USER GUIDE

HOLOGIC®
Diagenode

# D-Plex cell-free RNA-seq Kit

For Illumina sequencing platforms

24 rxns

**Cat. No. C05030040**

# Content



Please read this manual carefully before starting your experiment

# Introduction

The **D-Plex cell-free RNA-seq library preparation kit** is designed as a flexible, discovery-oriented solution for profiling **circulating cell-free RNA** from biofluids. The method is designed to be largely insensitive to RNA biotype, 5′/3′ end moieties, and secondary structure, thereby favoring incorporation based primarily on relative abundance and size, with a preferential inclusion of shorter sequences.

Circulating cell-free RNA in biofluids is naturally composed predominantly of short molecules (<200 nt) spanning degraded transcripts (rRNA, mRNA, lncRNA), intact sncRNAs (tRNA, miRNA, miscRNA, snRNA, snoRNA), and processed fragments (tRNA, snoRNA, and rRNA-derived fragments). In this context, D-Plex was designed to be insensitive to these different RNA classes and to incorporate all of them, providing an “as close as possible” representation of the transcriptome of origin. By minimizing class-specific biases while accepting the inherent limitations of any library preparation method, D-Plex offers a well-suited solution for NGS-based cell-free RNA profiling.

# Kit Method Overview

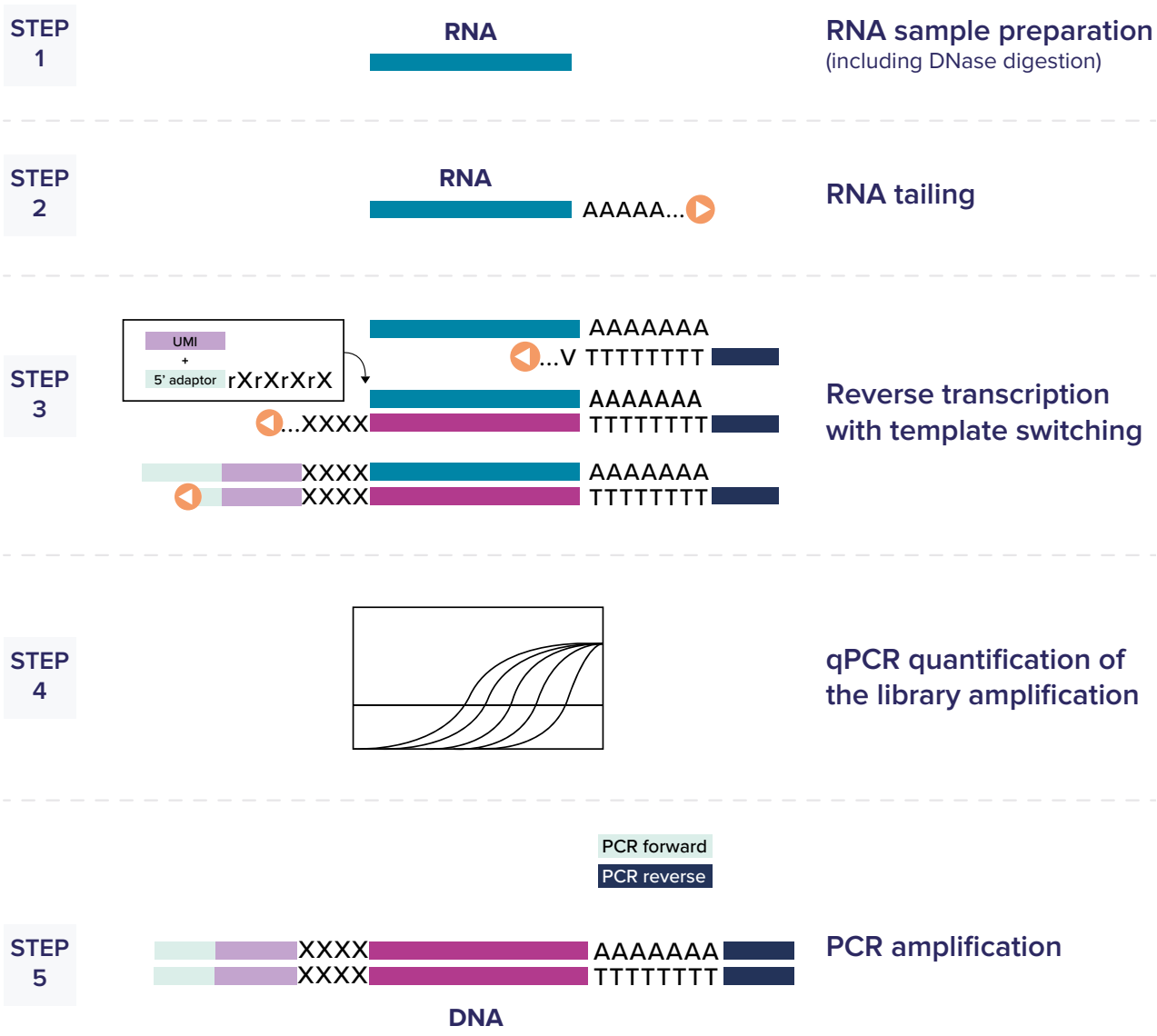


***Figure 1:*** *D-Plex cell-free RNA-seq kit workflow.*

Purified cell-free RNA (cfRNA) is treated with DNase I digestion to remove remaining traces of DNA from the sample. In a second step, the cfRNA is tailed with a 20-nt–long poly(A) tail starting from the 3' end of every RNA molecule in the sample. In step 3, an oligo(dT) primer bearing part of the Illumina P7 adapter is annealed to the complementary poly(A) tail. First-strand cDNA synthesis is then initiated with a reverse transcriptase (RTase). Once the RTase reaches the end of the RNA template, its terminal deoxynucleotidyl transferase (TdT) activity adds a series of non-templated nucleotides guided by the presence of a complementary template switch oligo (TSO). The RTase switches templates (template-switching effect) and synthesizes the complementary strand of the TSO incorporated into the library. The TSO has been designed to bear a 12-nt Unique Molecular Identifier (UMI) and part of the Illumina P5 adapter.

In step 4, an aliquot of the library obtained after reverse transcription and template switching is used to quantify the number of PCR cycles required to achieve a sufficiently concentrated library for sequencing. This step has been introduced in the protocol because it is not always possible to have a reliable quantification of the starting amount of cfRNA when starting a library. Finally, in step 5, the library is PCR-amplified using the number of PCR cycles determined in step 4.

After step 5, the library is ready to be purified, quality-controlled, and processed downstream for sequencing.

### Technical Specifications of the D-Plex cell-free RNA-seq Kit

***Table 1:*** *Technical Specifications of the D-Plex Cell-Free RNA-Seq Kit.*

| Parameter | Value |
|---|---|
| **Kit format** | 24 rxns |
| **Validated template** | Platelet-Free Plasma (PFP)<br>Platelet-Poor Plasma (PPP) |
| **Starting plasma volume (for the upstream extraction/purification)** | 200 µL – 500 µL |
| **Starting cell-free RNA input range for library preparation** | 200 pg – 1200 pg (quantification based on the FemtoPulse US RNA assay) |
| **Initial sample volume to start the library preparation** | 7 µL |
| **Library type** | Complete profiling of the cell-free transcriptome |
| **Library preparation technology** | 3'-end poly(A) tailing and 5'-end template switching-based |
| **Total duration of the library preparation protocol** | One day (process in a plate if ≥ 6 samples) |
| **Multiplexing capacity for sequencing** | Up to 48 samples/sequencing lane with D-Plex UDI for cell-free RNA-seq - Set A (Cat. No. C05030045) and Set B (Cat. No. C05030046) |

# Kit Materials

The following products are required to complete a D-Plex cell-free RNA-seq library preparation:

1. **D-Plex cell-free RNA-seq kit** (Cat. No. C05030040)
2. **D-Plex UDI for cell-free RNA-seq Set A** (Cat. No. C05030045) or **Set B** (Cat. No. C05030046)

The D-Plex cell-free RNA-seq kit contains enough reagents to perform 24 reactions. The kit components are listed in Table 2.

The PCR primer pairs required for library amplification and simultaneous sample indexing are not included in the D-Plex cell-free RNA-seq kit and must be purchased separately. Currently, two sets of primers are available (D-Plex UDI for cell-free RNA-seq, Set A and B), each containing 24 UDIs. The use of two sets simultaneously allows multiplexing of up to 48 samples per sequencing lane.

***Table 2.*** *Components Provided in the D-Plex Cell-Free RNA-Seq Kit (cat. no. C05030040).*

| Component | Cap Color | Quantity (µl) | Storage |
|---|---|---|---|
| DNase I | None | 3 µL | -20°C |
| DNase I Buffer | None | 40 µL | -20°C |
| Sample Buffer (SB) | Yellow | 48 µL | -20°C |
| Crowding Buffer (CB) | Yellow | 120 µL | 4°C |
| Tailing Reagent CF (TRCF) | Red | 24 µL | -20°C |
| Tailing Buffer CF (TBCF) | Red | 24 µL | -20°C |
| RT Buffer CF (RTBCF) | Purple | 120 µL | -20°C |
| RT Reagent (RTR) | Purple | 24 µL | -20°C |
| PCR Master Mix CF (PCRMMCF) | Green | 780 µL | -20°C |
| qPCR Primer Mix (qPCRPM) | None | 48 µL | -20°C |
| SYBR Green I 20x | None | 12 µL | -20°C |
| Nuclease-free Water | White | 1800 µL | -20°C |
| Positive Control miRNA (CTL+) | Black | 24 µL | -80°C |
| RT Primer CF (RTPCF) | Purple | 24 µL | -20°C |
| Template Switching Oligo CF (TSOCF) | Purple | 48 µL | -20°C |

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

# Required Materials Not Provided

### Reagents

- D-Plex UDI for cell-free RNA-seq, Set A or B (Hologic Diagenode, Cat. No. C05030045 or C05030046, respectively)
- RNase AWAY™ decontamination reagent (ThermoFisher Scientific, 10328011)
- Agencourt® AMPure® XP Beads (Beckman Coulter, A63881)
- Absolute ethanol (VWR, 20821.310)
- UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher, 10977035)

### Consumables

- Powder-free gloves
- A set of micropipettes with pipetting volumes ranging from 1.0 µL to 1,000 µL, in combination with filter tips (Mettler Toledo, RAININ Pipet-Lite LTS Pipettes)
- High-quality, contamination-free (DNA, RNA, nucleases) plasticware (microtubes) for highly sensitive applications (Sarstedt Biosphere® plus products)
- Eppendorf twin.tec® PCR Plate 96 LoBind® (Eppendorf, 0030129504) for the preparation of the libraries in plates, in combination with flat 12-cap strips (ThermoFisher Scientific, AB-0851)
- Individual PCR tubes with attached flat caps (Avantor, 20170-012) for the preparation of the libraries in tubes.

