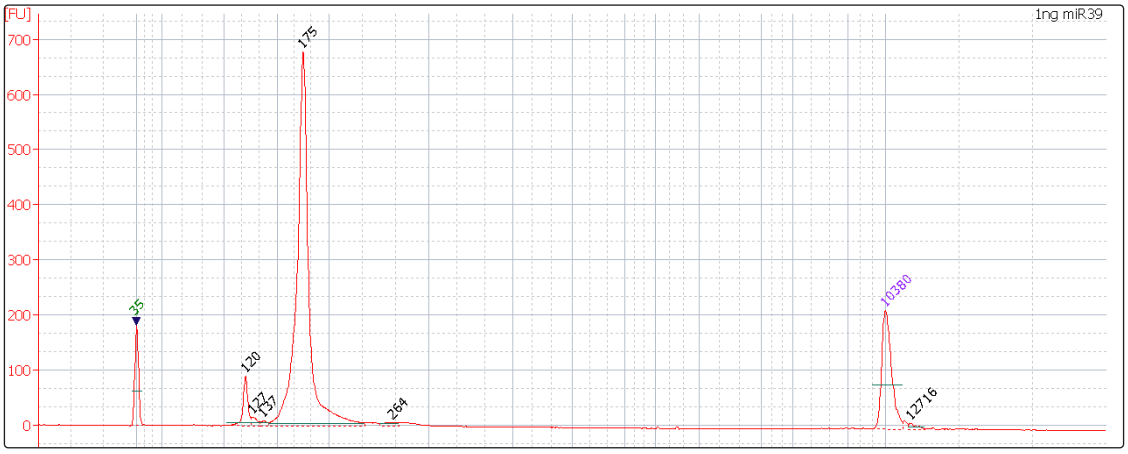
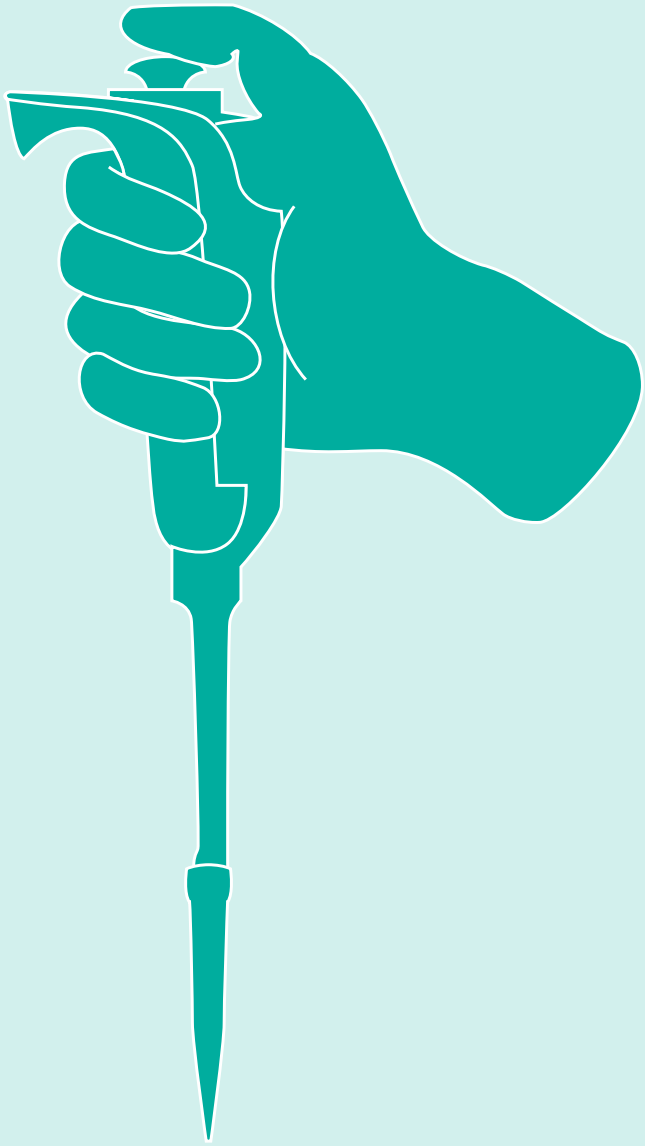


Figure 2: DNA electrophoregram of a D-Plex small RNA library prepared from 1 ng of miR39 (12X PCR cycles) after DiaPure purification. 1 ng of the library was loaded on the Bioanalyzer.

SHORT PROTOCOL



FOR EXPERIENCED USERS

Short protocol for experienced users

Notice before starting

The D-Plex small RNA library preparation incorporates the different classes of sncRNAs in the final library for sequencing. If coding RNA or lncRNA degradation products are of particular interest to the researcher, it is possible to perform an optional (step 4) that will help enrich for those products in the library.

Furthermore, it has also been noted that treating circulating RNA samples with DR tends to increase the abundance of sncRNA in general and decrease the representation of tRNA that may take up a certain percentage of the library (sample and input dependent).

