diagenode

A Hologic Company

D-Plex Small RNA-seq Kit

Small RNA library preparation kit for Illumina® sequencing

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

USER GUIDE

V2 03_2024

Please read this manual carefully before starting your experiment

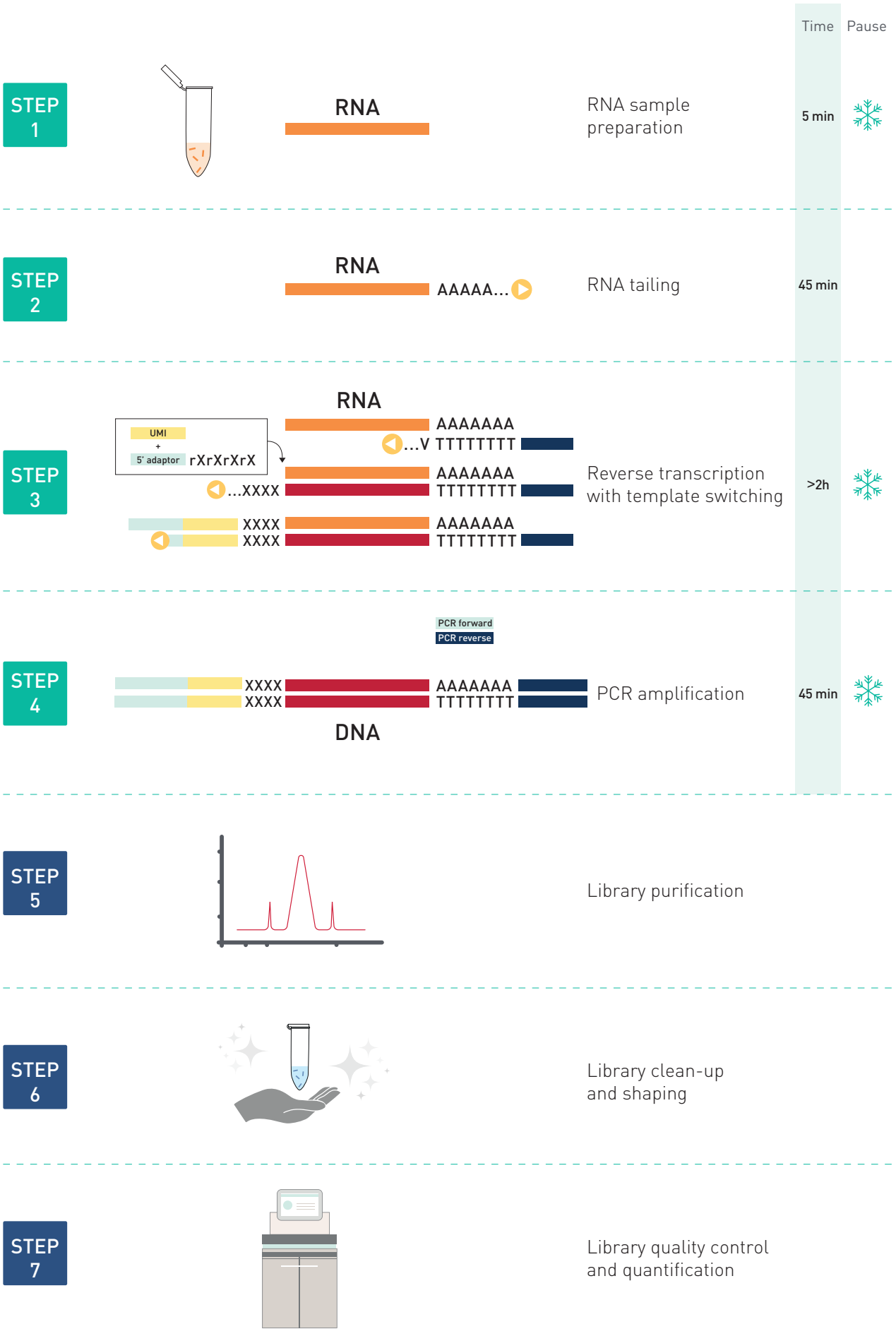
Indexes are not included in this kit and are available separately. Please check "Required materials not provided" section.

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

Kit Method Overview



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 incorporates the unique **D-Plex technology** to generate small RNA libraries for **Illumina sequencing**.

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 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 RNAs (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 small RNAs (< 200 nt) or circulating RNAs **up to 100 ng** of total RNAs.

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.

For optimal workflow flexibility, the library preparation is available in both **unique dual index (UDI)** and **single index (SI)** configurations. The D-Plex Unique Dual Indexes Modules (C05030021, C05030022, C05030023 and C05030024) and the D-Plex Single Indexes Modules (C05030010 and C05030011) are available separately from the library preparation kit, providing PCR primers for library multiplexing up to 96 (using the UDI sets). The use of UDI is highly recommended to mitigate errors introduced by read misassignment, including index hopping frequently observed with patterned flow cells such as Illumina's NovaSeq system.

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 transcript expression quantification.

Kit Materials

Table 1: Components of the D-Plex Small RNA-seq kit (C05030001)

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/+40°F
Small Tailing Reagent (STR)	Red	12 µL	-20°C/-4°F
Small Tailing Buffer (STB)	Red	24 µL	-20°C/-4°F
RT Primer H UDI (RTPH_UDI)	Purple	24 µL	-20°C/-4°F
RT Primer M UDI (RTPM_UDI)	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
Small Template Switching Oligo UDI (STSO_UDI)	Purple	48 µL	-20°C/-4°F
PCR Master Mix (PCRMM)	Green	1200 µL	-20°C/-4°F
Nuclease-free Water	Clear	2000 µL	20°C/-4°F
Positive Control miRNA (CTL+)	Black	12 µL (1 ng/µL)	-20°C/-4°F Long term storage: -80°C/-112°F

Important Notice

The **RT Primer H, RT Primer M** and **Small Template Switching Oligo** components included in the D-Plex Small RNA-seq kit (C05030001) are **only suitable for UDI library construction**. The tubes are labelled with a "UDI" tag. For SI library construction, the RT Primer H, RT Primer M and Small Template Switching Oligo components suitable for SI library construction are included in the D-Plex Single Indexes modules (C05030010 and C05030011). **You should use the components corresponding to the desired – UDI or SI – library construction**.

Storage

The components should be stored at temperatures indicated in Table 1.

