



duet 6-base mosaic User guide

Laboratory instructions

For the preparation of NGS-ready duet constructs for sequencing on Illumina NGS platforms.

Suitable for use with 8, 24 and 96 reaction kits.

 This user guide is specifically compatible with **Product Numbers: 6301-2 (8 reactions), 6302-2 (24 reactions) and 6303-2 (96 reactions)**. If you have a different product number, please refer to the appropriate guide or contact biomodal support for assistance.

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For Research Use Only. Not for use in diagnostic procedures.



duet 6-base mosaic User guide

This protocol is for the use of the duet 6-base mosaic kit.

Suitable for use with 8, 24 and 96 reaction kits.

Table of Contents

Table of Contents.....	1
A. Introduction.....	2
B. Kit Method Overview.....	3
C. Kit Information.....	4
1. Reagents Provided.....	4
2. Required Equipment and Reagents Not Provided.....	6
D. Experimental Protocol.....	7
1. Before Starting.....	7
2. DNA Sample Input Preparation.....	9
3. DNA Denaturation Step.....	11
4. Tailing and Hairpin Adapter Ligation.....	12
5. Bead-Mediated Purification 1 (with Isopropyl Alcohol).....	13
6. Strand Copying.....	15
7. Forkhead Adapter Ligation.....	16
8. Bead-Mediated Purification 2.....	17
9. Copy Methylation.....	19
10. Bead-Mediated Purification 3.....	20
11. Oxidation – Protection of Modified Cytosines.....	22
12. Bead-Mediated Purification 4.....	25
13. Deamination of Cytosines.....	27
14. Library Amplification.....	28
15. Bead-Mediated Purification 5.....	29
16. Library Quantification.....	31
E. Sequencing.....	33
1. Set-up.....	33
2. Index Sequences.....	33
F. Revision History	34

A. Introduction

The duet 6-base mosaic solution provides complete genetic information whilst also discriminating between the epigenetic modifications 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC), and is specifically optimized for the characteristics and technical requirements of cfDNA. The duet 6-base mosaic kit contains the necessary reagents for quantitative, site-specific conversion of unmodified cytosine bases. This enables the user to obtain single base resolution 6-base sequencing of human cell-free DNA into a single workflow using a standard Illumina NGS platform.

B. Kit Method Overview

The duet 6-base mosaic solution comprises 8 key steps, shown in Figure 1. A duet mosaic construct is formed by DNA denaturation, tailing and hairpin adapter ligation, strand copying and forkhead adapter ligation. The duet construct then undergoes copy methylation, oxidation, deamination, and PCR amplification to add unique dual indexes (UDIs) and NGS sequencing adapters. The final duet construct is then ready for NGS on Illumina Platforms.

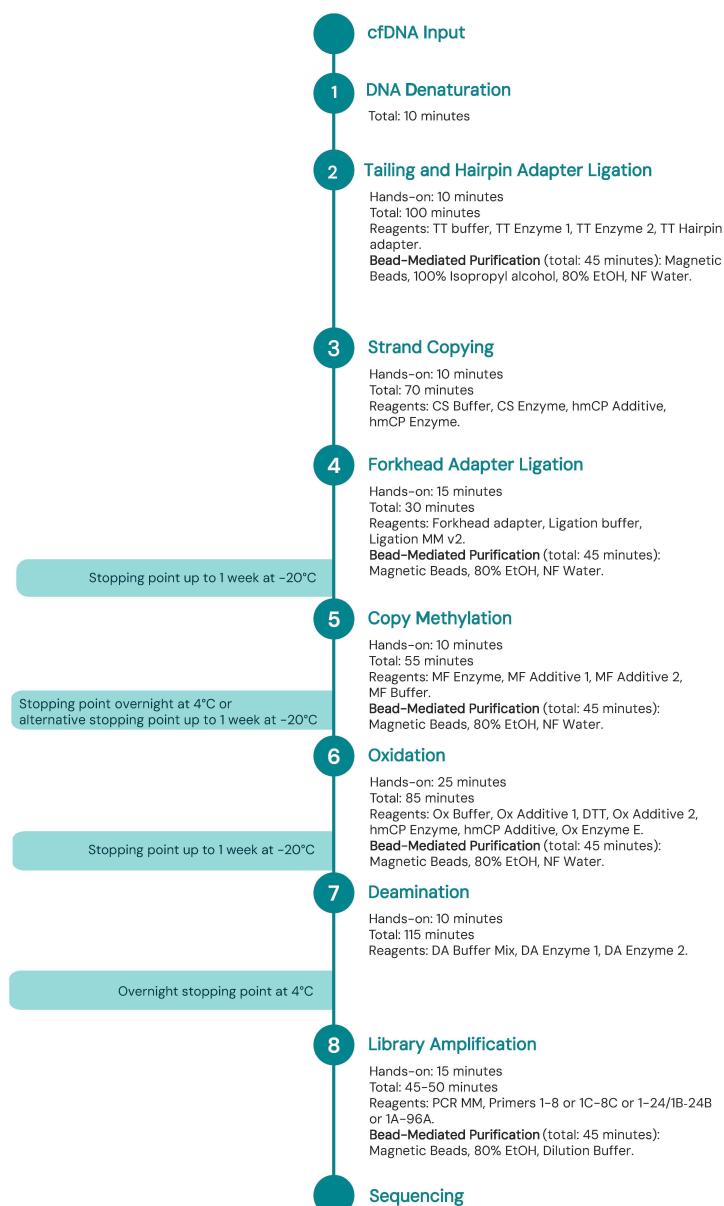


Figure 1. duet 6-base mosaic workflow

C. Kit Information

1. Reagents Provided

duet 6-base mosaic 6301-2 (8RXN) and 6302-2 (24RXN) and 6303-2 (96RXN).