> ***CAUTION**: At first time use, thaw the Template Switch Oligo (TSO) at 95°C for 2 minutes and then immediately cool down on ice. From then on, always keep the **denaturated TSO at -20°C or on ice**.*

1. Take the **Crowding Buffer (CB)** out of the -20°C freezer and let it warm up on the bench at room temperature for 30 minutes before using it at step 3. From then on, CB can be stored at +4°C.

2. Prepare the RNA sample in nuclease-free water to get a total volume of 8 µl.

3. Add **2 µL of Dephosphorylation Buffer (DB)** and **5 µL of CB** and incubate for 10 minutes at 70°C.Then immediately put on ice for 2 minutes.

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

5. Prepare the **Tailing Master Mix (TMM)** by mixing **1 µL of Small Tailing Buffer (STB)** and **0.5 µL of Tailing Reagent (TR)** in a nuclease-free PCR tube.

6. Add **1.5 µL of TMM** and incubate 40 minutes at 37°C + 20 minutes at 65°C and then put on ice to cool down.

7. Add **1 µL of Reverse Transcription Primer (RTP)** to the sample:

   - For 100 ng - 10 ng total RNA or 10 ng - 1 ng small RNA fraction use **RTPH**.
   - For 10 ng - 100 pg total RNA or 1 ng - 10 pg small RNA fraction use **RTPM**.

8. Incubate 10 minutes at 70°C, and then slowly decrease to 25°C for 2 minutes by ramping down at 0.5°C/second.

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

10. Add **6 µL of Reverse Transcription Reagent (RTR)** to the sample tube and incubate 15 minutes at 25°C.

11. Add 2µl of Template Switch Oligo (TSO) to the sample tube (N.B. dilute TSO 1/5 if working on very low input samples; refer to detailed protocol for more information).

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

13. Add **1 µL of Removal Reagent (RR)** to the sample tube and incubate 5 minutes at 37°C + 1 minute at 80°C.

14. Add **10 µL of Forward Primer (FP)** + **10 µL of indexed Reverse Primer (RP)** + **50 µL of PCR Master Mix (PCRMM)**.

15. 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:*

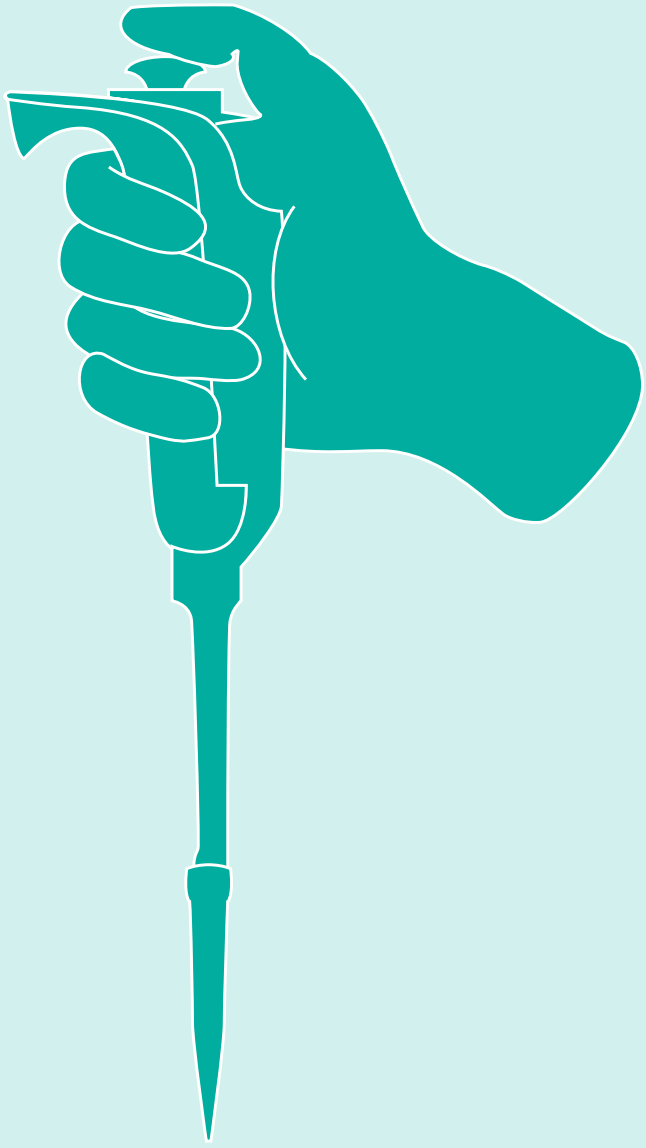
- *from **10 cycles**: for 100 ng total RNA or 10 ng small RNA*
- *up to **20 cycles**: for 100 pg total RNA or 10 pg small RNA*

***NOTE**: If you plan to proceed to the size-selection of the library for short inserts (< 50 nt) (see sections 5B-C-D of the detailed protocol), add 2 more cycles to the* n *cycles displayed above.*

16. 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 section 5 of the detailed protocol.