### Equipment

- DiaMag 0.2 mL tube magnetic rack (Hologic Diagenode, cat. no. B04000001)
- Tabletop centrifuge with strip rotor
- Centrifuge with a 96-well plate adapter
- Vortex agitator
- Tube holder for 0.2 mL and 1.5 mL tubes
- Plate holder compatible with 96-well plates
- Bio-Rad Thermal Cycler T100 (Bio-Rad, 1861096)
- Roche LightCycler 96 (Roche, 05815916001)
- Qubit 2.0 fluorometer (ThermoFisher Scientific) or a more recent version of the instrument, in combination with the Qubit HS dsDNA assay kit (ThermoFisher Scientific, Q32854)

- Agilent Fragment Analyzer 5300 (Agilent, M5311AA) in combination with the HS NGS Fragment Kit (Agilent, DNF-474)
- For cell-free RNA QC before library preparation: Agilent FemtoPulse (Agilent, M5330AA) in combination with the Ultrasensitivity RNA Kit (Agilent, FP-1201-0275)

# Remarks Before Starting

## General Recommendations

1. Clean the working area, the pipettes, and everything that will be used during the procedure (tip racks, tube holders, etc.) with RNase AWAY (ThermoFisher Scientific, 10328011).
2. Always keep the RNA on ice during handling and waiting times.
3. Return the RNA to −80°C as soon as it is no longer needed.
4. Avoid repeated freeze/thaw cycles of the RNA solution and D-Plex reagents; aliquot if needed.
5. Prepare the libraries on ice (with the help of a cooling block if preferred) until the PCR amplification step. Afterwards, libraries can be manipulated at room temperature (RT).
6. Reagents may be thawed at room temperature. Once thawed, keep reagents on ice until use. As a best practice, thaw reagents shortly before use to avoid unnecessary waiting time.
7. Before use, mix the kit reagents by gently pipetting up and down at least 10 times.
8. Set the lid of the thermal cycler to 105°C during the incubation steps.
9. The protocol is adapted for a 96-well plate format (validated with 48 samples in parallel):
    - 9.1 Account for 15% excess to prepare the master mixes.
    - 9.2 For each reagent added to the library preparation solution, calculate the volume needed per plate row based on the number of samples, add 15% excess, and dispense this total volume into a 12-tube strip. Use this strip as a reservoir and distribute the reagent one plate row at a time with a 12-channel pipette.
    - 9.3 Always mix by pipetting up and down several times until the library preparation solution appears completely homogeneous.
    - 9.4 **Never vortex** the reagents, RNA, or library preparation solution (except for the final step of bead clean-up).
    - 9.5 The crowding buffer (CB) is intentionally viscous. Thorough mixing by pipetting up and down is critically important during the tailing and reverse transcription reactions to ensure reproducibility.
    - 9.6 Use 12-strip caps rather than sealing foil to close the plate for reactions and incubations.
    - 9.7 After mixing and before incubating in the thermocycler, spin the plate down in a centrifuge to collect droplets at the bottom of the sample wells.

10. In the experimental design, account for three technical replicates of the positive control and the negative control:

    10.1 **Positive control:** Prepare a 10-fold dilution of the positive control miRNA (CTL+) in nuclease-free water to obtain a final concentration of 0.1 ng/µL. Use 1 µL of this diluted solution and mix with 6 µL of water to constitute the positive control.

    10.2 **Negative control (NTC):** Use 7 µL of nuclease-free water instead of the sample. The NTC is used to monitor whether contamination has occurred during the library preparation, given its highly sensitive nature. **It is strongly recommended to separate the NTCs from the samples in the plate/tube strip, leaving space between template libraries and NTCs** (typically, NTCs are placed in the bottom right corner of the plate and samples in the upper left corner). This will reduce the likelihood of cross-contamination of the NTCs.

## End-to-end cell-free RNA-seq workflow using the D-Plex cell-free RNA-seq kit

As for any library preparation, the D-Plex cell-free RNA-seq kit is embedded in a sample-to-result workflow, as illustrated in Figure 2. Some recommendations derived from the work led by key opinion leaders[1,2] and from internal experience are given in this section to achieve a high-quality outcome. These recommendations should be considered as helpful guidance only and do not constitute strict requirements for the library preparation kit to function properly.

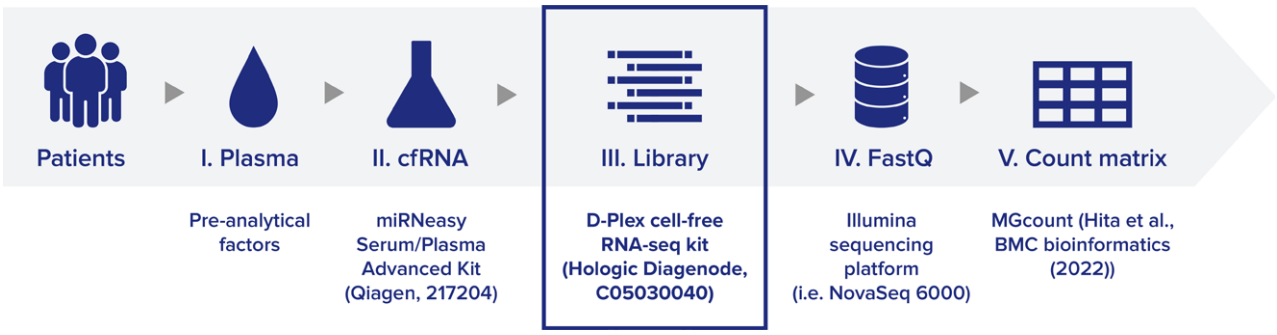


***Figure 2.*** *End-to-End Cell-Free RNA-Seq Workflow Using the D-Plex Kit.*

### I. Pre-Analytical Factors

Extracellular RNA (exRNA) profiling by next-generation sequencing (NGS) is highly

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[1] *exRNAQC Consortium. Blood collection tube and RNA purification method recommendations for extracellular RNA transcriptome profiling. Nat Commun. 2025 May 15;16(1):4513*

[2] *Van Der Schueren C et al., Subpar reporting of pre-analytical variables in RNA-focused blood plasma studies. Mol Oncol. 2025 Jul;19(7):1968-1978.*

dependent on a variety of factors, among them the pre-analytical factors used during blood draw and plasma processing. For quality control purposes, it is advised to record the following parameters at the time of blood draw and plasma processing:

**1. Blood draw**

1.1 Caliber of needle used to draw blood (recommendation: 21G – if narrower, the risk of hemolysis increases)

1.2 Site of the blood draw (e.g., antecubital)

1.3 Time before processing/after collection of blood (recommendation: less than 4 hours)

1.4 Type of blood collection tube

1.5 Nature of the anticoagulant (recommendation: K2-EDTA)

**2. Plasma processing**

2.1 Speed of centrifugation steps

2.2 Duration of each centrifugation

2.3 Temperature (°C) applied during centrifugation steps

2.4 Brake applied during centrifugation

2.5 Thickness of the buffy coat/pellet remaining between centrifugation steps to prepare plasma

2.6 Time before freezing the plasma at -80°C or in liquid nitrogen

2.7 Freezing method (-80°C or snap freezing in liquid nitrogen)

**3. Recommendations for processing plasma[3]**

3.1 Leave 0.5 cm of plasma above the buffy coat and pellet after each centrifugation to avoid carrying over blood cells or platelets into the plasma.

3.1.1 Processing platelet-poor plasma (PPP):

3.1.1.1 Centrifuge at 400 x g for 20 minutes.

3.1.1.2 Centrifuge at 800 x g for 10 minutes without brake.

3.1.1.3 Freeze at -80°C until further use.

3.1.2 Processing platelet-free plasma (PFP):

3.1.2.1 Centrifuge at 2,500 x g for 15 minutes.

3.1.2.2 Centrifuge at 2,500 x g for 15 minutes without brake.

3.1.2.3 Freeze at -80°C until further use.

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[3] *Everaert C, et al. Performance assessment of total RNA sequencing of human biofluids and extracellular vesicles. Sci Rep. 2019 Nov 26;9(1):17574)*

## II. Cell-free RNA extraction/purification

The miRNeasy Serum/Plasma Advanced Kit (Qiagen, 217204) is recommended for the extraction and purification of cell-free RNA from human plasma. Note that **it is imperative to follow the on-column DNase digestion** option during the procedure, otherwise there is a risk of significant contamination with cell-free DNA.

## III. Library preparation

1. The D-Plex cell-free RNA-seq library preparation kit has been developed and validated using both PPP and PFP. Usually, enough cfRNA can be recovered after extraction/purification from 0.2 mL of plasma. During method validation, the linearity was confirmed between 200 and 1,200 pg of starting cfRNA input. This usually represents an initial starting plasma volume of 0.2–0.5 mL.
2. Before starting the library preparation, it is recommended to perform quality control of the cfRNA purified using the FemtoPulse US RNA assay (Agilent, FP-1201-0275). Examples of quality cfRNA are given in section "Quality Control Results" of the manual. If, despite purification, the cfRNA does not appear totally pure (i.e, eluate is cloudy at 4°C, floating white “clouds”, etc.), it is strongly advised to repurify the cfRNA eluate using the Zymo RNA Clean & Concentrator-5 (Zymo Research, R1015).
3. The kit is provided with a positive control miRNA (CTL+) that is recommended to be used in combination with a negative control (7 µL of nuclease-free water) to ensure the quality of the experiment.
4. The method has a qPCR step before amplification to calculate the number of cycles to apply, considering that an accurate quantification of the starting RNA input is not always possible.
5. For the PCR amplification step, the expected maximum number of cycles is 16, which corresponds to a 200 pg cfRNA starting input. Above 16 cycles, the method is still valid but may start producing a larger empty library (~185 bp peak) that is detrimental to downstream read allocation on library fragments containing biological information.

## IV. Sequencing

1. The D-Plex cell-free RNA-seq kit has been developed for sequencing on Illumina platforms. Currently, the development and validation have been carried out on the NovaSeq 6000 and NovaSeq X instruments with the recommendations provided in the section “Sequencing Recommendations” of the manual.
2. If other sequencing platforms are considered, it is possible that some of the parameters will need to be adapted to obtain optimal results.

## V. Feature quantification

1. A dedicated bioinformatic pipeline has been developed to process D-Plex cell-free RNA-seq data. Details about the pipeline are given in the section “Data Analysis Recommendations” of the manual.
2. We recommend MGcount[4] as the counting software. MGcount was specifically designed to process whole-transcriptome datasets that closely resemble those obtained from extracellular RNA samples. Its strategy, developed to agglomerate multi-mapping reads and resolve multi-overlapping annotations, ensures a balanced and accurate representation of the features.

[4] *Hita, 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)*

# Short Protocol for Experienced Users

In this chapter, we present the short protocol for preparing next-generation sequencing (NGS) libraries for Illumina platforms using the D-Plex cell-free RNA-seq kit. For first-time use of the kit, or for operators who are not experienced in preparing RNA-seq libraries, it is strongly recommended to follow the detailed instructions provided later in this manual.