Frozen box (Qty1)

 **Note:** if using the 96RXN kit, it is recommended to perform a minimum of 24 reactions at a time.

Product Name	8RXN Volume (μ L)	24RXN Volume (μ L)	96RXN Volume (μ L)	Lid colour	Store at
Control DNA (CON)	16.0	55.0	191.0 ¹	White	-20°C
TT Buffer (TTB)	951.0	951.0	951.0 ¹	Yellow	-20°C
TT Hairpin Adapter (TTH)	10.0	25.0	68.0 ¹	Yellow	-20°C
TT Enzyme 1 (TTE1)	10.0	25.0	68.0 ¹	Yellow	-20°C
TT Enzyme 2 (TTE2)	22.0	68.0	254.0 ¹	Yellow	-20°C
CS Buffer (CSB)	27.0	83.0	317.0 ¹	Blue	-20°C
CS Enzyme (CSE)	53.0	159.0	634.0 ¹	Blue	-20°C
MF Buffer (MFB)	117.0	349.0	1394.0 ¹	Pink	-20°C
MF Additive 1 (MFA1)	16.0	25.0	68.0 ¹	Pink	-20°C
MF Additive 2 (MFA2)	16.0	54.0	191.0 ¹	Pink	-20°C
MF Enzyme (MFE)	22.0	68.0	254.0 ¹	Pink	-20°C
Forkhead Adapter (FA)	27.0	83.0	317.0 ¹	Purple	-20°C
Ligation Buffer (FLB)	106.0	317.0	1268.0 ¹	Purple	-20°C
Ligation MM v2 (FLMM)	37.0	111.0	444.0 ¹	Purple	-20°C
Ox Buffer (OxB)	500.0	1600.0	1600.0 ²	Brown	-20°C
Ox Additive 1 (OxA1)	Powder (0.584 mg) – 1 tube	Powder (0.584 mg) – 3 tubes	Powder (0.584 mg) – 4 tubes	Brown	-20°C
Ox Additive 2 (OxA2)	127.0	127.0	127.0 ¹	Brown	-20°C
DTT (DTT)	127.0	127.0	127.0 ¹	Brown	-20°C
hmCP Additive (hmCPA)	52.0	111.0	444.0 ¹	Brown	-20°C

hmCP Enzyme (hmCPE)	22.0	64.0	254.0 ¹	Brown	-20°C
Ox Enzyme E (OxEE)	23.0	64.0	254.0 ¹	Brown	-20°C
DA Enzyme 1 (DAE1)	23.0	68.0	254.0 ¹	Red	-20°C
DA Enzyme 2 (DAE2)	27.0	80.0	317.0 ¹	Red	-20°C
DA Buffer Mix (DBM)	111.0	333.0	1331.0 ¹	Red	-20°C
PCR MM (PMM)	160.0	476.0	1901.0 ¹	Clear	-20°C
Dilution Buffer (DB)	1350.0	1350.0	1350.0 ¹	White	-20°C

¹ 1 tube provided, volumes in each tube are enough for 96 reactions

² 2 tubes provided, volumes in each tube are enough for 48 reactions

duet magnetic beads mosaic 8RXN 4001-1, 24RXN 4002-1 and 96 RXN 4003-1



Note: for kits ordered with magnetic beads only. If not, refer to the Required reagents not “provided” below.

Product Name	8RXN Volume (mL)	24RXN Volume (mL)	96RXN Volume (mL)	Lid colour	Store at
duet magnetic beads mosaic	2.65	8.0	8.0 (4 tubes)	White	15°C to 30°C

duet UDIs 1 – 8 4101 (Qty 1) or duet UDIs 1C–8C 4103 (Qty 1)

Product Name	8RXN Volume (µL)	Lid colour	Store at
duet UDIs 1–8 or 1C–8C*	5.0 of each	Clear	-20°C

duet UDIs 1 – 24/1B–24B 4102 (Qty 1)

Product Name	24RXN Volume (µL)	Lid colour	Store at
duet UDIs 1–24/1B–24B*	5.0 of each	Clear	-20°C

- * duet UDIs 1–8 (4101): identical to the first 8 UDIs in 4102
- * duet UDIs 1–24/1B–24B (4102): first 8 UDIs are identical to 4101
- * duet UDIs 1C–8C (4103): unique and differ from 4102

duet UDIs 1A – 96A 4104 (Qty 1)

Product Name	96RXN Volume (µL)	Lid colour	Store at
duet UDIs 1A–96A*	5.0 of each	N/A	-20°C

*duet UDIs 1A – 96A are supplied in a 96 well plate

Please note that the 96 UDI format is unique and differs from the UDIs 1–8/1C–8C and UDIs 1–24/1B–24B, except for three i5-only index sequences. Please refer to the [UDI sequence Excel sheets](#) on the customer documentation portal if you are planning to combine UDI kits.