17. 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 Take the **Crowding Buffer (CB)** out of the -20°C freezer and let it warm up on the bench at room temperature for 30 minutes before using it at step 1.3. From then on, CB can be stored at +4°C

1.2 Dilute the RNA sample in **nuclease-free water** to get a total volume of **8 µL.**

1.3 To the **8 µL RNA solution**, add **2 µL of Dephosphorylation Buffer (DB)** and **5 µL of Crowding Buffer (CB)**. Pipet slowly as CB is viscous. Mix by pipetting up and down thoroughly but gently until solution is homogenous.

1.4 Incubate 10 minutes at 70°C and then immediately put the sample 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 15 minutes at 37°C then put the sample on ice.**]**

2.2 Prepare the **Tailing Master Mix (TMM)** by mixing **1 µL of Small Tailing Buffer (STB)** and **0.5 µL of Tailing Reagent (TR)** in a nuclease-free PCR 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 during 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*

***CAUTION**: At first time use, thaw the Template Switch Oligo (TSO) at 95°C for 2 minutes and then immediately cool down on ice. From then on, always keep the **denaturated TSO at -20°C or on ice**.*

3.1 Add **1 µL of Reverse Transcription Primer (RTP)** to the reaction tube. Choose the right RTP according to your starting RNA amount:

Total RNA		Small RNA fraction / biofluid-isolated RNA	
100 pg – 10 ng	10 ng – 100 ng	10 pg – 1 ng	1 ng – 10 ng
RTPM	RTPH	RTPM	RTPH

3.2 Mix by pipetting up and down until solution is homogeneous. Incubate during 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 put on ice until further processing.

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

***IMPORTANT NOTICE:** in case of ultra-low input sample processing, please dilute the TSO 1/5:*

- *for total RNA samples <200 pg*
- *for small RNA (<200nt) such as circulating RNA <50 pg*

3.4 Add **6 µL of Reverse Transcription Reagent (RTR)** to the reaction tube and mix by pipetting up and down until the solution is homogenous.

3.5 Incubate 15 minutes at 25°C and then put the sample on ice.

3.6 Add **2 µL of Template Switch Oligo (TSO)** to the reaction tube and mix by pipetting up and down until the solution is homogeneous. Store the TSO back in the freezer after use.

3.7 Incubate the sample during for 120 minutes at 42°C + 10 minutes at 70°C, and then let the sample sit on ice until further processing.

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

3.8 Spin down the reaction tube to collect the library at the bottom.

3.9 Add **1 µL of Removal Reagent (RR)** to the library and incubate 5 minutes at 37°C + 1 minute at 80°C.

3.10 Put the sample back on ice to cool down and then spin down the reaction tube to collect the sample at the bottom.

STEP 4

PCR amplification - *1 hour*

4.1 Add **10 µL of Forward Primer (FP)** and **10 µL of indexed Reverse Primer (RP)** to the reaction tube. Add **50 µL of PCRMM** and mix directly by pipetting up and down until solution is homogenous.

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

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 5B-C-D), add 2 more cycles to the n cycles determined earlier.*

STEP 5

DNA library purification & shaping

5.1 Purify the amplified library using the MicroChIP DiaPure kit (Diagenode C03040001), according to the kit instructions or 1.5X AMPure XP Beads. Note that the library volume to start the purification is around **100 µL**. You can check the "Example of results" section (page 38) to see what you should expect as DNA quantity after purification.

5.2 Perform the elution in **50 µL** in a 1.5 mL tube for further clean-up or size-selection of the library using **Solid Phase Reversible Immobilization (SPRI) beads** (section 5A-B). For a precise size-selection of the library by a gel-cut procedure (see section 5C-D), the elution should be done in **20 µL** in a 1.5 mL tube:

Library processing	DiaPure elution volume
SPRI beads clean up (Section 5A)	50 µL
SPRI beads size-selection (Section 5B)	50 µL
Agarose gel-cut size-selection (Section 5C)	20 µL
Polyacrylamide gel-cut size-selection (Section 5D)	20 µL

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

- **SECTION 5A**: SPRI beads clean-up to retain all the library fragments.
- **SECTION 5B**: SPRI beads size selection to enrich the library in small inserts (<50 nt).
- **SECTION 5C & 5D**: precise gel-cut size selection for the accurate selection of any length of fragments.

5A - BEADS CLEAN UP

5A.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.

5A.2 Add **75 µL of beads** to the 50 µL of eluted DNA from step 5.2 and mix thoroughly by pipetting up and down or vortexing.

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

5A.4 Spin the tube down and put it in a magnetic rack for 5 minutes until the beads are collected to the side of the tube and the solution is clear.

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

5A.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 then remove carefully the supernatant without disturbing the beads.

5A.7 Spin down the tube, place it back in the magnetic rack and remove any remaining ethanol.

5A.8 Leave the tube open to let the beads pellet dry for 2-3 minutes and then remove it from the magnetic rack.

5A.9 Resuspend the beads in **20 µL nuclease-free water** by slowly pipetting up and down.

5A.10 Incubate the beads in water during 2 minutes at room temperature under mild agitation.

5A.11 Spin the tube down and put it in a magnetic rack for 5 minutes until the beads are collected to the side of the tube and the solution is clear.

5A.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.

5B - BEADS SIZE SELECTION

To enrich the library for short inserts (<50 nt), perform the following bead-based size-selection after library purification with the DiaPure Kit (step 5.2).