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: D-Plex Indexes Modules and their corresponding references

Unique Dual Indexes Modules	Format	Reference
D-Plex Unique Dual Indexes Module – Set A	24 rxns	C05030021
D-Plex Unique Dual Indexes Module – Set B	24 rxns	C05030022
D-Plex Unique Dual Indexes Module – Set C	24 rxns	C05030023
D-Plex Unique Dual Indexes Module – Set D	24 rxns	C05030024
Single Indexes Modules	**Format**	**Reference**
D-Plex Single Indexes Module – Set A	24 rxns	C05030010
D-Plex Single Indexes Module – Set B	24 rxns	C05030011

For complete workflow, the D-Plex Small RNA-seq kit (C05030001) should be ordered together with D-Plex Indexes modules (Table 2). The final D-Plex library construction will either bear UDI or SI (Table 3).

Table 3: D-Plex library construction

D-Plex UDI Library Construction		D-Plex SI Library Construction	
D-Plex Small RNA-seq Kit	C05030001	D-Plex Small RNA-seq Kit	C05030001
AND		**AND**	
D-Plex Unique Dual Indexes Module	C05030021 C05030022 C05030023 C05030024	D-Plex Single Indexes Module	C05030010 C05030011

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)
- Cruched ice
- 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)
- Thermal cycler

Post-PCR libraries purification

- MicroChIP DiaPure kit (Diagenode, C03040001)
- DNase-free, RNase-free ultrapure water

Post-PCR libraries clean-up

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

Post-PCR libraries size selection

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

Remarks Before Starting

General recommendations

- Read the complete manual before first time use.
- The D-Plex Small RNA-seq protocol is essentially the same for both UDI and SI library constructions. However, the **RT Primer H, RT Primer M** and **Small Template Switching Oligo are specific to the desired – UDI or SI – library construction**. The RT Primer H, RT Primer M and Small Template Switching Oligo components included in the D-Plex Small RNA-seq kit (C05030001) are only suitable for UDI library construction. The tubes are labelled with a "UDI" tag. For SI library construction, the RT Primer H, RT Primer M and Small Template Switching Oligo components only suitable for SI library construction are included in the D-Plex Single Indexes Modules (C05030010 and C05030011). **You should carefully use the components corresponding to the desired – UDI or SI – library construction**.
- 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 kit.
- Unless otherwise stated, the lid of the thermal cycler used to carry the reactions should be set at 105°C.

Template

Both total RNA and small RNA (<200 nt) templates can be used as input for the D-Plex Small RNA-seq 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). Please be aware that not all extraction kits on the market are able to efficiently isolate RNAs under 200 nt.

The D-Plex Small RNA-seq kit has also been developed with high-quality total RNAs (RIN > 8), but the kit can also perform well on partially and even highly degraded samples (e.g. FFPE-derived samples). However, sample degradation may result in the underrepresentation of the small non-coding RNAs in the final library. For total RNA templates, we recommend to assess the RNA integrity and quality using the Agilent RNA 6000 Pico kit (Agilent, 5067-1531) before library preparation.

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 (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.

Starting material

The D-Plex Small RNA-seq protocol has been validated for starting amounts ranging **from 10 pg to 10 ng for small RNAs (< 200 nt) or circulating RNAs** and ranging **from 100 pg to 100 ng for total RNAs (RIN ≥ 8)** (Table 4).

Table 4: Input recommendations

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

A **starting volume of 8 µl** is used in the library preparation process. RNA templates should be dissolved in nuclease-free water in this volume before starting the protocol.

Multi-sample protocol

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

Multiplexing advices

The PCR primers can be used for library multiplexing up to 48. In case of a multiplexing scenario, it is recommended to follow Illumina's library pooling guidelines that are explained in the D-Plex Unique Dual Indexes and the D-Plex Single Indexes manuals.

Positive control: cel-miR-39-3p

A positive control is supplied in the kit for 12 reactions at a ready-to-use 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'

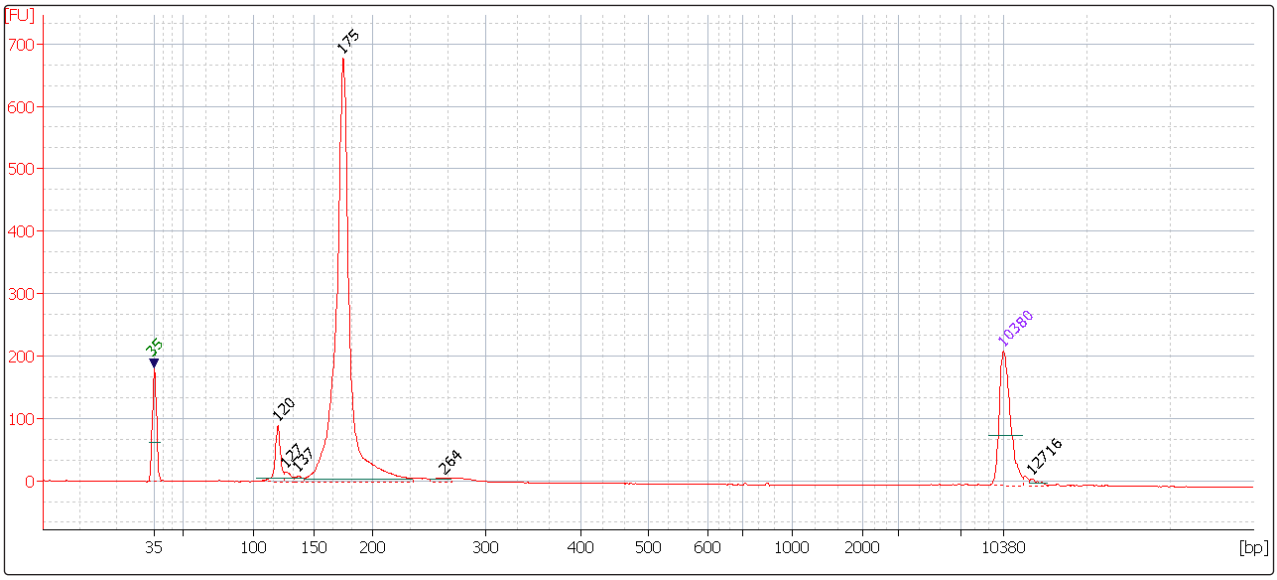
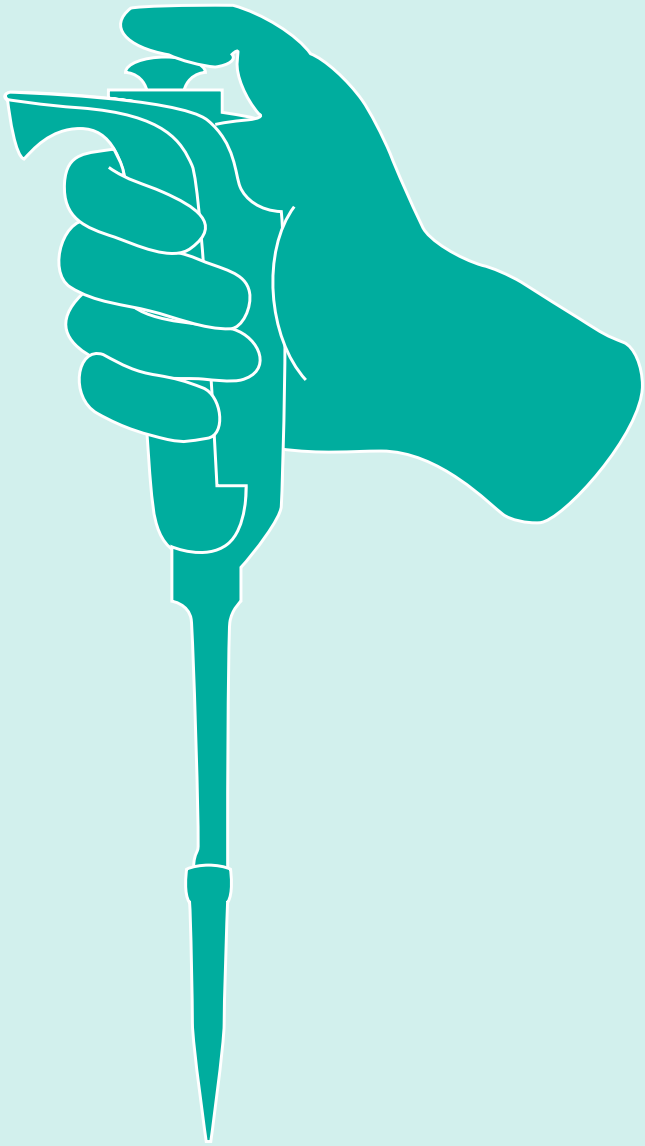