1. Put **7 µL** (200–1,200 pg) of **purified cell-free RNA** (cfRNA) (on-column DNase-digested) in a clean 0.2 mL microwell/tube.
2. Add **1 µL** of **DNase I**, **diluted 10-fold in DNase I buffer**.
3. Incubate the cfRNA solution:
   - at **37°C** for 10 minutes
   - at **75°C** for 10 minutes
   - cool **on ice** for 2 minutes.
4. Prepare the Tailing Master Mix (**TMM**):

| Reagent | Volume for 1 sample |
|---|---|
| Crowding buffer (CB) | 5 µL |
| Sample buffer (SB) | 2 µL |
| Tailing buffer CF (TBCF) | 1 µl |
| Tailing reagent CF (TRCF) | 1 µl |
| **Total volume** | **9 µl** |

5. Add **9 µL** of **TMM** to the cfRNA solution.
6. Incubate the solution for exactly 40 minutes at **37°C**, then cool it **on ice** for 2 minutes.
7. Add **1 µL** of **RTPCF** to the tailed cfRNA solution.
8. Incubate the cfRNA solution:
   - at **70°C** for 10 minutes
   - at **25°C** for 2 minutes with a ramping down of 0.5°C/s from 70°C to 25°C
   - at **4°C** for a maximum of 10 minutes before proceeding to the next step.
9. Prepare the RT Master Mix (**RTMM**):

| Reagent | Volume for 1 sample |
|---|---|
| RT Buffer CF (RTBCF) | 5 µL |
| RT Reagent (RTR) | 1 µL |
| **Total volume** | **6 µl** |

10. Add **6µL** of **RTMM** to the tailed cfRNA solution.
11. Incubate the solution at **25°C** for 15 minutes and return **on ice** for no longer than 10 minutes before proceeding to the next step.
12. Add **2 µL** of **TSOCF** to the cfRNA solution.
13. Incubate the solution:
    - at **42°C** for 120 minutes
    - at **70°C** for 10 minutes
    - at **4°C** for no longer than 18 hours (overnight) before proceeding to the next step.

***PAUSE POINT:*** *Libraries prepared up to that point can be left at 4°C overnight (18 hours) or stored at -20°C for a maximum of 3 days (over the weekend).*

14. Aliquot **2.5 µL** of the **cDNA library** and mix it with **7.5 µL** of the **Amplification Master Mix** prepared as shown below.

| **Reagent** | **Volume for 1 sample** |
|---|---|
| qPCR Primer Mix (qPCRPM) | 2 µL |
| PCR Master Mix CF (PCRMMCF) | 5 µL |
| SYBR Green I 20X | 0.5 µl |
| **Total volume** | **7.5 µl** |

15. Run the following qPCR program (fluorescence acquired after the extension step of each cycle):

| **qPCR Step** | **Temperature** | **Duration of the Step** |
|---|---|---|
| Initial Denaturation | 98°C | 30 seconds |
| Amplification (30 cycles) | 98°C | 10 seconds |
| | 62°C | 30 seconds |
| | 72°C | 30 seconds |
| | Acquisition of Fluorescence | |
| Holding Step | 4°C | Until Further Processing |

Upon completion of the qPCR (30 cycles), retrieve the Cq values associated with the sample and calculate the number of PCR amplification cycles by rounding the Cq value up to the nearest integer and subtracting 3:

***PCR amplification cycles = rounded Cq value − 3***

16. Reconstitute the **PCR amplification** mix as follows:

| **Reagent** | **Volume for 1 sample** |
|---|---|
| PCR Master Mix CF (PCRMMCF) | 27.5 µL |
| Nuclease-Free Water | 2.5 µL |

17. Add **30 µL** of **PCR amplification mix** and **2 µL** of **D-Plex UDI for cell-free RNA-seq** to the 23.5 µL of cDNA.

    17.1 Incubate the sample for the number of cycles determined in step 15.

***PAUSE POINT:*** *Libraries prepared up to that point can be left at 4°C overnight (18 hours) or stored at -20°C for a maximum of 6 months.*

18. Run the AMPure XP beads purification of the libraries so that the **bead: library ratio is 1.0X**.

    18.1 Mix the solution thoroughly before and during the binding step to ensure optional capture of the library fragments.

    18.2 Avoid over-drying the beads and ensure that no residual ethanol remains on the beads after the washing steps.

    18.3 Elute the final library in **10 µL** of **nuclease-free water** and transfer **9.5 µL** of **clear supernatant** to a new tube/microwell. This transferred eluate constitutes the **purified library**.

# Detailed Protocol

## DNase I Digestion

1. Put **7 µL** of **purified cell-free RNA** (cfRNA) in a clean 0.2 mL microwell/tube.
   - Validated method range: **200-1200 pg** of starting **cfRNA input**.
   - It is imperative that the cfRNA has been DNase-digested during the extraction/purification process to ensure minimal carryover of cell-free DNA (cfDNA) into library preparation.
   - Ideally, cfRNA has been quantified and quality-controlled before starting the library preparation using the FemtoPulse Ultrasensitivity Kit (Agilent, FP-1201-0275).
2. Add **1 µL** of **DNase I diluted 10-fold in DNase I buffer** to the sample. For convenience, a DNase I master mix (DNase I diluted 10-fold in DNase I buffer) can be prepared in advance and kept on ice until dispensed to the samples.
3. Incubate the cfRNA solution as follows:
   - at **37°C** for 10 minutes
   - at **75°C** for 10 minutes
   - cool **on ice** for 2 minutes

## Tailing Reaction

4. Prepare the Tailing Master Mix (**TMM**):

| **Reagent** | **Volume for 1 sample** |
|---|---|
| Crowding buffer (CB) | 5 µL |
| Sample buffer (SB) | 2 µL |
| Tailing buffer CF (TBCF) | 1 µl |
| Tailing reagent CF (TRCF) | 1 µl |
| **Total volume** | **9 µl** |

5. Add **9 µL** of the **TMM** to the cfRNA solution. The TMM is very viscous due to the presence of the CB. It is therefore very important to mix the solution well by pipetting up and down until it appears completely homogeneous. This is a **critical step** to ensure reproducibility across the sample set.
6. Incubate the solution for exactly 40 minutes at **37°C**, then cool it **on ice** for 2 minutes.

### Reverse-Transcription Reaction

7. Add **1 µL** of **RT Primer CF** (RTPCF) to the tailed cfRNA solution. Mix well by pipetting up and down until the solution appears completely homogeneous. This is a critical step to ensure reproducibility across the sample set.
8. Incubate the cfRNA solution:
   - at **70°C** for 10 minutes for priming
   - at **25°C** for 2 minutes with a ramping down of 0.5°C/s from 70°C to 25°C
   - at **4°C** for a maximum of 10 minutes before proceeding to the next step.
9. Prepare the RT Master Mix (**RTMM**):

| Reagent | Volume for 1 sample |
|---|---|
| RT Buffer CF (RTBCF) | 5 µL |
| RT Reagent (RTR) | 1 µL |
| **Total volume** | **6 µl** |

10. Add **6 µL** of the **RTMM** to the tailed cfRNA solution. Mix well by pipetting up and down until the solution appears completely homogeneous. This is a critical step to ensure reproducibility across the sample set.
11. Incubate the solution at **25°C** for 15 minutes and place it **on ice** for no longer than 10 minutes before proceeding to the next step.
12. Add **2 µL** of the **Template Switching Oligo CF (TSOCF)** to the cfRNA solution. Mix well by pipetting up and down until the solution appears completely homogeneous. This is a critical step to ensure reproducibility across the sample set.
13. Incubate the solution:
    - at **42°C** for 120 minutes
    - at **70°C** for 10 minutes
    - at **4°C** for no longer than 18 hours (overnight) before proceeding to the next step.

***PAUSE POINT:*** *Libraries prepared up to that point can be left at 4°C overnight (18 hours) or stored at -20°C for a maximum of 3 days (over the weekend).*

### qPCR Quantification of the Library Amplification

14. Aliquot **2.5 µL** of the **cDNA library** and mix it with **7.5 µL** of the **amplification master mix** prepared as follows:

| Reagent | Volume for 1 sample |
|---|---|
| qPCR Primer Mix (qPCRPM) | 2 µL |
| PCR Master Mix CF (PCRMMCF) | 5 µL |
| SYBR Green I 20X | 0.5 µl |
| **Total volume** | **7.5 µl** |

15. Run the following qPCR program (fluorescence acquired after the extension step of each cycle):

| qPCR Step | Temperature | Duration of the Step | Amplification Sub-Step |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | / |
| Amplification (30 cycles) | 98°C | 10 seconds | Denaturation |
| | 62°C | 30 seconds | Annealing |
| | 72°C | 30 seconds | Extension |
| | Acquisition of Fluorescence | | |
| Holding Step | 4°C | Until Further Processing | / |

Upon completion of the qPCR (30 cycles), retrieve the Cq values associated with the sample and calculate the number of PCR amplification cycles by rounding the Cq value to the nearest integer (down if < 0.5 and up if ≥ 0.5) and subtracting 3:

***PCR amplification cycles = rounded Cq value − 3***

For example, for a Cq value of 18.4 obtained after qPCR quantification, the number of PCR cycles to apply will be:

| | |
|---|---|
| 18.4 | Cq Value |
| 18.0 | Cq value rounded to the nearest integer (.4 is rounded down because it is below the .5 mark) |
| 18.0 – 3 | Application of the formula |
| 15 | Number of required PCR amplification cycles |

Total number of PCR cycles **should not exceed** the values shown in **Table 3**.

***Table 3.*** *PCR cycle recommendations for library amplification.*

| Starting cfRNA input for library preparation | Recommended/Expected number of PCR cycles to apply |
|---|---|
| 200 pg | 16 |
| 400 – 600 pg | 15 |
| 1200 pg | 14 |

### PCR Amplification

16. For PCR amplification, reconstitute the **PCR amplification mix** as follows:

| Reagent | Volume for 1 sample |
|---|---|
| PCR Master Mix CF (PCRMMCF) | 27.5 µL |
| Nuclease-Free Water | 2.5 µL |

17. Add **30 µL** of **PCR amplification mix** and **2 µL** of **D-Plex UDI for cell-free RNA-seq** to the 23.5 µL of cDNA.

17.1 Each sample pooled within the same sequencing lane must be assigned a unique UDI.

17.2 Upon assembling the PCR amplification solution, incubate for the number of cycles determined in step 15, following the PCR protocol below.

| qPCR Step | Temperature | Duration of the Step | Amplification Sub-Step |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | / |
| Amplification (x calculated number of cycles) | 98°C | 10 seconds | Denaturation |
| | 62°C | 30 seconds | Annealing |
| | 72°C | 30 seconds | Extension |
| | Acquisition of Fluorescence | | |
| Final Extension | 72°C | 10 minutes | / |
| Holding Step | 4°C | Until Further Processing | / |

17.3 Amplify the **positive** and **negative controls** (0.1 ng Positive Control miRNA (CTL+) and NTC) in your experiment to 15 PCR cycles.

***PAUSE POINT:*** *Libraries prepared up to that point can be left at 4°C overnight (18 hours) or stored at -20°C for a maximum of 6 months.*

The Cq difference between the positive and negative controls after qPCR quantification should be at least 5, indicating a clean library preparation.