5B.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.

5B.2 Add **55 µL of beads** to the 50 µL of eluted DNA from step 5.2 and mix thoroughly by pipetting up and down or vortexing.

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

5B.4 Spin the tube down and put it in a magnetic rack for 5 minutes until the beads are collected to the side of the tube and the solution is clear.

5B.5 Carefully collect the supernatant without taking up any beads and transfer it to a new 1.5 mL tube. Discard the previous tube containing the used-up beads.

5B.6 Add **75 µL of fresh AMPure XP beads** to the supernatant and mix well by pipetting up and down or vortexing.

5B.7 Incubate the suspension at least 5 minutes at room temperature under mild agitation.

5B.8 Spin the tube down and put it in a magnetic rack for 5 minutes until the beads are collected to the side of the tube and the solution is clear.

5B.9 Carefully remove and discard the supernatant without disturbing the beads.

5B.10 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 then remove carefully the ethanol without disturbing the beads.

5B.11 Spin down the tube, place it back in the magnetic rack and remove any remaining ethanol.

5B.12 Leave the tube open to let the beads dry for 2-3 minutes and then remove the tube from the magnetic rack.

5B.13 Resuspend the beads in **20 µL of nuclease-free water** by slowly pipetting up and down.

5B.14 Incubate the beads in water for 2 minutes at room temperature under mild agitation.

5B.15 Spin the tube down and put it in a magnetic rack for 5 minutes until the beads are collected to the side of the tube and the solution is clear.

5B.16 Carefully collect the supernatant without taking up any beads. Transfer the size-selected library in a nuclease-free tube and store at -20°C for further use.

5C - AGAROSE GEL-CUT SIZE-SELECTION

5C.1 Load your **20 µL eluted DNA library** from step 5.2 (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. 25-100 bp mixed DNA ladder Bionneer D-1020).

5C.2 Run the electrophoresis until the migration front reaches the end of the gel.

5C.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 ~180 bp.

5C.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**.

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

5C.6 After gel dissolution, spin down the tube, collect the solution to transfer it in a MinElute® column.

5C.7 Centrifuge for 20 seconds at 16,000 g and discard the flow-through.

5C.8 Add **0.75 mL of buffer PE** to the MinElute® column, incubate for 1 minute and centrifuge for 1 minute at 16,000 g.

5C.9 Discard the flow-through and centrifuge the column for 1 minute at 16,000 g.

5C.10 Place the MinElute® column in a clean 1.5 mL tube, add **20 µL of Buffer EB** and incubate 2 minutes at room temperature.

5C.11 Centrifuge for 1 minute at 10,000 g and collect the eluted DNA library. Store at -20°C for further use.

5D - POLYACRYLAMIDE GEL-CUT SIZE-SELECTION

5D.1 Load your 20 µL eluted DNA library from step 5.2 (mixed with appropriate electrophoresis loading buffer) in a well of a **4-6% polyacrylamide gel soaked in 0.5X TBE buffer**. Use a **gel stain** of your choice (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. 25-100 bp mixed DNA ladder Bioneer; D-1020).

5D.2 Run the electrophoresis until the migration front reaches the end of the gel.

5D.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 ~180 bp.

5D.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**.

5D.5 Crush the gel slice in the tube with a DNase-free pestle or cut it into very small pieces, 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.

5D.6 Spin down the tube at full speed, collect supernatant and transfer it in a MinElute® column.

5D.7 Centrifuge 20 seconds at 16,000 g and discard flow-through.

5D.8 Add **0.75 mL of buffer PE** to the MinElute® column, incubate for 1 minute and centrifuge 1 minute at 16,000 g.

5D.9 Discard the flow-through and centrifuge the column for 1 minute at 16,000 g.

5D.10 Place the MinElute® column in a clean 1.5 mL tube, add **20 µL of Buffer EB** and incubate 2 minutes at room temperature.

5D.11 Centrifuge 1 minute at 10,000 g and collect the eluted DNA library. Store at -20°C for further use.

Step 6

Library quality control and quantification

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

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

Sequencing recommendations

The D-Plex small RNA-seq library construct bears the TruSeq small RNA adapters. It is therefore recommended to submit the D-Plex libraries as TruSeq small RNA libraries to your sequencing provider.

The indexes (up to 48) provided in the different indexes modules are also the TruSeq small RNA indexes as indicated in the D-Plex 24 Single Indexes manual.

The complete architecture of the D-Plex small RNA construct is provided hereafter in the manual (Figure 3). The empty library size (adapters + template switch + UMI + A tail) is equal to 155 bp.