***Figure 2**: DNA electrophoregram of a D-Plex Small RNA-seq SI library construction prepared from 1 ng of miR39 (12X PCR cycles) after DiaPure purification. 1 ng of the library was loaded on the Bioanalyzer.*

SHORT PROTOCOL



FOR EXPERIENCED USERS

Short Protocol For Experienced Users

Notice before starting

- The D-Plex Small RNA-seq protocol is essentially the same for both UDI and SI library constructions. However, the **RT Primer H, RT Primer M** and **Small Template Switching Oligo are specific to the desired – UDI or SI – library construction**. The RT Primer H, RT Primer M and Small Template Switching Oligo components included in the D-Plex Small RNA-seq kit (C05030001) are only suitable for UDI library construction. The tubes are labelled with a "UDI" tag. For SI library construction, the RT Primer H, RT Primer M and Small Template Switching Oligo components only suitable for SI library construction are included in the D-Plex Single Indexes modules (C05030010 and C05030011). **You should carefully use the components corresponding to the desired – UDI or SI – library construction**.
- The D-Plex Small RNA-seq 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, we recommend to use the Dephosphorylation Reagent (DR) in step 3 to enrich the final library with those products.

1. Take the **Crowding Buffer (CB)** out of the freezer and let it warm up at room temperature for 30 minutes before using it at step 2. From then on, CB can be stored at +4°C.

2. Add **2 µl of Dephosphorylation Buffer (DB)** and **5 µl of CB** to the **8 µl of RNA sample.**

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

4. Prepare the **Small Tailing Master Mix (STMM)** by mixing **1 µl of Small Tailing Buffer (STB)** and **0.5 µl of Small Tailing Reagent (STR)** in a RNase-free tube.

5. Add **1.5 µl of STMM** to the sample, incubate for 40 minutes at 37°C, and then cool down on ice.

6. Add **1 µl of Reverse Transcription Primer (RTP)** to the sample **on ice**:

   - 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**.

***CAUTION**: Carefully use **RTPH** and **RTPM** corresponding to the **desired - UDI or SI - library construction**.*

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

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

   ***CAUTION**: Carefully use **STSO** corresponding to the **desired - UDI or SI - library construction**.*

9. Prepare the **Reverse Transcription Master Mix (RTMM)** by mixing **5 µl of Reverse Transcription Buffer (RTB)** and **1 µl of Reverse Transcription Reagent (RTR)** in a RNase-free tube. Place the RTMM on ice until addition to the sample.

10. Add **6 µl of RTMM** to the sample and incubate for 15 minutes at 25°C.

11. Add **2 µl of STSO** to the sample.

    ***NOTE**: Dilute STSO 1/5 if working on very low input samples; refer to detailed protocol for more information.*

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

13. **For UDI library construction**: Add **20 µl of D-Plex Primer UDI** and **50 µl of PCR Master Mix (PCRMM)** to the sample.

    For SI library construction: Add **10 µl of D-Plex Formard Primer**, **10 µl of indexed D-Plex Reverse Primer**, and **50 µl of PCR Master Mix (PCRMM)** to the sample.

14. Proceed to PCR reaction and amplify the library according to the following program:

    For UDI library construction:

<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="2">n cycles*</td></tr>
<tr><td>72°C</td><td>60 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>

For SI library construction:

Temperature	Time & Cycles	
98°C	30 seconds	
98°C	15 seconds	n cycles*
62°C	30 seconds	
72°C	30 seconds	
72°C	10 minutes	
Hold at 4°C or freeze until further processing		