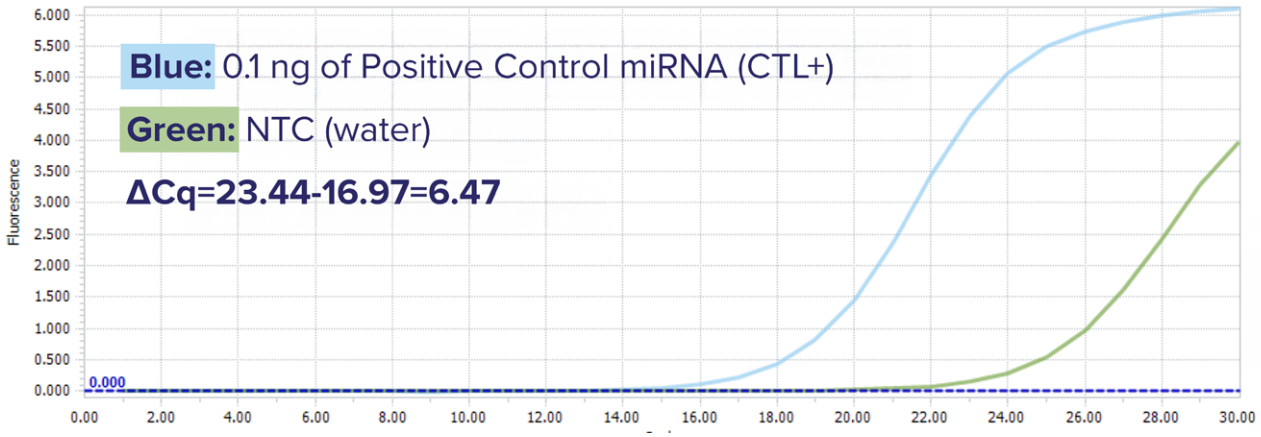


***Figure 3.*** *qPCR amplification curves of a positive control (blue) and a negative control (green).*

### AMPure XP Beads Purification

18. Measure the volume of 2-3 samples after PCR amplification to confirm the remaining reaction volume as significant evaporation might have occurred.
19. Adjust the volume of AMPure XP to add to the library solution to maintain a **1.0x** bead-to-sample ratio.
20. Vortex the library-bead mixture using 5-10 short pulses.
21. Incubate the library-bead mixture for 5 minutes at room temperature (RT) with gentle agitation (rotating wheel, rocking station).
22. Place the library-bead mixture on a magnet and incubate for at least 5 minutes, until the beads have fully precipitated and the solution is clear. Carefully remove and discard the supernatant.
23. Wash the bead pellet twice for 30 seconds with **200 µL** of **80% (v/v) ethanol**, **freshly prepared from absolute ethanol**.
24. Allow the beads to air-dry for 2 minutes. Ensure that all residual ethanol has evaporated and that the bead pellet is completely dry before elution. Do not over-dry the pellet (cracks in the pellet), as it may hinder its resuspension.
25. Add **10 µL** of **nuclease-free water** to the dried bead pellet, pulse vortex 5 times to resuspend the beads, and incubate for 5 minutes at room temperature (RT).
26. Place the bead mixture on a magnet and incubate until the beads have fully precipitated. Transfer **9.5 µL** of **clear supernatant** to a new tube/microwell. This transferred eluate constitutes the **purified library**.

***PAUSE POINT:*** *Libraries prepared up to that point can be left at 4°C overnight (18 hours) or stored at -20°C for a maximum of 6 months.*

### Library Quantification and Quality Control

27. Perform library quantification according to the detailed Qubit HS dsDNA Assay Kit user protocol.
28. Acquire the library electropherogram following Agilent's HS NGS Fragment Kit user protocol.

### OPTIONAL: Final Library Pool Clean-Up

Analysis of the library electropherogram may indicate the presence of residual PCR primers following bead clean-up. Libraries containing excess PCR primers are not suitable for sequencing and therefore require an additional clean-up step.

29. Pool the libraries according to their **gated** (150-1000 bp) Fragment Analyzer quantification values expressed in nanomolar (nM).

30. Adjust the final volume of the pooled libraries to **50 µL**.
    If the pooled volume exceeds 50 µL, adjust the volume of AMPure XP beads accordingly to maintain a bead-to-sample ratio of 1.5x.

31. Perform an AMPure XP bead clean-up with a bead ratio of **1.5x** (e.g., add 75 µL of beads to a 50 µL pool) by repeating steps 20 to 26.

32. Analyze the cleaned pooled libraries on the Fragment Analyzer using the HS NGS Fragment Kit to assess the library profile and confirm the absence of residual PCR primers.

***PAUSE POINT:*** *Libraries prepared up to that point can be left at 4°C overnight (18 hours) or stored at -20°C for a maximum of 6 months.*

# Quality Control Results

### Platelet-Poor Plasma (PPP) Cell-Free RNA Profile

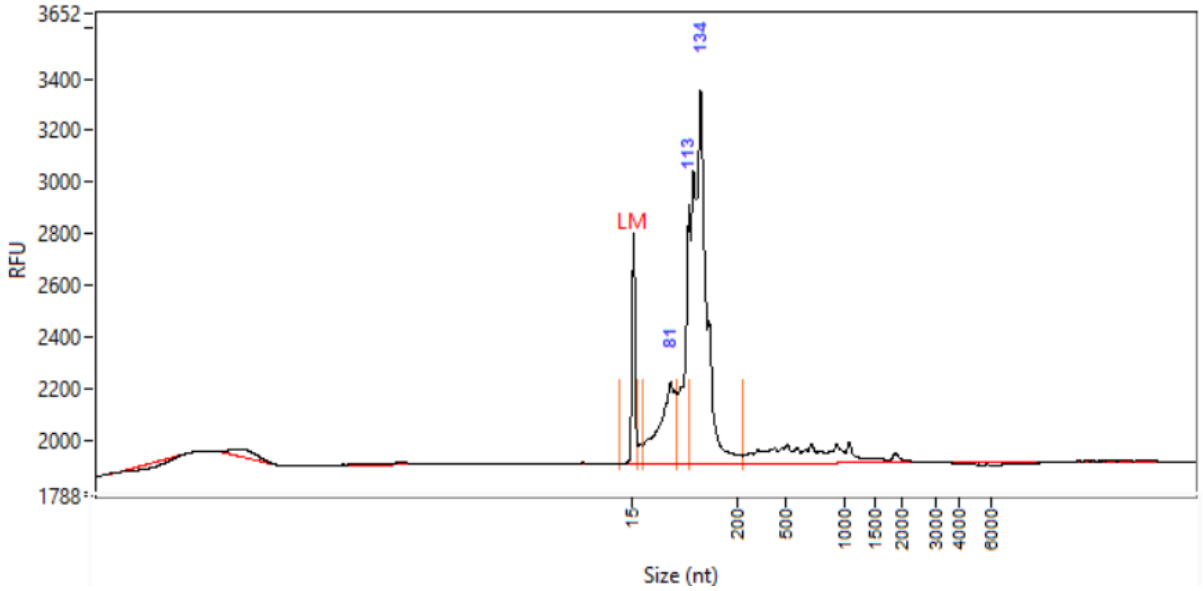


***Figure 4.*** *Electropherogram of a cell-free RNA sample extracted from platelet-poor plasma (PPP).*

### Platelet-Free Plasma (PPP) Cell-Free RNA Profile

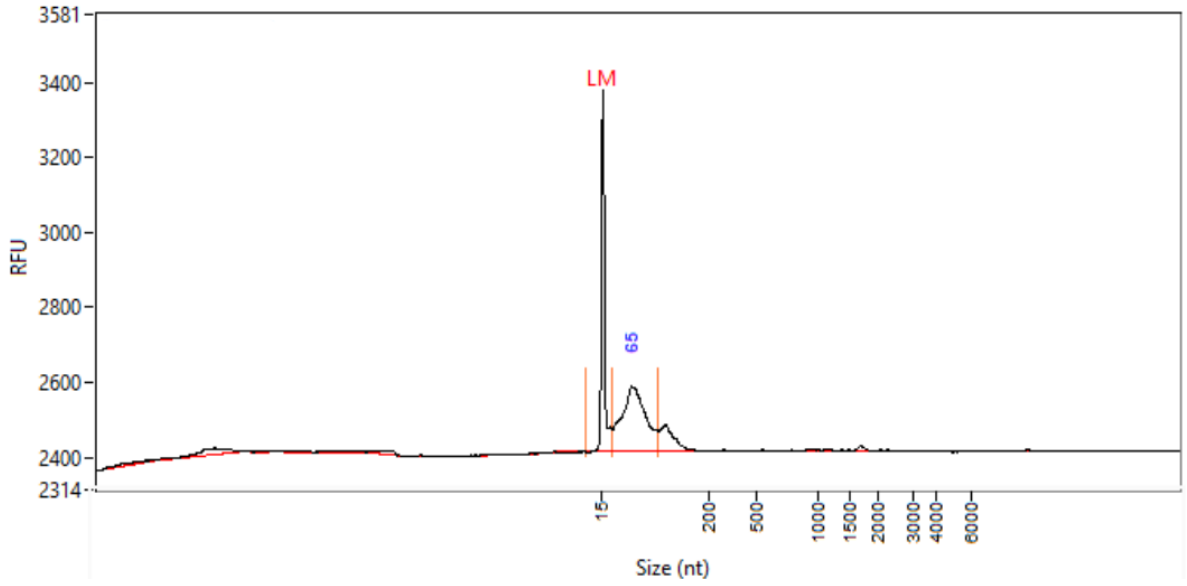


***Figure 5.*** *Electropherogram of a cell-free RNA sample extracted from platelet-free plasma (PFP).*

To properly assess the importance and impact of plasma preparation on the cell-free RNA profile after isolation, it should be noted that the profiles shown in Figures 4 and 5 were generated from the same blood pool, which was split to evaluate differences arising solely from plasma preparation.