***IMPORTANT NOTICES**:*

- *Given the high complexity of D-Plex Small RNA-seq 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.*
- *The D-Plex small RNA libraries can be sequenced as regular Illumina libraries, meaning that they don't require any specific considerations regarding phiX spike-in or cluster density optimization. However, it is recommended to spike ~1% of phiX in the library pool that is about to be sequenced for a quality monitoring purpose.*
- *As the D-Plex Small RNA-seq library should contain short sequences (primarily small non-coding RNA), and a UMI at the 5' end of the forward strand, it is not recommended to use paired-end sequencing. It makes the data analysis difficult, and in some cases, unreliable, and due to the predominantly short fragments it generates mostly redundant information. Instead, if you are interested in longer fragments, we recommend increasing the read length with single-end sequencing, as it greatly increases the chance of reading the complete length of the whole fragment.*

D-Plex Construct

P5/P7 = Illumina adapters

UMI = Unique Molecular Identifier (12 nt)

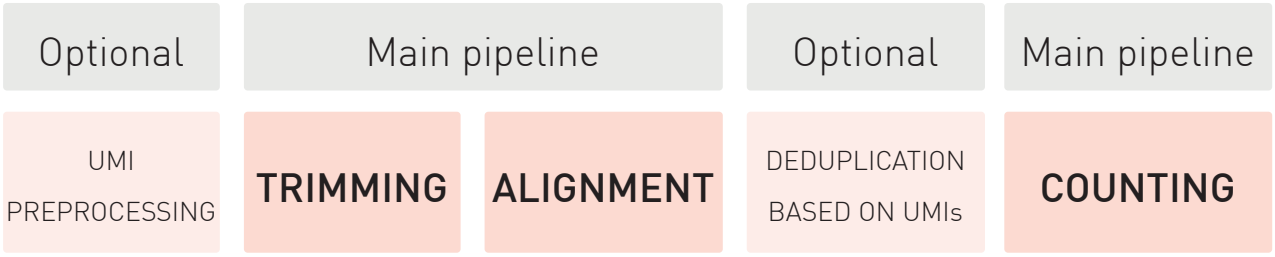
TSM = Template-Switch Motif (4 nt)

I = Index

Poly A/T = PolyA and PolyT tail

Data analysis recommendations

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 UMI, the polyA tail, and the template switch motif. This guide will take you through the basic processes of trimming, alignment and counting, complemented with an optional UMI processing, 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. If the UMIs are not of interest, these two optional steps can safely be skipped and the rest of the pipeline will not change. In such a case, the UMI sequences will be only removed from the reads during trimming and will be ignored for the rest of the analysis. The recommended software package for UMI processing can be downloaded from the link at the end of this chapter (or can be installed with conda; please see the readme file of the software package for the installation instructions). In our example commands we assume that the necessary software tools are in the PATH.



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

UMI-preprocessing (optional)

If you want to process the UMIs, the first thing you need to do is to copy the UMI sequence to the read ID. The first 12 bases in the read (from the 5' end) are the Unique Molecular Identifier. In the fumi-tools package the copy_umi command can copy (not move!) these bases to the correct lines in the fastq file. You don't need to use all the 12 bases if you don't want to and you can copy less, which can reduce the data processing time and resource need at the expense of a reduced UMI complexity. The tool expects a fastq file as input (which can be gzip compressed), and it will output a fastq file, which will be either gzip compressed or uncompressed, based on the extension set on the commandline (.gz for compression).

Besides the input, the command needs one mandatory option: the length of the UMI (to copy from the read to the ID line). A typical example is below, using all the 12 UMI bases and 10 threads for compression:

```
fumi_tools copy_umi --threads 10 --umi-length 12 -i
reads.fastq.gz -o reads_w_umi.fastq.gz
```

Trimming

Trimming is mandatory for the reads generated with the D-Plex small RNA-seq kit, because the read length is often longer than the targeted small RNAs, so in many cases the polyA tail and the 3' adapter are also sequenced. In addition, the 5' end contains other sequences that were originally not part of the RNA: the UMI and the template switch motif. 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 readset as clean as possible. To minimize the effect of ambiguously read bases, we trim the Ns and allow IUPAC codes in the reads. We cut off 16 bases from the 5' end (12 for the UMI, 4 for the template switch motif) and then from the 3' end we remove first the 3' adapter, then the polyA tail. For the former, we use the standard Illumina TruSeq Universal Adapter sequence; for the latter we are looking for a pattern consisting of 8 As and we allow a three times repetition of this pattern. We found that this combination of parameters prevents excessive overtrimming and read loss, while reliably and fully cleans up the readset coming from an average Illumina sequencing run. Finally we filter the remaining reads by length, and we discard the ones below 15 nucleotides, as they might be difficult to map uniquely. The example command with cutadapt is below. Note that the input file raw_reads.fastq.gz can mean both the UMI-preprocessed reads – see the previous step – or the reads directly coming from the sequencer without the UMI pre-processing. The trimming command is the same in either case, with or without considering the UMIs.

```
cutadapt --trim-n --match-read-wildcards -u 16 -n
3 -a AGATCGGAAGAGCACACGTCTG -a AAAAAAAA -m 15 -o
trimmed_reads.fastq.gz raw_reads.fastq.gz
```