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

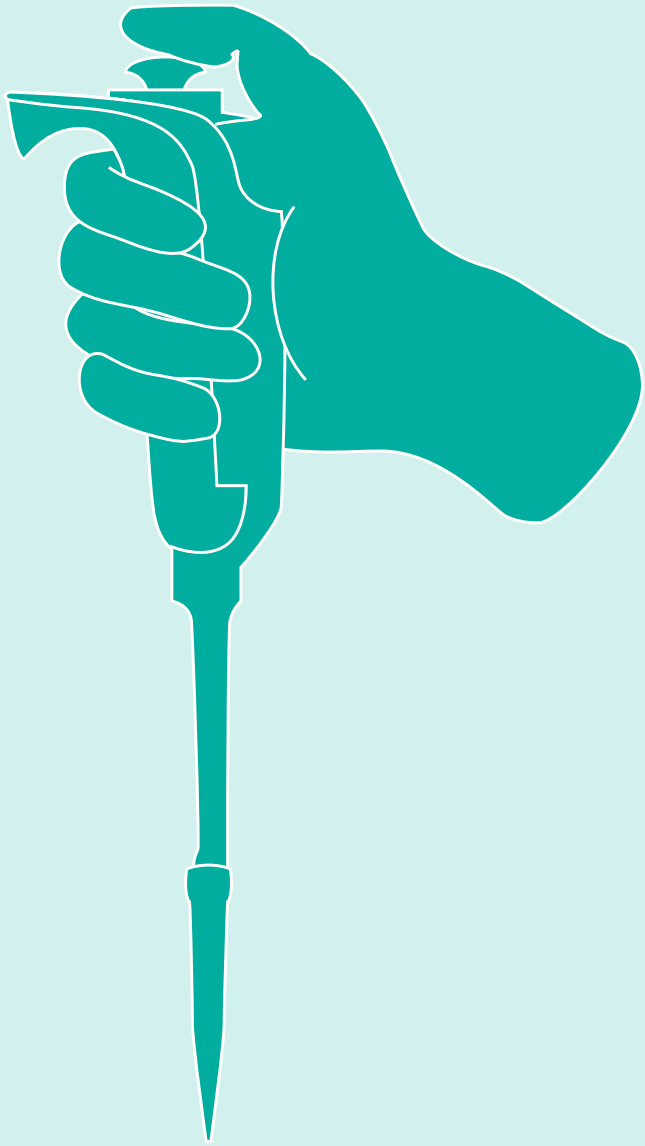
- *from* ***10 cycles****: for 100 ng total RNAs or 10 ng small RNAs*
- *up to* ***20 cycles****: for 100 pg total RNAs or 10 pg small RNAs*

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

15. Purify the PCR product using the MicroChIP DiaPure kit (Diagenode C03040001) according to the kit's instructions (follow the "Protocol for ChIP samples"). To perform further clean-up or size-selection of the library, refer to section 6 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 -  *5 minutes*

1.1 Take the **Crowding Buffer (CB)** out of the freezer and let it warm up at room temperature for 30 minutes before using it at step 1.2. From then on, CB can be stored at +4°C.

1.2 Add **2 µl of Dephosphorylation Buffer (DB)** and **5 µl of CB** to the **8 µl of RNA sample.** Pipet slowly as CB is viscous.

1.3 Mix by pipetting up and down until solution is homogenous.

STEP 2

RNA tailing - *45 minutes*

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

2.2 Prepare a **Small Tailing Master Mix (STMM)** in a RNase-free tube according as follows:

Component	Volume
Small Tailing Buffer (STB)	1 µl
Small Tailing Reagent (STR)	0.5 µl
Total Volume	**1.5 µl**

2.3 Mix by pipetting up and down and use immediately.

2.4 Add **1.5 µl of STMM** to the sample.

2.5 Mix by pipetting up and down until solution is homogeneous.

2.6 Spin down the sample briefly (1-2 seconds) in a table top centrifuge.

2.7 Incubate for 40 minutes at 37°C on thermal cycler and then cool down on ice for 2 minutes.

2.8 Spin down the sample in a table top centrifuge and place on ice until further processing.

STEP 3

Reverse transcription with template switching -  *> 2 hours*

***CAUTION**: Carefully use the **RT Primer H**, **RT Primer M** and **Small Template Switching Oligo** components corresponding to **the desired - UDI or SI – libray construction**:*

- *The components suitable for the UDI library construction are included in the D-Plex Small RNA-seq Kit (C05030001). The tubes are labelled with a "UDI" tag.*
- *The components suitable for the SI library construction are included in the D-Plex Single Indexes Modules (C05030010 and C05030011).*

3.1 Add **1 µL of Reverse Transcription Primer (RTP)** to the sample **on ice**. Choose the right RTP according to your starting RNA input:

Total RNA		Small RNA / Circulating 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.

3.3 Place the sample in a thermal cycler and run the following program:

Temperature	Time
70°C	10 min
70°C - 25°C	0.5°/sec
25°C	2 min hold

3.4 Spin down the sample in a table top centrifuge and place on ice until further processing.

3.5 Take the **Small Template Switch Oligo (STSO)** tube out of the freezer and let it thaw on ice.

3.6 Prepare a **Reverse Transcription Master Mix (RTMM)** in a RNase-free tube according as follows:

Component	Volume
Reverse Transcription Buffer (RTB)	5 µl
Reverse Transcription Reagent (RTR)	1 µl
Total Volume	**6 µl**

3.7 Mix by pipetting up and down and use immediately.

3.8 Add **6 µl of RTMM** to the sample.

3.9 Mix by pipetting up and down until the solution is homogenous.

3.10 Incubate for 15 minutes at 25°C and then place the sample on ice.

3.11 Add **2 µl of STSO** to the sample. **STSO** should be stored at -20°C after use.

NOTE: *In case of ultra-low input sample processing, please dilute the TSO 1/5:*

- *for total RNAs < 200 pg*
- *for small RNAs and circulating RNAs < 50 pg*

3.12 Mix by pipetting up and down until the solution is homogeneous.

3.13 Incubate for 120 minutes at 42°C + 10 minutes at 70°C, and then cool down the sample 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 additional hold at +4°C once the first two steps are completed.*

3.14 Spin down the sample in a table top centrifuge to collect the library at the bottom and proceed with next steps or store the sample at -20°C until further use.

STEP 4

PCR amplification - *1 hour*

4.1 **For UDI Library construction**: Add **20 µl of D-Plex Primer UDI** (Primer UDI#) to the sample.

For SI library construction: Add **10 µl of D-Plex Forward Primer** and **10 µl of indexed D-Plex Reverser Primer** to the sample.

4.2 Add **50 µl of PCR Master Mix (PCRMM)** to the sample.

4.3 Mix by pipetting up and down until solution is homogeneous.

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

Template	Starting amount	UDI library construction n cycles	SI library construction n cycles
Total RNA	100 ng	10	10
	10 ng	12	12
	1 ng	14	15
	100 pg	18	20
Small RNA / Circulating RNA	10 ng	10	10
	1 ng	13	12
	100 pg	15	15
	10 pg	18	20

4.5 Incubate for PCR amplification according to the following program:

For UDI library construction:

Step	Temperature	Time & Cycles	
1. Initial denaturation	98°C	30 seconds	
2. Denaturation	98°C	15 seconds	*n* cycles
3. Annealing & extension	72°C	60 seconds	
4. Final extension	72°C	10 minutes	
Hold at 4°C or freeze until further processing			

For SI library construction:

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), add 2 more cycles to the n cycles determined earlier.*

STEP 5

DNA 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.*

5.1 Purify the amplified library using the MicroChIP DiaPure kit (Diagenode, C03040001) according to the kit's instructions (follow the "Protocol for ChIP samples"). 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 5: Elution volume after MicroChIP DiaPure purification

Next library processing step	DiaPure elution volume
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

DNA library clean-up and shaping

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 **For UDI library construction**: Add **50 µl of beads** to the **50 µl of eluted DNA library** from section 5 and mix thoroughly by pipetting up and down or vortexing.