### Positive Control miRNA Library

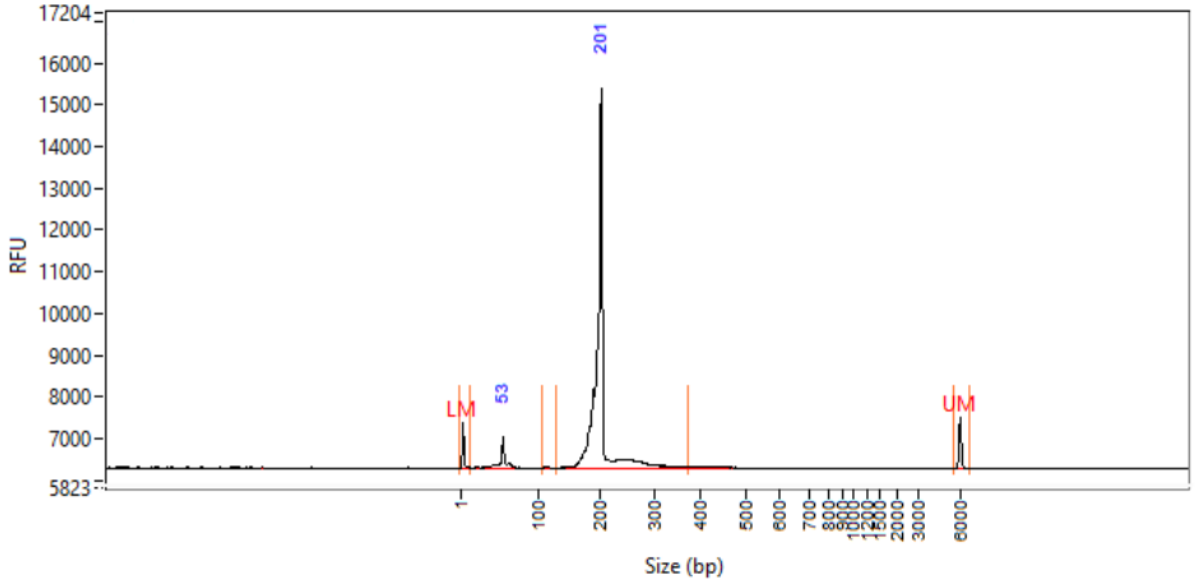


***Figure 6.*** *Electropherogram of a Positive Control miRNA (0.1 ng) at 15x amplification.*

### Negative Control (Water) Library

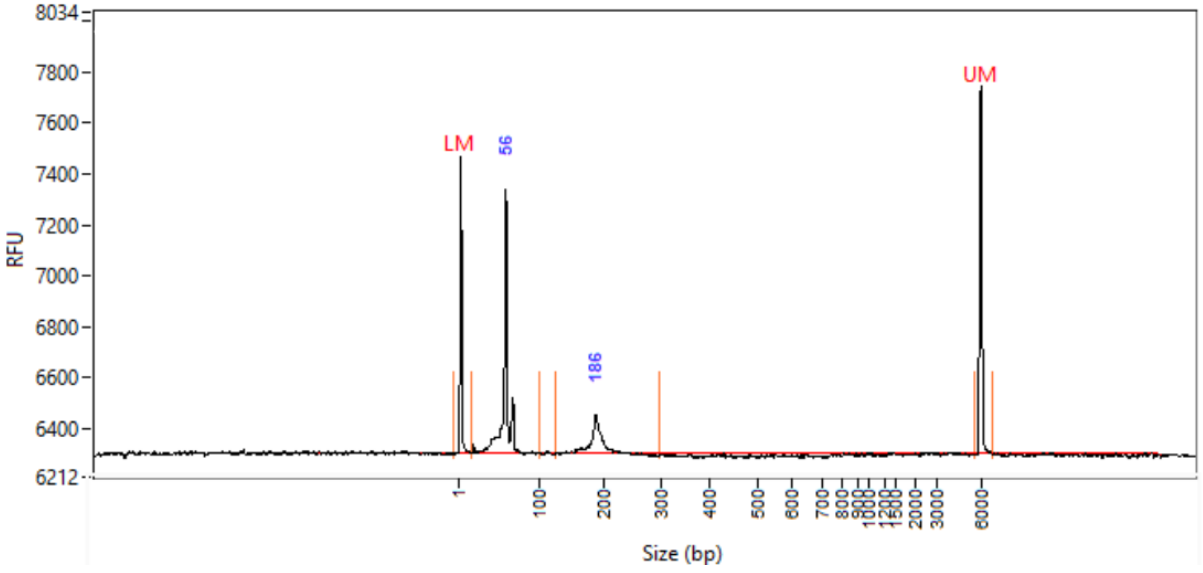


***Figure 7.*** *Electropherogram of a NTC at 15x amplification.*

A small peak at ~186 bp, corresponding to the empty library, is visible on the electropherogram. As long as this peak remains minimal, as shown in Figure 7, it does not pose an issue, and the reads associated with it will be removed at the trimming/filtering step during data analysis. However, the presence of a higher empty-library peak, additional peaks, a smear, or a tail extending beyond the empty-library peak should be considered a warning sign of potential contamination of the experiment or reagents. If such features are observed, careful consideration should be given before proceeding with sequencing.

### Platelet-Free Plasma (PFP) Library

Based on the recommended amplification, libraries prepared from PFP will typically reach concentrations between 3 and 15 nM when measured on the Fragment Analyzer using the 150–1000 bp gated region.

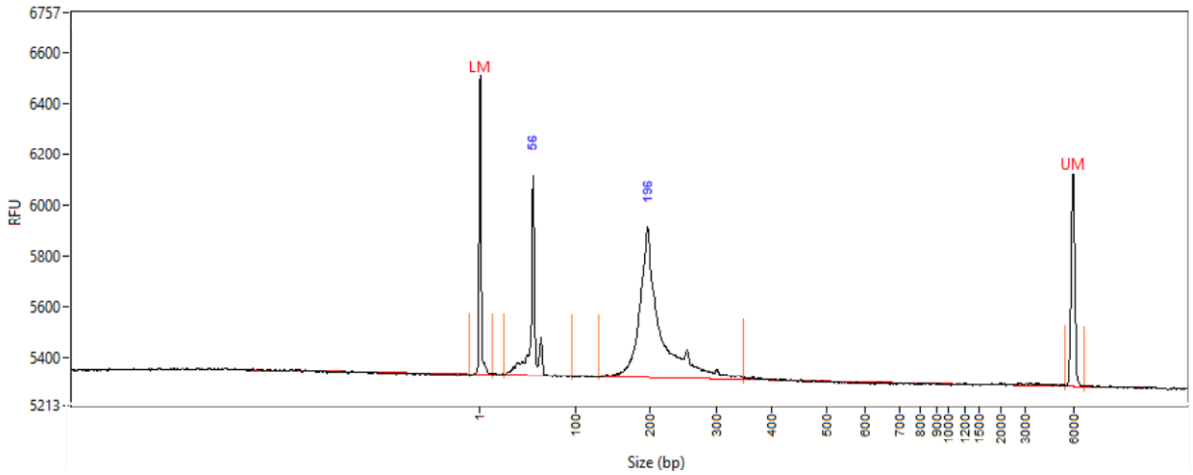


***Figure 8.*** *Electropherogram of a D-Plex cell-free RNA library prepared from 0.5 mL of platelet-free plasma (PFP).*

### Platelet-Poor Plasma (PPP) Library

Based on the recommended amplification, libraries prepared from PPP will typically reach concentrations between 3 and 5 nM when measured on the Fragment Analyzer using the 150–1000 bp gated region.

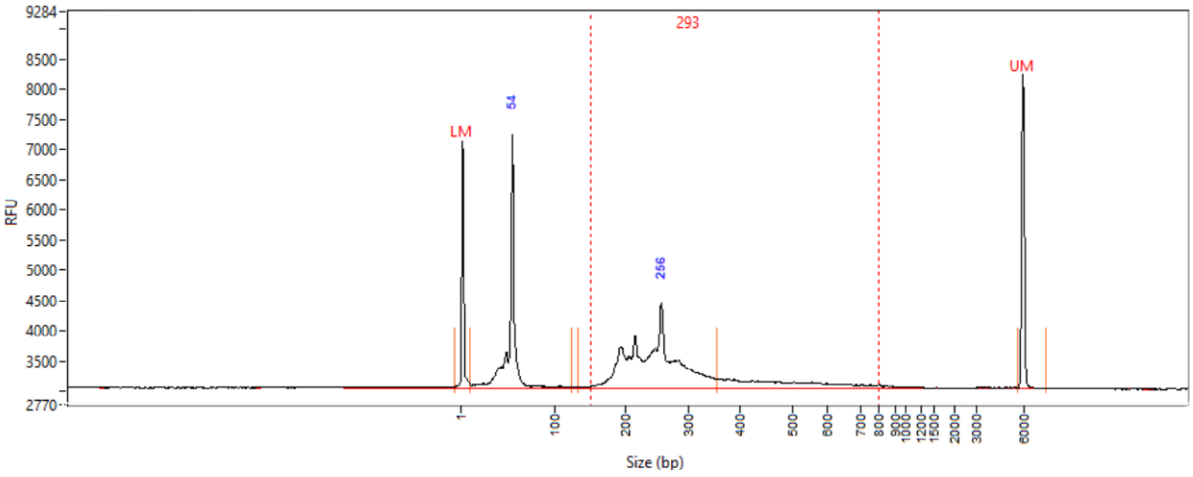


***Figure 9.*** *Electropherogram of a D-Plex cell-free RNA library prepared from 0.5 mL of platelet-poor plasma (PPP).*

A clear difference in library profile is observed between Figures 8 and 9, corresponding to the different plasma types used (platelet-free versus platelet-poor). These differences are consistent with the initial cell-free RNA electropherograms shown in Figures 4 and 5. This observation supports the conclusion that D-Plex cell-free RNA-seq library preparation faithfully reflects the original cell-free RNA content of the input sample.

# D-Plex cell-free RNA Construct

Figure 10 illustrates the D-Plex cell-free RNA-seq library construct after completion of the library preparation. Read 1 sequences the Unique Molecular Identifier (UMI; 12 cycles), followed by the Template Switch Motif (TSM; 3 cycles) before reading into the library insert. In many cases, Read 1 will extend beyond the insert length and may therefore sequence part of the poly(A) tail in some clusters. This does not pose a problem, as specific trimming commands have been implemented to remove all library construct-derived sequences before alignment to the reference genome.


***Figure 10.*** *D-Plex cell-free RNA-seq library construct after preparation.*

**Legend:**

- **P5/P7 =** TruSeq HT Illumina adapter sequences
- **UMI =** Unique Molecular Identifier (12 bp)
- **TSM =** template switch motif (3–4 bp)
- **ILMN R1 =** Illumina Read 1 sequencing primer and i5 sequencing primer hybridization location
- **ILMN R2 =** Illumina Read 2 sequencing primer and i7 sequencing primer hybridization location
- **i5/i7 =** dual indexes (8 bp each)
- **A-tail =** artificial poly(A) tail synthesized during the D-Plex cell-free RNA library preparation (20 bp)

# Sequencing Recommendations

The cell-free RNA-seq method was developed and validated using Illumina NovaSeq 6000 and NovaSeq X instruments. Consequently, the recommendations below are directly applicable only to these platforms. When using other sequencing systems, users are advised to consult their sequencing provider regarding PhiX spike-in percentages and flow cell loading concentrations, considering the guidelines outlined below.

| **Sequencing Paramenters for the D-Plex cfRNA-seq Libraries** | |
|---|---|
| **Read Length** | Minimum 75 cycles<br>Recommendation: 100 to 150 cycles |
| **Number of Reads** | 50 million reads per sample |
| **Validated Sequencing Chemistry** | NovaSeq 6000 chemistry v1.5<br>NovaSeq X |
| **Sequencing Mode** | • **Single-end:** It is the recommended sequencing mode, as the entire length of the library insert will be completely covered by Read 1 of 100 to 150 cycles.<br>• **Paired-end:** Paired-end sequencing is discouraged and unnecessary for D-Plex cell-free RNA libraries. Read 2 initiates sequencing of the 20-base-long poly(T) homopolymer tail located at the 5′ end of the reverse DNA strand. This can lead to reduced base-calling quality for that segment and may negatively impact the accuracy of downstream base calling. If paired-end sequencing is set for a given experiment, it is strongly recommended to combine D-Plex cell-free RNA libraries with balanced libraries using compatible indexes. |
| **PhiX Spike-In Proportion** | • NovaSeq 6000: 10–20% PhiX spiked into the library pool<br>• NovaSeq X: 20% PhiX spiked into the library pool |
| **Final Library Pool Clean-Up Before Sequencing (after excess primer digestion/blocking)** | 1.5x AMPure beads-to-sample ratio |
| **Indexing (Barcoding)** | Unique Dual Indexing<br>Index length: 2 × 8 bp (i5/i7) |
| **Loading Concentration** | Library loading on NovaSeq 6000 and NovaSeq X = 0.7 nM |
| **Adapter Sequences** | TruSeq HT |
| **Flow Cell Type** | SP, S1, S2, S4 on NovaSeq 6000<br>10B on NovaSeq X |

# Unique Dual Index Sequences

The D-Plex cell-free RNA method produces unique dual-indexed (UDI) libraries compatible with Illumina sequencing platforms. The index sequences listed in Table 4 cover the two distinct index-reading workflows used across Illumina platforms. While the i7 index is read in the same orientation on all Illumina sequencers, the i5 index is read in different orientations depending on the instrument model and sequencing chemistry.