Alignment

Aligning the trimmed reads needs no special treatment as you can use any aligner that is suitable for mapping RNA-seq reads. First and foremost, we recommend aligning to the genome (instead of the transcriptome). Indeed, D-Plex tends to generate a very high-complexity library 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 below, we will use the software STAR which does exactly that: it aligns the reads to the hg19 genome (assuming we are dealing with a human sample, and the hg19 genome index for STAR has already been prepared), then assigns them to known transcripts, using the gtf annotation file. We also assume that the reads were 50 bases long and STAR was run in multithreading mode on 10 cores.

```
STAR --runThreadN 10 --readFilesCommand zcat
--genomeDir /genomes/hg19/ --sjdbGTFfile /genomes/
hg19/hg19.gtf --sjdbOverhang 49 --readFilesIn
trimmed_reads.fastq.gz --quantMode TranscriptomeSAM
--quantTranscriptomeBAMcompression -1 --outSAMtype
BAM SortedByCoordinate --outSAMunmapped Within
--outFileNamePrefix ./MySample_
```

Deduplication based on UMIs (optional)

This is where you can exploit the UMIs (if you have performed the UMI pre-processing at the beginning of the pipeline). They enable the distinction between duplicate reads that are coming from different molecules, thus having different UMIs (e.g. the same gene transcribed multiple times in the sample), and duplicates that are essentially artifacts, such as the ones resulting from PCR amplification. These have the same UMIs in their read IDs (after the pre-processing), and consequently they can be recognized and removed until only one copy remains. This way the subsequent counting will be more precise and you will get a more realistic picture of the expression levels, which will not be biased by artificial duplicates.

You can deduplicate both the genome and the transcriptome alignments

of course. The example commands below show you how to do it in both cases, using the STAR outputs from the previous step. Do not forget however, that the transcriptome alignment is not sorted (though the reads with the same names, e.g. multimapping reads follow each other in consecutive lines, which is required by most counting software). As the UMI deduplication tool needs a coordinate sorted alignment for input, you need to sort the transcriptome alignment first, for example with samtools (assuming 10 cores, just as in the STAR example):

```
samtools sort -@ 10 -o MySample_Aligned.
toTranscriptome.sorted.out.bam MySample_Aligned.
toTranscriptome.out.bam
```

The genome alignment from the above STAR command is already sorted by coordinates and it needs no more sorting. Now you can deduplicate both the genome and the transcriptome alignment with the UMI deduplication command of fumi-tools called dedup (again running it on 10 cores and assigning 10 GB RAM):

```
fumi_tools dedup --threads 10 --memory 10G -i
MySample_Aligned.toTranscriptome.sorted.out.bam -o
MySample_deduplicated_transcriptome.bam
```

```
fumi_tools dedup --threads 10 --memory 10G -i
MySample_Aligned.sortedByCoord.out.bam -o MySample_
deduplicated_genome.bam
```

Note that fumi-tools outputs name-sorted bam files, which can be directly used for example with counting software tools, but remember to sort them if you want to use them for another application that needs a different sorting, e.g. sorting by coordinates.

Counting

The counting, or expression level calculation is the last step in this guide. As written above, counting software usually requires that the reads in the input bam file with the same name follow each other on consecutive lines. Therefore both the STAR output (transcriptome alignment) and the fumi-tools output (deduplicated transcriptome alignment) are ready to use with counting tools without further modifications. So this step is again independent of the UMI processing and it is the same whether you considered UMIs or not.

We provide an example command below, using RSEM for counting, continuing our example pipeline. We assume that the hg19 index for RSEM has been prepared beforehand, and we run the counting on 10 cores. The other options specify that we do not want a bam output and we start from an alignment file (RSEM can also do the alignment, but we have already done it with STAR); the minimum read length is 15 (as we specified during trimming), and the reads are originating from the forward strand (D-Plex produces stranded libraries). In the end (among other RSEM outputs) we will obtain the expression levels of known genes as TPM/FPKM values.

```
rsem-calculate-expression -p 10 --strandedness
forward --seed-length 15 --no-bam-output
--alignments MySample_transcriptome_alignment.bam /
transcriptomes/hg19/hg19 MySample
```

Links for the tools used in the example pipeline

Tool	Link
Website	https://www.diagenode.com/en/
Fumi-tools	https://ccb-gitlab.cs.uni-saarland.de/tobias/fumi_tools/
Cutadapt	https://github.com/marcelm/cutadapt
STAR	https://github.com/alexdobin/STAR
Samtools	http://www.htslib.org/download
RSEM	http://deweylab.github.io/RSEM

Example of results

Quality requirements

When the D-Plex small RNA-seq protocol is followed precisely and the proper conditions for the particular RNA input are used, the following standards of quality can be expected:

Table 10: Minimum yield expected from a D-Plex library after DiaPure purification for different inputs and templates.