For SI library construction: Add **75 µl of beads** to the **50 µl of eluted DNA library** from section 5 and mix thoroughly by pipetting up and down or vortexing.

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

6A.4 Spin down the tube and place 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 minutes and then remove it from the magnetic rack.

6A.9 Resuspend the beads in **15 µl of nuclease-free water** by slowly pipetting up and down.

6A.10 Incubate the beads in water during 2-3 minutes at room temperature under mild agitation.

6A.11 Spin down the tube 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 RNase-free tube and store it at -20°C until further use.

6B – AGAROSE GEL-CUT

6B.1 Load the **20 µl eluted DNA library** from section 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 ~195 bp for UDI library construction whereas it is ~180 bp for SI library construction.

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's 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 down the tube, collect the solution and 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 tube, add **20 µl of Buffer EB** and incubate for 2 minutes at room temperature.

6B.11 Centrifuge for 1 minute at 10.000g and collect the DNA library in a RNase-free tube. Store it at -20°C until further use.

6C – POLYACRYLAMIDE GEL-CUT

6C.1 Load the **20 µl eluted DNA library** from section 5 (mixed with appropriate electrophoresis loading buffer) in a well of a **4-6% polyacrylamide gel soaked in 0.5xTBE 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 ~195 bp for UDI library construction whereas it is ~180 bp for SI library construction.

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. Incubate overnight at 37°C under an agitation of 300 rpm to let DNA diffuse out of the gel.

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

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

6C.8 Add **0.75 ml of buffer PE** to the MinElute® column, incubate for 1 minute and centrifuge for 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 tube, add **20 µl of Buffer EB** and incubate for 2 minutes at room temperature.

6C.11 Centrifuge for 1 minute at 10.000g and collect the DNA library in a RNase-free tube. Store it at -20°C until further use.

STEP 7

Library QC 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 Construct

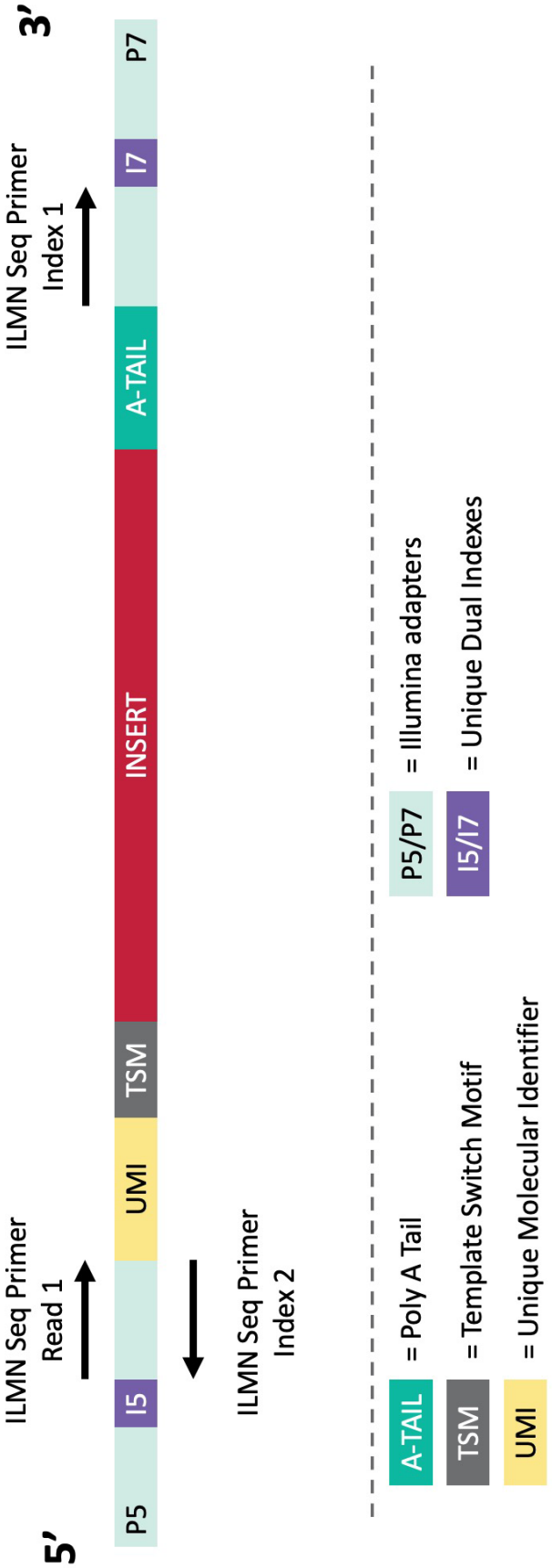


***Figure 3**: The D-Plex Small RNA-seq UDI library construct bears the Truseq (Illumina) HT adapters with unique dual indexes (UDI) and unique molecular identifiers (UMI). Read 1 = UMI – TSM– Insert – A-tail – 3' adapter.*