The i5 index sequence is read in the forward orientation on MiSeq. In contrast, the i5 index sequence is read in the reverse-complement orientation on the iSeq 100, NextSeq 500/550, NextSeq 1000/2000, NovaSeq 6000 (v1.5 reagents), and NovaSeq X/X Plus.

During demultiplexing, the sample sheet provided to bcl2fastq for FASTQ generation must have the i5 index in the correct orientation for the platform used. However, most Illumina sample sheet–generation software handles this automatically. These tools assume that the i5 index sequence is always provided in the forward orientation and will automatically generate the reverse complement when required, based on the selected instrument type. More information can be found on: https://help.connected.illumina.com/run-set-up/overview/index-orientation-guide.

**Table 4.** UDI sequences used in the D-Plex cell-free RNA library preparation method.

| **Unique Dual Index Name** | **i5 barcode forward orientation** | **i5 barcode reverse complement orientation i7 barcode** | **i7 barcode** |
|---|---|---|---|
| D-Plex CF UDI#1 | AGCGCTAG | CTAGCGCT | CCGCGGTT |
| D-Plex CF UDI#2 | GATATCGA | TCGATATC | TTATAACC |
| D-Plex CF UDI#3 | CGCAGACG | CGTCTGCG | GGACTTGG |
| D-Plex CF UDI#4 | TATGAGTA | TACTCATA | AAGTCCAA |
| D-Plex CF UDI#5 | AGGTGCGT | ACGCACCT | ATCCACTG |
| D-Plex CF UDI#6 | GAACATAC | GTATGTTC | GCTTGTCA |
| D-Plex CF UDI#7 | ACATAGCG | CGCTATGT | CAAGCTAG |
| D-Plex CF UDI#8 | GTGCGATA | TATCGCAC | TGGATCGA |
| D-Plex CF UDI#9 | CCAACAGA | TCTGTTGG | AGTTCAGG |
| D-Plex CF UDI#10 | TTGGTGAG | CTCACCAA | GACCTGAA |
| D-Plex CF UDI#11 | CGCGGTTC | GAACCGCG | TCTCTACT |
| D-Plex CF UDI#12 | TATAACCT | AGGTTATA | CTCTCGTC |
| D-Plex CF UDI#13 | AAGGATGA | TCATCCTT | CCAAGTCT |
| D-Plex CF UDI#14 | GGAAGCAG | CTGCTTCC | TTGGACTC |
| D-Plex CF UDI#15 | TCGTGACC | GGTCACGA | GGCTTAAG |

| Unique Dual Index Name | i5 barcode forward orientation | i5 barcode reverse complement orientation i7 barcode | i7 barcode |
|---|---|---|---|
| D-Plex CF UDI#16 | CTACAGTT | AACTGTAG | AATCCGGA |
| D-Plex CF UDI#17 | ATATTCAC | GTGAATAT | TAATACAG |
| D-Plex CF UDI#18 | GCGCCTGT | ACAGGCGC | CGGCGTGA |
| D-Plex CF UDI#19 | ACTCTATG | CATAGAGT | ATGTAAGT |
| D-Plex CF UDI#20 | GTCTCGCA | TGCGAGAC | GCACGGAC |
| D-Plex CF UDI#21 | AAGACGTC | GACGTCTT | GGTACCTT |
| D-Plex CF UDI#22 | GGAGTACT | AGTACTCC | AACGTTCC |
| D-Plex CF UDI#23 | ACCGGCCA | TGGCCGGT | GCAGAATT |
| D-Plex CF UDI#24 | GTTAATTG | CAATTAAC | ATGAGGCC |
| D-Plex CF UDI#25 | AACCGCGG | CCGCGGTT | ACTAAGAT |
| D-Plex CF UDI#26 | GGTTATAA | TTATAACC | GTCGGAGC |
| D-Plex CF UDI#27 | CCAAGTCC | GGACTTGG | CTTGGTAT |
| D-Plex CF UDI#28 | TTGGACTT | AAGTCCAA | TCCAACGC |
| D-Plex CF UDI#29 | CAGTGGAT | ATCCACTG | CCGTGAAG |
| D-Plex CF UDI#30 | TGACAAGC | GCTTGTCA | TTACAGGA |
| D-Plex CF UDI#31 | CTAGCTTG | CAAGCTAG | GGCATTCT |
| D-Plex CF UDI#32 | TCGATCCA | TGGATCGA | AATGCCTC |
| D-Plex CF UDI#33 | CCTGAACT | AGTTCAGG | TACCGAGG |
| D-Plex CF UDI#34 | TTCAGGTC | GACCTGAA | CGTTAGAA |
| D-Plex CF UDI#35 | AGTAGAGA | TCTCTACT | AGCCTCAT |
| D-Plex CF UDI#36 | GACGAGAG | CTCTCGTC | GATTCTGC |
| D-Plex CF UDI#37 | AGACTTGG | CCAAGTCT | TCGTAGTG |
| D-Plex CF UDI#38 | GAGTCCAA | TTGGACTC | CTACGACA |
| D-Plex CF UDI#39 | CTTAAGCC | GGCTTAAG | TAAGTGGT |
| D-Plex CF UDI#40 | TCCGGATT | AATCCGGA | CGGACAAC |
| D-Plex CF UDI#41 | CTGTATTA | TAATACAG | ATATGGAT |
| D-Plex CF UDI#42 | TCACGCCG | CGGCGTGA | GCGCAAGC |
| D-Plex CF UDI#43 | ACTTACAT | ATGTAAGT | AAGATACT |
| D-Plex CF UDI#44 | GTCCGTGC | GCACGGAC | GGAGCGTC |
| D-Plex CF UDI#45 | AAGGTACC | GGTACCTT | ATGGCATG |
| D-Plex CF UDI#46 | GGAACGTT | AACGTTCC | GCAATGCA |
| D-Plex CF UDI#47 | AATTCTGC | GCAGAATT | GTTCCAAT |
| D-Plex CF UDI#48 | GGCCTCAT | ATGAGGCC | ACCTTGGC |

# Data Analysis Recommendations

This section provides an overview of the trimming, alignment, and feature quantification (counting) processes, and includes an optional but recommended UMI-processing workflow, using software tools and parameter settings that have been validated (Figure 11). While alternative software tools may be used, users are responsible for selecting and optimizing appropriate parameters to ensure equivalent data quality and performance.

In the example commands provided, it is assumed that the required software tools have been downloaded from the links listed at the end of this section, have been installed, and are available in the system PATH.

Bioinformatics analysis is also available as a service, with both Standard and Advanced options. For more information, please contact us (www.diagenode.com).

**MAIN ANALYSIS STEPS**
**OPTIONAL**
**QUALITY CONTROL**


***Figure 11.*** *Overview of the bioinformatics pipeline for D-Plex cell-free RNA-seq data analysis.*

### UMI Extraction

To enable UMI-based deduplication at later stages of the analysis, the UMI sequence must first be copied from the read sequence to the read identifier. In D-Plex cell-free RNA-seq, the first 12 bases at the 5′ end of Read 1 correspond to the UMI (see Figure 10).

Within the **fumitools** package, the **copy_umi** command is used to extract these bases and append them to the appropriate fields in the FASTQ read header. The command accepts a FASTQ file as input, which may be either compressed (.gz) or uncompressed, and produces a FASTQ output file in either format depending on the specified output filename extension.

In addition to the input and output files, the command requires one mandatory parameter specifying the UMI length (i.e., the number of bases to copy from the read sequence to the read ID). Optionally, computational performance can be improved by increasing the number of CPU threads via the --threads parameter.

The example command below demonstrates the processing of single-end reads using all 12 UMI bases (recommended) and 4 CPU threads.

```
fumi_tools copy_umi --threads 4 \
    --umi-length 12 \
    -i Sample1_R1.fastq.gz \
    -o Sample1_with_UMI_R1.fastq.gz
```

***UMI processing is strongly recommended but optional.*** *If UMI information is not required, this step may be safely omitted. In this case, UMI sequences will be removed from the reads during the trimming step and ignored for the remainder of the analysis, without affecting the rest of the pipeline.*

**Trimming**

In addition to the 12 nt UMI, D-Plex cell-free RNA-seq libraries contain a template switch motif (TSM) of 3–4 nucleotides and a poly(A) tail (Figure 10), which requires specialized trimming procedures to ensure optimal data processing.

The command below uses **Cutadapt** to remove these library-derived artifacts. Specifically, the command:

- removes the fixed 5′ UMI/TSM segment (-u)
- trims 3′ poly(A) sequence and adapter/motif remnants (-a ...)
- performs up to four sequential trimming rounds per read (-n 4)
- discards reads shorter than 17 bases after trimming (-m 17)

The trimming command can be applied either to the raw input files (Sample1_R1.fastq.gz) generated directly by the sequencer, or to the UMI-preprocessed files (Sample1_with_UMI_R1.fastq.gz) when UMI information is retained.

```
cutadapt --cores 4 \
    --trim-n --match-read-wildcards -u 16 -n 4 \
    -a AGATCGGAAGAGCACACGTCTG -a AAAAAAAA -a GAACTCCAGTCAC \
    -e 0.2 --nextseq-trim 20 -m 17 \
    -o Sample1_R1_trimmed.fastq.gz \
    <Sample1_R1.fastq.gz | Sample1_with_UMI.fastq.gz>
```

*Explanation of the key parameters:*

- ***-u 16:*** *Unconditionally removes 16 bases from the 5' end of every read, corresponding to the 12nt UMI and the template switch motif (TSM; 3–4 nt, depending on the library construct). Using -u 16 ensures complete removal of the UMI and TSM across all constructs.*
- ***-n 4:*** *Performs up to four sequential adapter-trimming rounds per read, enabling removal of multiple 3' sequences from the same read (e.g., Illumina adapter, poly(A), and additional adapter/motif remnants). By default (-n 1), Cutadapt trims at most one match and then stops after removal of the first matching adapter.*
- ***-m 17:*** *Discards reads shorter than 17 bp after all trimming steps, as these are considered too short for reliable downstream analysis.*

**Quality Control**

We recommend running **FastQC** both **before and after trimming** to assess overall read quality, detect sequencing issues, and confirm that trimming successfully removed the UMI, TSM sequence, poly(A) tail, adapter sequences, and low-quality bases.