Template	Input	DNA quantity after DiaPure purification (ng)
Total RNA (e.g. human brain RNA)	100 pg	~50
	100 ng	~50
Small RNA (< 200 nt)	1 ng	~20

Electrophoregrams

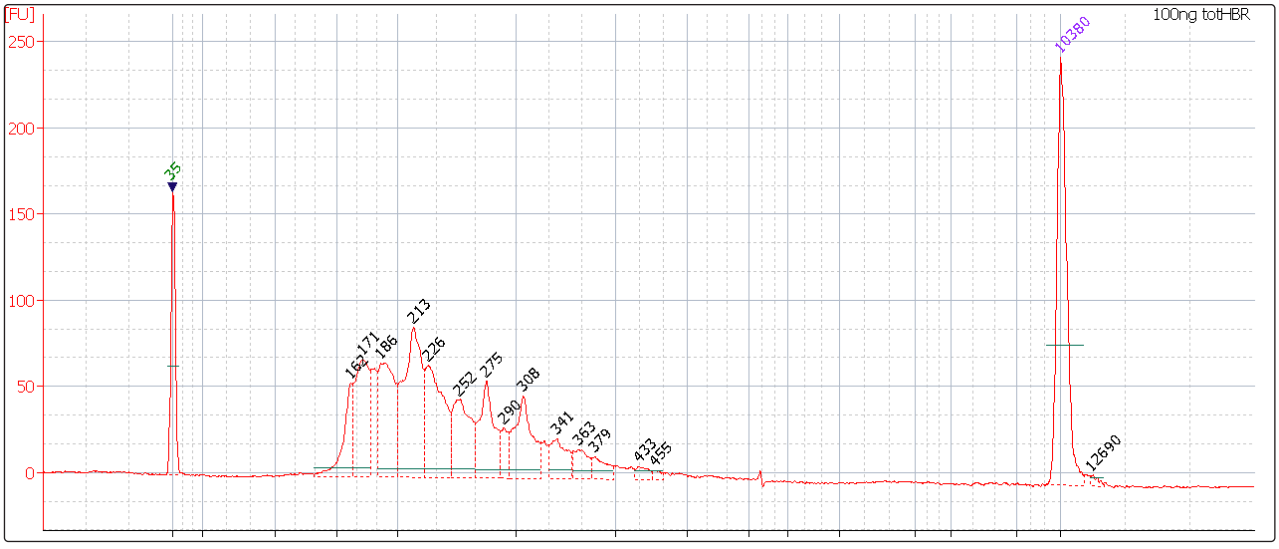


Figure 4: DNA electrophoregram of a D-Plex small RNA library (1.5 X clean up) made from 100 ng of commercial human brain RNA (Thermo Fisher Scientific, AM7962). 1 ng of the library was loaded on the Bioanalyzer.

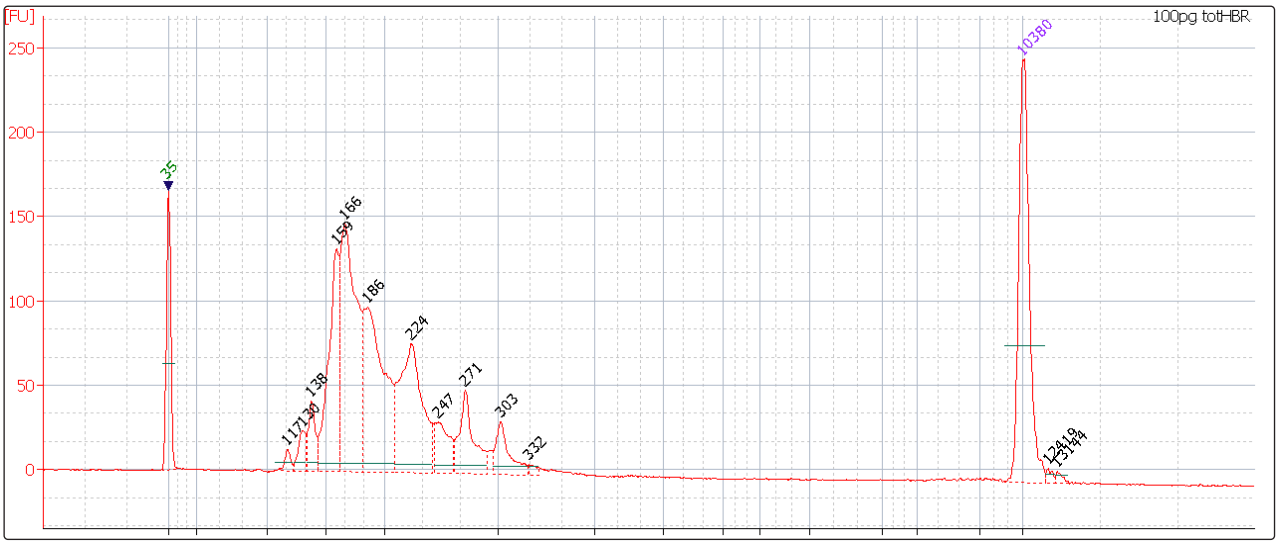


Figure 5: DNA electrophoregram of a D-Plex small RNA library (1, 5 X clean up) made from 100 pg of commercial human brain RNA (Thermo Fisher Scientific, AM7962). 1 ng of the library was loaded on the Bioanalyzer.