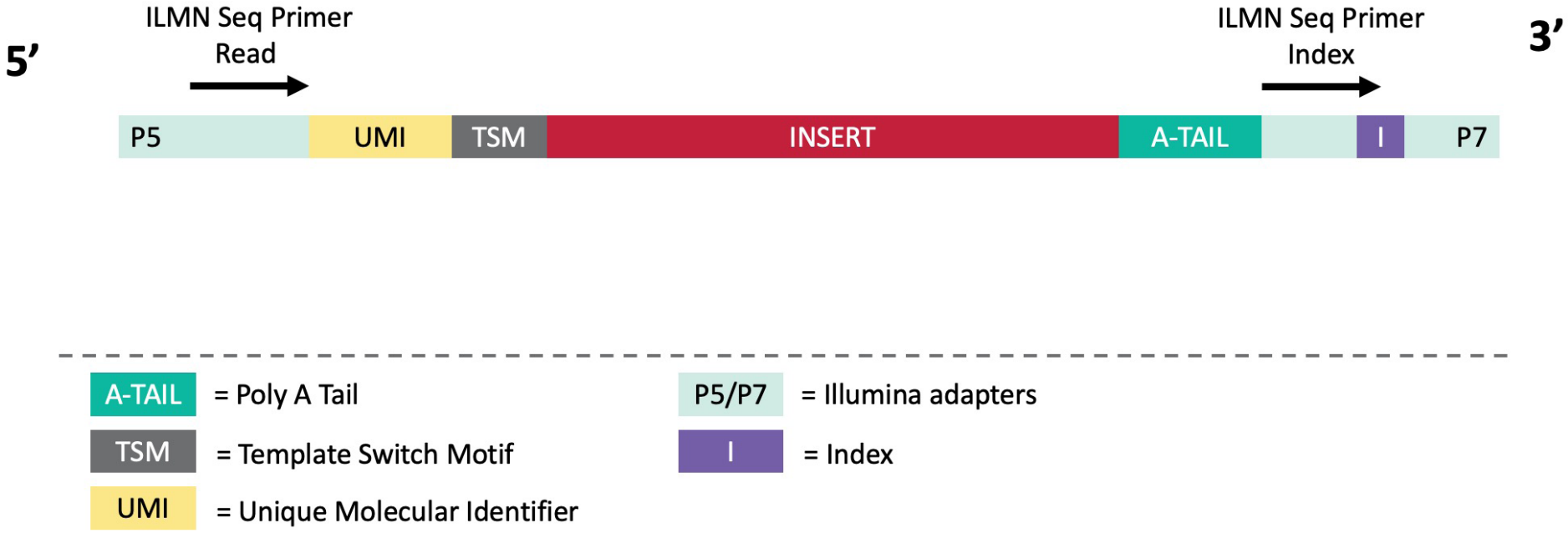


Figure 4: The D-Plex Small RNA-seq SI library construct bears the Truseq (Illumina) Small RNA adapters with single index (SI) and unique molecular identifiers (UMI). Read = UMI – TSM– Insert – A-tail – 3’ adapter.

Sequencing Recommendations

Given the high complexity of D-Plex Small RNA-seq libraries, it is advised to sequence one library with a **minimum of 20 million reads** in order to sufficiently cover the library content for downstream analysis.

D-Plex small RNA-seq 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, we recommend 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 RNAs) and a UMI at the 5' end of the forward strand, we highly recommend to use **single-end sequencing**.

Paired-end sequencing makes the data analysis difficult, unreliable in some cases, and due to the predominantly short fragments it generates mostly redundant information. If you are interested in longer fragments, we recommend to increase the read length with single-end sequencing, as this parameter should be suitable to read the complete length of the whole fragment.

UDI library construction

The D-Plex Small RNA-seq UDI library construct bears the TruSeq (Illumina) HT adapters. In case of a multiplexing scenario, it is therefore recommended to submit the D-Plex libraries as TruSeq HT libraries to your sequencing provider.

The complete architecture of the D-Plex Small RNA-seq UDI construct is provided in Figure 1. The empty library size (adapters + template switch + UMI + A tail) is equal to 172 bp.

SI library construction

The D-Plex small RNA-seq SI library construct bears the TruSeq (Illumina) Small RNA adapters. In case of a multiplexing scenario, it is therefore recommended to submit the D-Plex libraries as TruSeq small RNA libraries to your sequencing provider.

The complete architecture of the D-Plex small RNA-seq SI construct is provided in Figure 2. The empty library size (adapters + template switch + UMI + A tail) is equal to 155 bp.

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. The D-Plex construct holds special sequences namely the UMI, the A-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. In our example commands, we assume that the necessary software tools have been downloaded from the links provided at the end of the section and are in the PATH.

We recommend the fumi-tools software package for UMI processing. If the UMIs are not of interest, their processing 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.

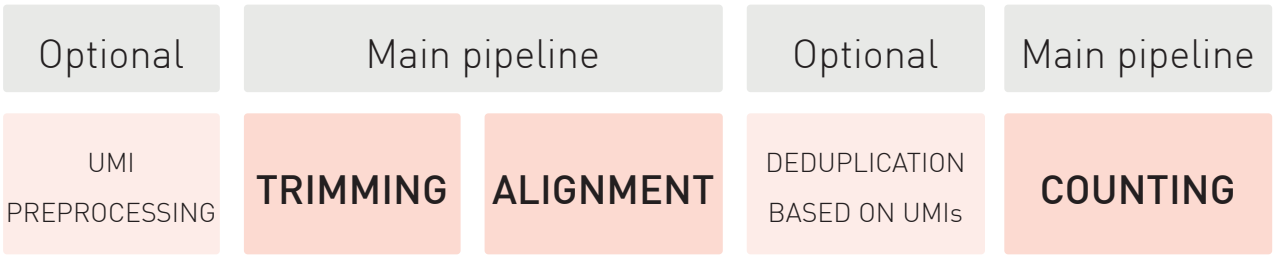


***Figure 5**: D-Plex Small RNA-seq bioinformatics pipeline*

UMI-preprocessing (optional)

To process the UMIs, the first thing to do is to copy the UMI sequence to the read ID. The first 12 bases in the read (from the 5' end) correspond to the UMI sequence. In the fumi-tools package, the copy_umi command can copy these bases to the correct lines in the fastq file. 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) as input. Optionally, the computational time can be reduced by increasing the number of CPUs to be used via the threads parameter. The example command below uses all the 12 UMI bases (recommended) and 10 threads.

Single-end processing

```
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. The aim of trimming is to remove these artificial sequences typical of D-Plex construct (UMI, A-tail, and template switch motif) that will likely hamper downstream analyses.

In our example command below, we use cutadapt package to properly trim the reads and obtain a read set as clean as possible. The command removes the UMI, A-tail, template switch motif and adapters sequences from all reads and discard reads which result shorter than 15 bases (these are considered too short to be included in the analysis). The trimming command can be applied on the input raw files (reads.fastq.gz) coming directly from the sequencer or alternatively, on the UMI pre-processed files (reads_w_umi.fastq.gz) if the UMIs are of interest.

Single-end processing

```
cutadapt --trim-n --match-read-wildcards -u 16
-n 4 -a AGATCGGAAGAGCACACGTCTG -a AAAAAAAA -a
GAACTCCAGTCAC -e 0.2 --nextseq-trim 20 -m 15 -o
trimmed_reads.fastq.gz raw_reads_w_umi.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 as for expression analysis of the known genes.

We recommend using the STAR alignment software. To run STAR, you need to provide the following arguments to the program: the input trimmed reads data file, the length at which reads were sequenced minus 1, the folder containing the STAR indexed genome (see STAR user guide for more information on how to prepare the genome), and optionally the number of CPUs to be used and the output format.