Below are example FastQC commands applied to a sample FASTQ file, both before and after trimming.

**Before trimming:**

```
fastqc --noextract --nogroup \
Sample1_R1.fastq.gz \
-o QC/
```

**After trimming:**

```
fastqc --noextract --nogroup \
Sample1_R1_trimmed.fastq.gz \
-o QC/
```

FastQC reports can be inspected individually or summarized with MultiQC.

Representative examples of the “Per base sequence content” of a D-Plex cell-free RNA-seq library before and after trimming - illustrating the successful removal of UMI, TSM, and poly(A) tail - are shown in Figures 12 and 13, respectively. Additionally, the “Adapter content” section of the FastQC report can be checked to confirm successful removal of adapter sequences.

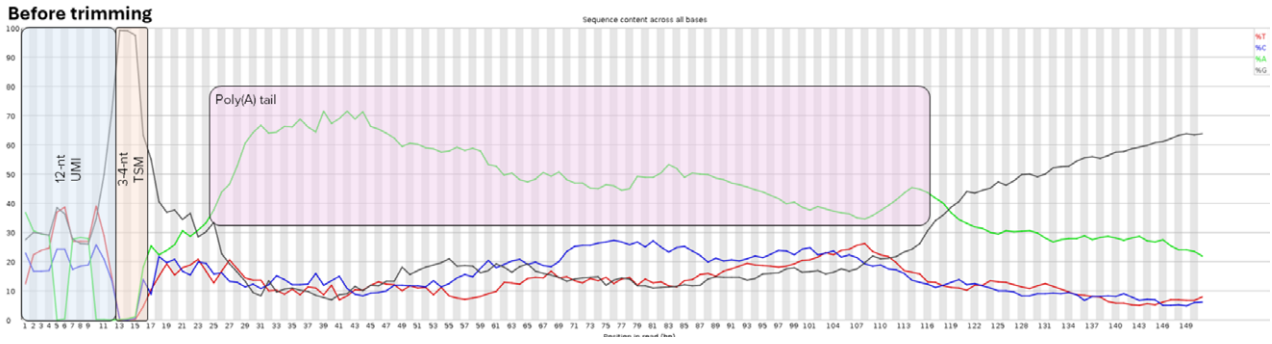


***Figure 12.*** *Representative example of “Per Base Sequence Content” of a D-Plex cell-free RNA-seq library before trimming, as reported by FastQC.*

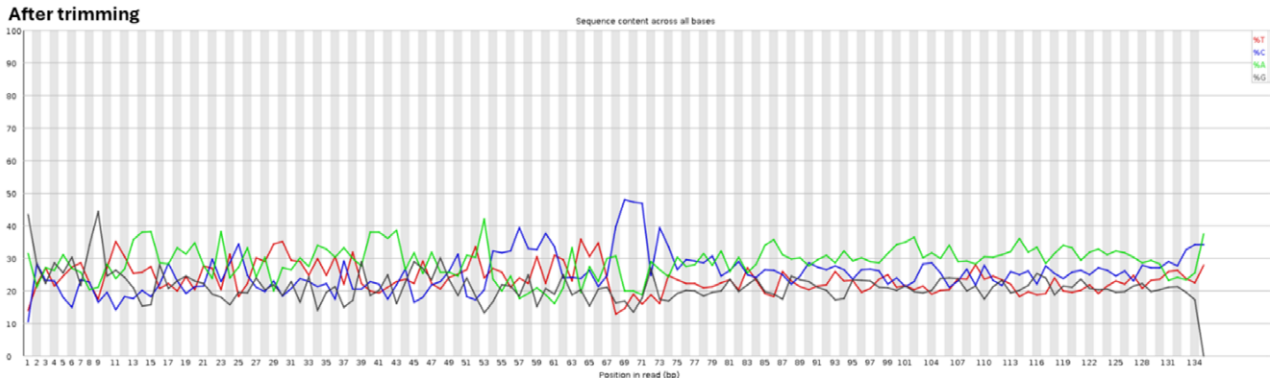


***Figure 13.*** *Representative example of ‘Per Base Sequence Content’ of a D-Plex cell-free RNA-seq library after trimming, as reported by FastQC.*

**Alignment**

Trimmed reads generated from the D-Plex cell-free RNA-seq workflow can be aligned using any standard RNA-seq aligner. No library-specific handling is required at the alignment step. However, **alignment to the reference genome is strongly recommended rather than alignment to a transcriptome-only reference**.

D-Plex cell-free RNA-seq libraries typically exhibit high complexity and frequently contain short or unannotated RNA fragments. As a result, a substantial fraction of reads may not correspond to currently annotated transcripts and would therefore fail to align when using a transcriptome-only reference. Genome-based alignment ensures that such reads are retained and enables comprehensive downstream analysis.

For genome alignment, we recommend the use of **STAR**, a widely adopted and high-performance RNA-seq aligner. To run STAR, the following inputs and parameters are required:

- the trimmed FASTQ file
- a directory containing the pre-indexed reference genome (see the STAR user guide for genome index generation)*

** The STAR genome index is generated without a gene annotation (GTF). This annotation-agnostic indexing strategy is intentional, as D-Plex cell-free RNAseq libraries frequently contain short or unannotated RNA fragments.*

The example command below illustrates STAR alignment for a **human sample** on the **hg38 reference genome**, assuming the use of 4 CPU threads. For datasets generated from non-human samples, the corresponding reference genome (-genomeDir) should be adjusted accordingly.

```
STAR --runThreadN 4 \
    --genomeDir /genomes/hg38 \
    --outFilterMultimapScoreRange 0 \
    --outFilterMultimapNmax 50 \
    --outFilterMismatchNoverLmax 0.05 \
    --outFilterMatchNmin 17 \
    --outFilterScoreMinOverLread 0 \
    --outFilterMatchNminOverLread 0 \
    --alignIntronMax 1 \
    --readFilesCommand zcat\
    --readFilesIn Sample1_R1_trimmed.fastq.gz \
    --outSAMtype BAM SortedByCoordinate \
    --outSAMunmapped Within \
    --outFileNamePrefix ./alignment/Sample1_
```

***Note:*** *STAR is configured to retain reads that map to multiple genomic locations (--outFilterMultimapNmax 50). This is intentional, as downstream read counting is*

*performed using MGcount, a software capable of appropriately handling multimapped reads. Retaining multimappers at the alignment stage ensures maximal data retention and flexibility for downstream analysis. This approach is particularly relevant for cell-free RNA-seq data, where short fragments and repetitive regions increase the prevalence of multimapping reads.*

After the alignment process is completed, the resulting sorted BAM file should be indexed to enable efficient access by downstream tools (e.g., for quantification, visualization, or QC). This can be done using **samtools index** as shown below:

```
samtools index ./alignment/Sample1_Aligned.sortedByCoord.out.bam
```

### UMI-Based Deduplication

UMIs allow the distinction between identical reads originating from different RNA molecules and identical reads resulting from PCR amplification. When UMI-based deduplication is performed, **it is essential that UMI extraction has been applied before trimming**, so that UMI sequences are preserved and correctly associated with each read.

Below is an example illustrating how to remove PCR duplicates from genome-aligned reads using the dedup function of **fumi-tools**. Deduplication is performed based on alignment coordinates and UMI sequences. Because STAR produces a **coordinate-sorted BAM file** when using the option --outSAMtype BAM SortedByCoordinate, no additional sorting is required before performing UMI deduplication. The computational resources used by fumi-tools can be adjusted via the --threads (number of CPUs) and --memory parameters.

```
fumi_tools dedup --memory 10G --threads 4 \
    -i ./alignment/Sample1_Aligned.sortedByCoord.out.bam \
    -o ./alignment/Sample1_name_sorted_dedup.bam
```

fumi-tools outputs a **name-sorted** BAM file after deduplication. Most counting software packages require a **coordinate-sorted BAM** as input. Therefore, the deduplicated BAM file must be coordintes-sorted before downstream quantification.

```
samtools sort --threads 4 \
    -o ./alignment/Sample1_dedup.bam \
    ./alignment/Sample1_name_sorted_dedup.bam
```

***Tip:*** *For convenience and improved performance, deduplication and coordinate sorting can be performed in a single step by piping the output of fumi_tools dedup directly into samtools sort. In this configuration, fumi-tools writes the deduplicated, name-sorted BAM stream to standard output (-), which is immediately consumed by samtools sort. This approach avoids generating an intermediate name-sorted BAM file and may reduce disk usage and runtime.*

```
fumi_tools dedup --memory 10G --threads 4 \
    -i ./alignment/Sample1_Aligned.sortedByCoord.out.bam \
    -o - | samtools sort --threads 4 \
    -o ./alignment/Sample1_dedup.bam
```

After deduplication, the coordinate-sorted BAM file should be indexed to allow efficient random access by counting tools, QC software, and genome browsers. This can be done using **samtools index** as shown below:

```
samtools index ./alignment/Sample1_dedup.bam
```

**Feature Quantification – Counting**

The final step of the workflow is the quantification of transcript abundance to generate an expression matrix. For cell-free RNA-seq data, we recommend using **MGcount**, a counting tool developed at Hologic Diagenode, together with an annotation file that includes the broad range of RNA biotypes typically present in cell-free RNA. Cell-free RNA libraries are inherently heterogeneous, containing mixtures of short RNA fragments, partially degraded mRNAs, non-coding RNAs of various classes, and other transcript remnants. Many of these molecules may overlap within the genome or occur in high copy number, which complicates straightforward read assignment.

Reads derived from cell-free RNA frequently map:

- to multiple genomic locations (due to repeated sequences or short fragments)
- to regions annotated with multiple transcript types
- to unannotated loci.

To address these challenges, MGcount implements a flexible quantification strategy built on top of featureCounts and tailored for complex, multi-biotype datasets such as cell-free RNA-seq. MGcount employs two complementary methods:

**1. Hierarchical read assignment:**

Reads are assigned in prioritized rounds, giving precedence to shorter and/or highly specific RNA biotypes before assigning reads to longer transcripts. This prevents overattribution to host genes when reads originate from embedded or overlapping non-coding RNAs, and ensures unbiased quantification across the diverse RNA fragments present in cell-free RNA.

**2. Graph-based locus collapsing:**

Genomic loci where reads consistently multi-map are grouped into *communities* using a graphbased clustering approach. Each community represents a set of related loci with nearly identical sequences. Quantification is then performed at the community level, reducing ambiguity caused by multi-mapping while preserving biological signal.