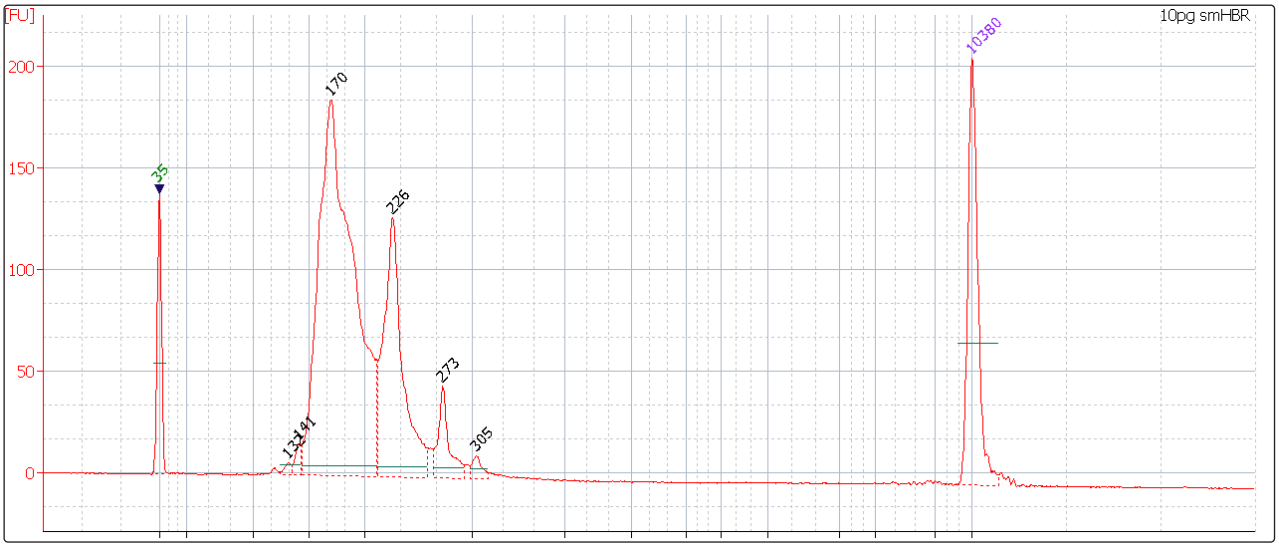


Figure 6: DNA electrophoregram of a D-Plex small RNA library (1, 5 X clean up) made from 10 pg of the small RNA fraction (< 100 nt) of commercial human brain RNA (Thermo Fisher Scientific, AM7962). 1 ng of the library was loaded on the Bioanalyzer.

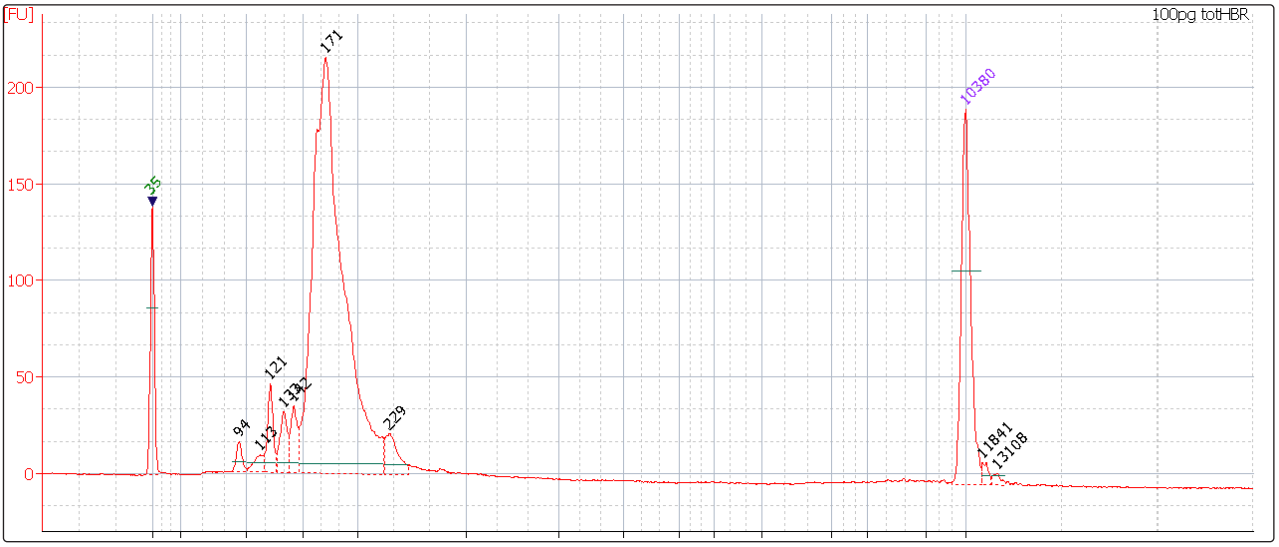


Figure 7: DNA electrophoregram of a D-Plex small RNA library, bead-based size selected for inserts < 50 bp, made from 100 pg of commercial human brain RNA (Thermo Fisher Scientific, AM7962). 1 ng of the library was loaded on the Bioanalyzer.

Related products

Product	Reference
D-Plex 24 Single Indexes - Set #A	C05030010
D-Plex 24 Single Indexes - Set #B	C05030011
DiaMag 0.2 mL tube magnetic rack	B04000001
DiaMag 1.5 mL tube magnetic rack	B04000003
MicroChIP DiaPure columns	C03040001

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