The example command below shows how to run STAR on the trimmed reads data. We assume we are dealing with a human sample, that reads were sequenced at 50 bases long and that we want to use 10 CPUs. Please adapt the read length if it is longer than 50 bp and change the hg19 genome if your samples are not human.

Single-end processing

```
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)

UMI processing enables the distinction between identical reads that are coming from different RNA molecules and identical reads resulting from PCR amplification. (If UMI deduplication is chosen, notice that you should have applied the UMI pre-processing step before trimming). Below we provide the command example to use fumi-tools to remove PCR clones from the alignment files. Computational resources to be used may be changed as desired using threads parameter (number of CPUs) and memory.

To remove duplicates from the genome alignment, no sorting is needed before UMI deduplication. However, to remove duplicates from the transcriptome alignment output, since the alignments are not sorted, please use samtools command provided below before UMI deduplication:

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

Now you can deduplicate both the genome and the transcriptome alignment with the UMI deduplication command of fumi-tools called dedup:

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

Note that fumi-tools outputs name-sorted bam files, which can be directly used as input for counting software tools. For another application, you may want to sort the bam files differently (e.g. sorting by coordinates).

Counting

The counting or expression level calculation is the last step of the processing to generate an expression level matrix. We recommend using MGcount*, a counting tool developed at Diagenode with a suitable annotations file that include the small RNA transcripts that are object of study. MGcount, built on top of featureCounts, employs a flexible quantification approach to deal with datasets containing multiple RNA biotypes such as D-plex libraries. Non-coding RNAs varying in length, biogenesis, and function, may overlap in a genomic region, and are sometimes present in the genome with a high copy number. Consequently, reads may align equally well to more than one position in the reference genome or/and align to a position where more than one annotated transcript is located. MGcount employs two strategies to quantify these reads. Firstly, it assigns reads in rounds prioritizing small-RNAs over long-RNAs ensuring the unbiased read attribution to intergenic and intragenic small RNAs while preventing host-genes transcription over-estimation. Secondly, MGcount collapses loci where reads consistently multi-map into communities of loci with a graph-based approach. The resultant communities are groups of biologically related loci with nearly identical sequence. Subsequently, quantification of reads is performed at the loci-community level, reducing multi-alignments ambiguity at individual locus. Ultimately, this strategy maximizes the transcriptome information analysed and improves the interrogation of non-coding RNAs. MGcount software repository provides annotation files for A. thaliana, H. sapiens, M. musculus and C. elegans. The required input files for MGcount are a .txt file listing the paths to the alignment input files (.bam format) and the annotations file (.gtf format). The output directory path has to be provided as an input as well.

Example command:

```
MGcount -T 2 --gtf Homo_sapiens.GRCh38.gtf --
outdir outputs --bam_infiles input_bamfilenames.txt
```

MGcount provides the choice to enable/disable the quantification of all RNA biotypes included in the annotation file in the form of "communities" as optional parameters for small-RNA (--ml_flag_small) and long-RNA (--ml_flag_long). Both are enabled by default. The main output of MGcount is the count_matrix.csv file containing an

expression matrix that can be imported to R or any other software for downstream analyses. A full user guide for MGCount is available here: https://filedn.com/lTnUWxFTA93JTyX3Hvbdn2h/mgcount/UserGuide.html.

Other standard counting tools such as featureCounts or HTSeq-counts can also be used alternatively. Given the high complexity of D-Plex libraries, we recommend having a clear understanding of the scientific question and the goal of the project before proceeding to the choice of the counting method as this will strongly impact downstream analyses. Diagenode provides bioinformatics support as a service (please, contact us if you need help with data analysis).

Notice that D-Plex produces forward-stranded data. Stranded libraries have the benefit that reads map to the genome strand where they were originated from. Therefore, when estimating transcript expression, reads aligned to the forward strand should be assigned only to transcript features in the forward strand whereas reads aligned to the reverse strand should be assigned only to transcript features in the reverse strand. For this, make sure you select "stranded mode" in any tool of choice. Stranded mode is selected by default in MGcount.

Hita, A., Brocart, G., Fernandez, A. et al. MGcount: a total RNA-seq quantification tool to address multi-mapping and multi-overlapping alignments ambiguity in non-coding transcripts. BMC Bioinformatics 23, 39 (2022). https://doi.org/10.1186/s12859-021-04544-3

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/releases
Cutadapt	https://github.com/marcelm/cutadapt
STAR	https://github.com/alexdobin/STAR
Samtools	http://www.htslib.org/download
featureCounts	http://subread.sourceforge.net
Htseqcount	https://htseq.readthedocs.io/en/master/count.html
MGcount	https://github.com/hitaandrea/MGcount

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

Template	Starting amount	DNA quantity after DiaPure purification (ng)
Total RNA	100 pg	~50
	100 ng	~50
Small RNA (< 200 nt)	1 ng	~20

D-Plex Small RNA-seq UDI library construct

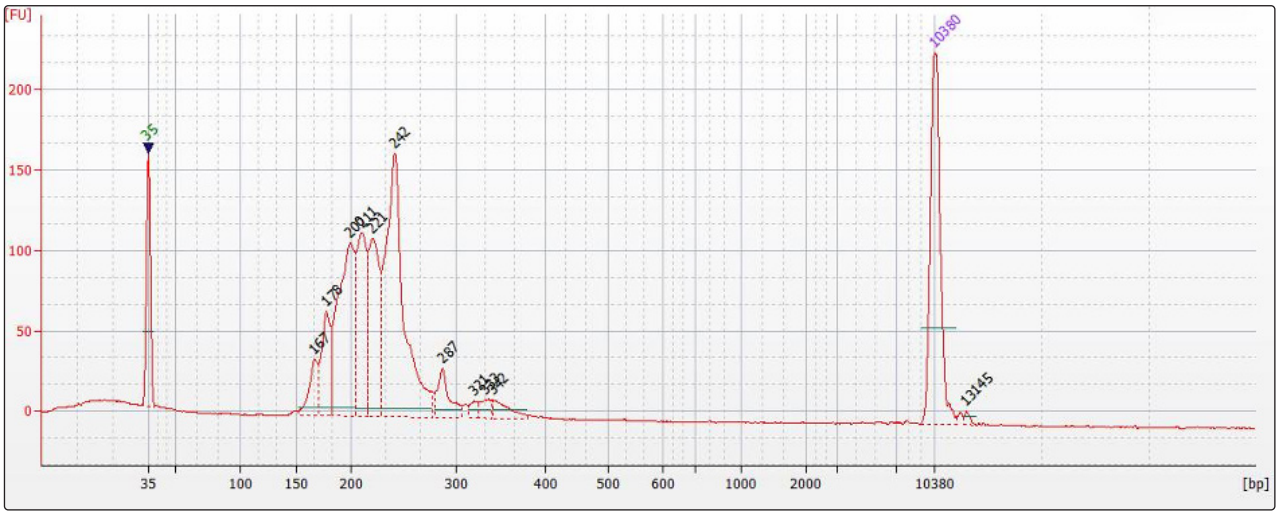


Figure 6: DNA electropherogram of a D-Plex Small RNA-seq UDI library construction (1.0X clean-up) made from 50 pg of circulating RNA, isolated from human plasma. 1 ng of the library was loaded on the Bioanalyzer.

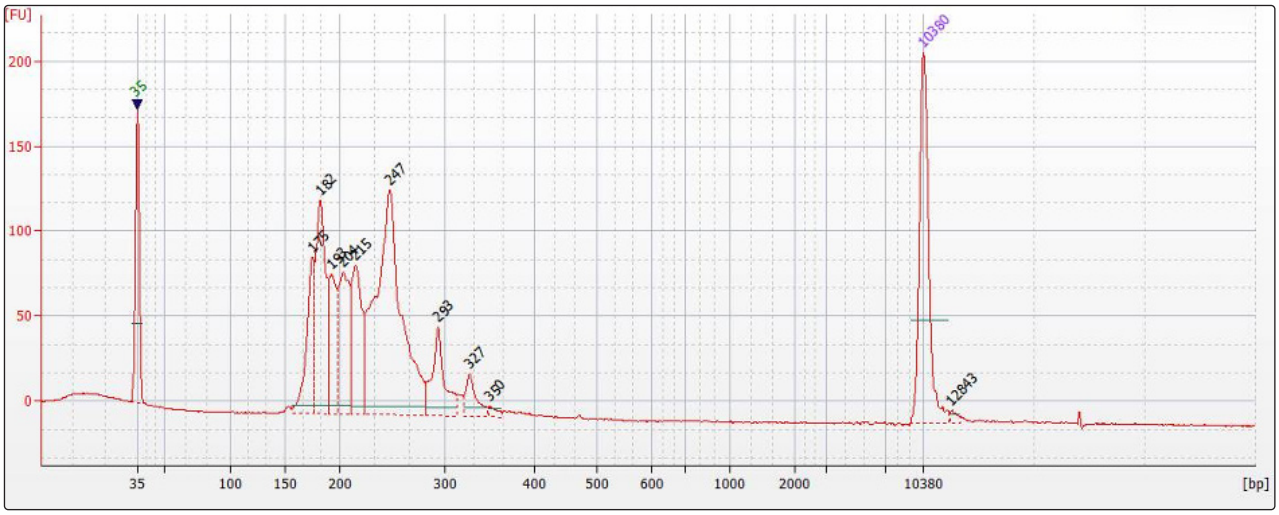


Figure 7: DNA electrophoregram of a D-Plex Small RNA-seq UDI library construction (1.0X clean-up) made from 100 pg of commercial human brain RNA (ThermoFisher Scientific, AM7962). 1 ng of the library was loaded on the Bioanalyzer.

D-Plex Small RNA-seq SI library construct

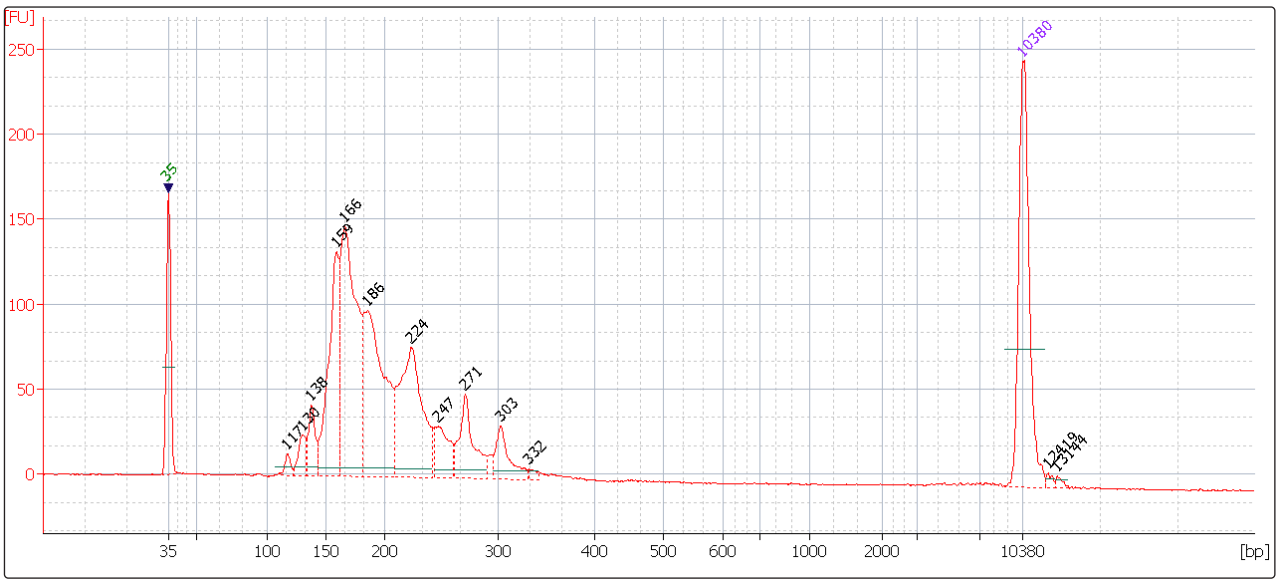


Figure 8: DNA electrophoregram of a D-Plex Small RNA-seq SI library construct (1,5X clean-up) made from 100 pg of commercial human brain RNA (Thermo Fisher Scientific, AM7962). 1 ng of the library was loaded on the Bioanalyzer.

Related Products

Product	Reference
D-Plex Unique Dual Indexes Module – Set A	C05030021
D-Plex Unique Dual Indexes Module – Set B	C05030022
D-Plex Unique Dual Indexes Module – Set C	C05030023
D-Plex Unique Dual Indexes Module – Set D	C05030024
D-Plex Single Indexes Module - Set A	C05030010
D-Plex Single Indexes Module - Set B	C05030011
DiaMag 0.2 mL tube magnetic rack	B04000001
MicroChIP DiaPure Kit	C03040001

Revision history

Version	Date of modification	Description of modifications
V2 03_2024	March 2024	- Page 22 - Typo correction in the step 4.4
V2 08_2023	August 2023	- Update of the paragraph "Counting", page 39 - Addition of new references and removal of obsolete references
V2 03_2021	March 2021	/

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