Together, these approaches maximize the amount of information recovered from cell-free RNA-seq libraries and improve the quantification of both coding and non-coding RNA fragments.

MGcount requires:

- a text file listing **full paths to the input BAM files**
- a **GTF annotation** file
- the **output directory** location

Most parameters in the MGcount example command below correspond to MGcount's default settings; however, they are included here for clarity and completeness:

```
MGcount --gtf hg38_annotation_file.gtf \
    --outdir ./matrix/ \
    --featureCounts_path /path/to/featureCounts \
    -T 4 \
    --min_overlap_small 0.7 \
    --min_overlap_long 0.7 \
    --feature_small transcript \
    --feature_biotype_long gene_biotype \
    --feature_biotype_small transcript_biotype \
    --feature_output_long gene_name \
    --feature_output_small transcript_name \
    --strand_option 1 \
    --ml_flag_small 1 \
    --ml_flag_long 1 \
    --bam_infiles ./alignment_infiles.txt
```

**Notes:**

1. Comprehensive annotation files for ***Homo sapiens***, ***Mus musculus***, ***Arabidopsis thaliana***, and ***Caenorhabditis elegans*** are provided in the MGcount repository.
2. If UMI extraction and deduplication were not performed, use the coordinate-sorted BAM produced by STAR (Sample1_Aligned.sortedByCoord.out.bam) as input to MGcount. If UMI deduplication was performed, use the coordinate-sorted deduplicated BAM (Sample1_dedup.bam).
3. MGcount allows enabling or disabling the processing of RNA biotype communities via:
   - --ml_flag_small (short/small RNA–dominated loci)
   - --ml_flag_long (long RNA–dominated loci)
   - Both flags are enabled by default.
4. D-Plex generates forward-stranded libraries. In a stranded protocol, sequencing reads map to the same genomic strand as the RNA molecule from which they

originate. This information helps to correctly distinguish overlapping or antisense transcripts.

For expression quantification, reads aligned to the forward (sense) strand should only be assigned to forward-strand transcript annotations, and reads aligned to the reverse (antisense) strand should only be assigned to reverse-strand annotations. MGcount uses forward-stranded mode (--strand_option 1) by default, which is the appropriate setting for D-Plex cell-free RNA-seq data.

The **primary output** of MGcount is the file **count_matrix.csv**, which is the expression matrix generated at the locus-community or transcript level, depending on the annotation and quantification options selected. This matrix is formatted as a standard table of features (rows) by samples (columns) and can be readily imported into **R**, Python, or any downstream data analysis environment.

For additional details on MGcount parameters, use and output files, please refer to the complete MGcount User Guide: https://filedn.com/lTnUWxFTA93JTyX3Hvbdn2h/mgcount/UserGuide.html.

### Tools References in this Pipeline

| Tool | Version | Link |
|---|---|---|
| FastQC | 0.11.9 | https://github.com/s-andrews/FastQC |
| fumi-tools | 0.17.0 | https://ccb-gitlab.cs.uni-saarland.de/tobias/fumi_tools/releases |
| cutadapt | 5.0 | https://github.com/marcelm/cutadapt |
| STAR | 2.7.9a | https://github.com/alexdobin/STAR |
| samtools | 1.15 | http://www.htslib.org/download |
| MGcount | 1.1.0 | https://github.com/hitaandrea/MGcount |
| featureCounts | 2.0.3 | http://subread.sourceforge.net |

# Examples of Results

Examples of typical results generated with the D-Plex cell-free RNA-seq kit following the guidelines described in this manual are displayed below. The factors that most strongly influence data quality are pre-analytical variables associated with blood collection, plasma preparation, cell-free RNA isolation, and storage. **It is paramount that these factors are documented, understood, and, where possible, controlled to ensure reliable and reproducible results.**

As illustrated in Figure 14, plasma preparation (platelet-poor versus platelet-free plasma) has a substantial impact on the resulting library composition. This observation demonstrates that the D-Plex cell-free RNA-seq method faithfully reflects the original cell-free RNA content of the input sample.

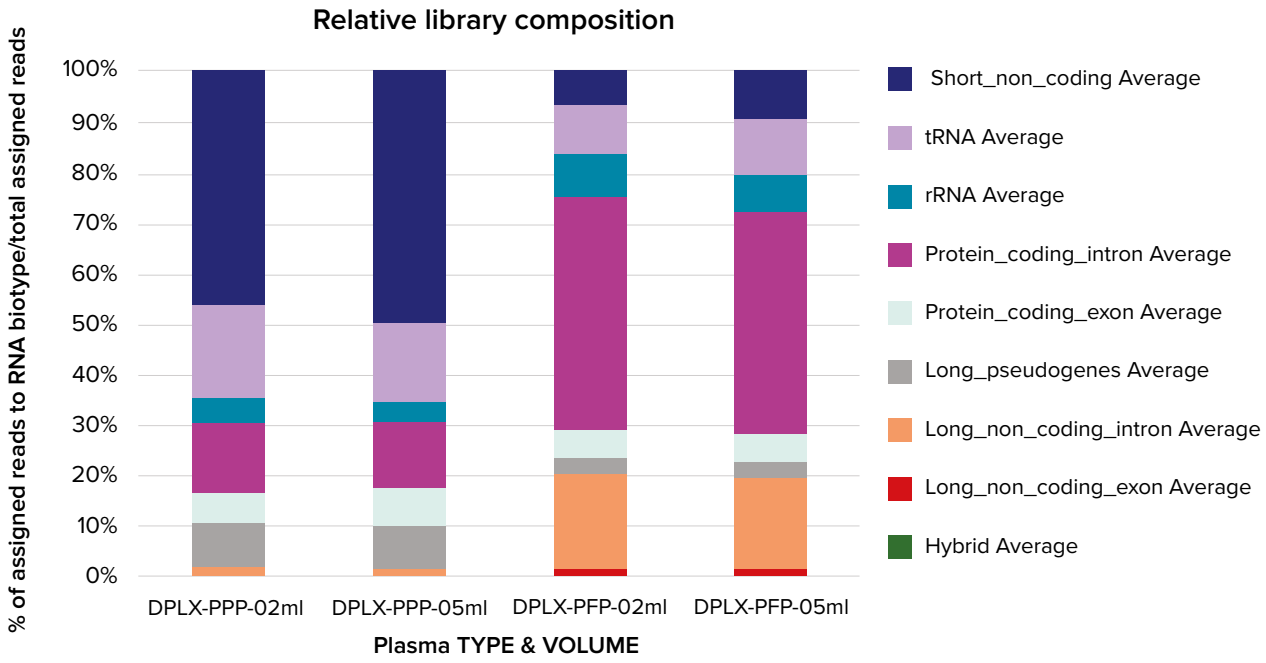


***Figure 14.*** *Relative library composition for 0.5 mL and 0.2 mL of platelet-poor plasma (PPP) and platelet-free plasma (PFP) using the D-Plex cell-free RNA-seq kit.*

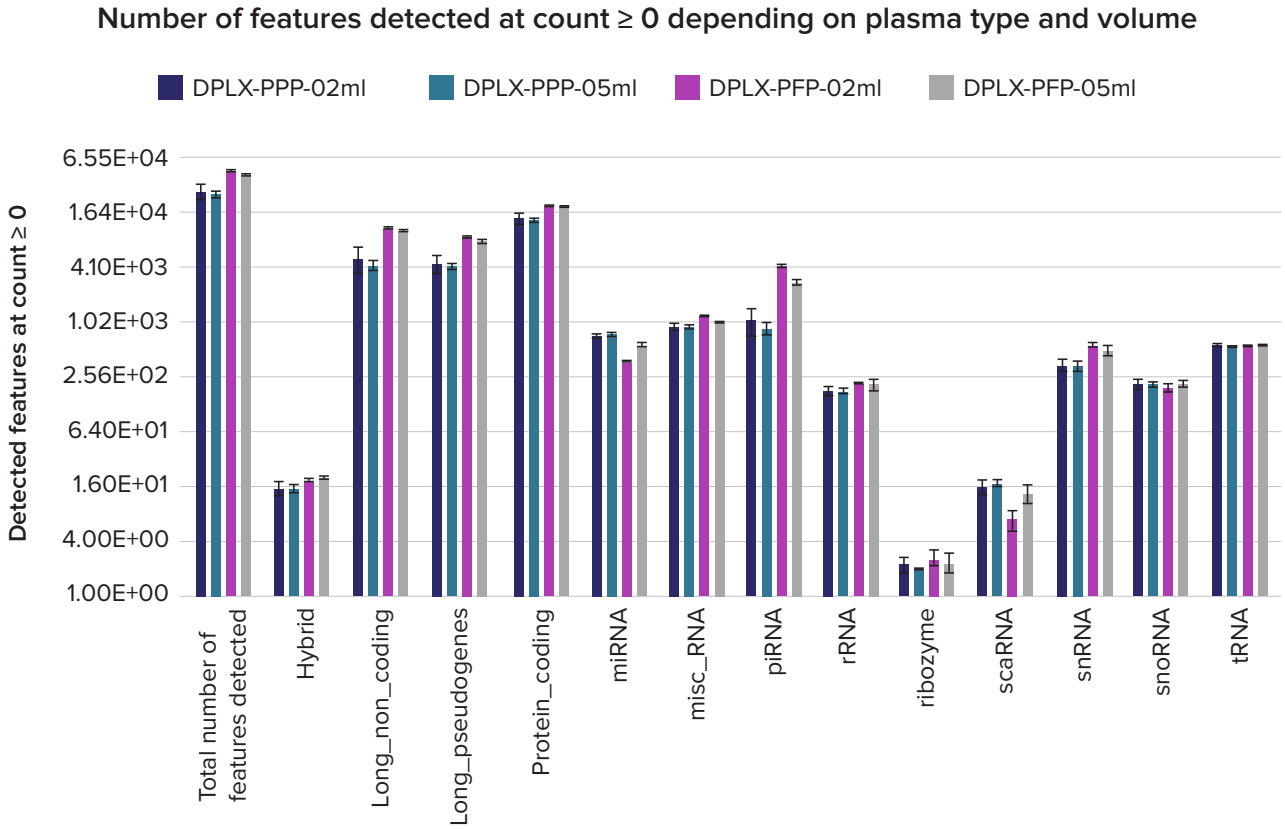


***Figure 15.*** *Average number of detected features at counts above 0 for 0.5 mL and 0.2 mL of platelet-poor plasma (PPP) and platelet-free plasma (PFP) using the D-Plex cell-free RNA-seq kit.*

# Related Products

| Product | Cat. No. |
|---|---|
| D-Plex UDI for cell-free RNA-seq - Set A (24 rxns) | C05030045 |
| D-Plex UDI for cell-free RNA-seq - Set A (24 rxns) | C05030046 |
| DiaMag 0.2ml tube magnetic rack | B04000001 |
| Bioinformatics Analysis - Services | Contact us |

## Revision history

| Version | Date of modification | Description of modifications |
|---|---|---|
| Version 1  04_2026 | April 2026 | Manual Creation |

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