2. Required Equipment and Reagents Not Provided

In addition to the reagents supplied in the kit the following equipment and consumables are required:

Consumable/Equipment	Supplier
Qubit [®] Fluorometer	Thermo Fisher
Qubit [®] dsDNA HS Assay Kit	Thermo Fisher, cat # Q32851 (100 Assays) or Q32854 (500 Assays)
Bioanalyzer / TapeStation with associated reagents and consumables or equivalent fragment analyser with associated reagents and consumables	Agilent General lab supplier
Thermal cycler with heated lid suitable for use with $\geq 70 \mu\text{L}$ volumes	General lab supplier
*DiaMagO2 magnetic rack or Magnetic rack (for 8-strip tubes or 96 well plates)	Protocol validated with the Diagenode, cat # kch-816-001 General lab supplier
Vortexer	General lab supplier
P2, P10, P20, P200, P1000 Pipettes and Tips	General lab supplier
1.5 mL microcentrifuge tubes	General lab supplier
2.0 mL microcentrifuge tubes	General lab supplier
1.5 mL microcentrifuge tubes, DNA low bind	General lab supplier
0.2 mL PCR strip tubes, high profile	General lab supplier
(Optional) Cold Blocks for 0.2 mL PCR strip tubes and 1.5 mL microcentrifuge tubes	General lab supplier
Nuclease-free water (NF water)	General lab supplier
100% Absolute Ethanol (EtOH), molecular biology grade	General lab supplier
Isopropyl Alcohol (iPrOH), Extra Pure, molecular biology grade (also known as propan-2-ol, isopropanol)	General lab supplier
Low TE Buffer that contains: 10 mM Tris HCl (pH 8.0), 0.1 mM EDTA	Thermo Fisher, cat # 12090015
Magnetic beads (not needed if magnetic beads were ordered with the kit)*	duet magnetic beads mosaic (8RXN 4001-1, 24RXN 4002-1, 96RXN 4003-1) or Beckman SPRI beads (B23317 / B23319 / B23318)

*Choice of magnet may impact assay performance.

D. Experimental Protocol

1. Before Starting

It is recommended that you read the entire protocol before starting in order to plan the experiment accordingly. The protocol can be performed across 2 days, and safe stopping points are described in the protocol.

Please read and follow these recommendations.

Mixing Advice

- We recommend assembling all reactions on ice. Alternatively, cold blocks may be used instead of wet ice. If using cold blocks, they must be removed from the freezer at least 10 minutes prior to use to prevent samples from re-freezing inside the blocks.
- We recommend using a low retention 1.5 mL tube when preparing master mixes and adding component in the order listed in the table.
- Thaw buffers at room temperature and place on ice immediately after thawing.
- Vortex buffers, adapters, and additives for 2–4 seconds after thawing and pulse spin to collect all volume at the bottom of the tube. If there is precipitate in a buffer, vortex until the precipitate fully dissolves.
- Keep enzymes directly on ice.
- **Do not** vortex stock enzymes. To mix enzymes, flick tubes or pipette up and down 10–15 times using ~75% of the total volume, then pulse spin to collect the liquid at the bottom of the tube.
- Vortex all user prepared master mixes containing enzymes for 2–4 seconds and pulse spin to collect all volume at the bottom of the tube.
- Do not vortex Ligation MM v2 and PCR MM. Instead, flick tubes or pipette up and down 10–15 times using ~75% of the total volume, then pulse spin to collect the liquid at the bottom of the tube.
- Make sure to close tube lids properly before mixing.
- For quick/pulse spin, use either a Mini Centrifuge or spin at a maximum of 7000 rpm for a maximum of 5 seconds.
- When performing pulse spin to pellet magnetic beads, make sure the pellet is facing towards the exterior of the rotor.

Storage Advice

- Store and use Magnetic Beads at room temperature.
- Room temperature is considered to be between 15°C and 30°C.

General Advice

- Volumes are stated for a single reaction. Master mix volumes for 8 samples (8RXN kit) and 24 samples (24RXN and 96RXN kits) are provided. Please use the calculation below if preparing a master mix for a different number of samples. The calculation includes 10% overage.

Component	Volume Calculation for Master Mix
Reagent	Reagent volume x no. of samples x 1.1

- If using the 96RXN kit, it is recommended to perform a minimum of 24 reactions at a time.
- Unless at a safe stopping point, begin the next step of the protocol immediately.

Bead-mediated purification steps

- For the bead-mediated purification steps, 80% ethanol solution should be freshly prepared with lab supplied nuclease-free water. You may prepare enough volume for all the purification steps you are planning to perform for the day (500 µL per reaction per purification). Discard the unused 80% ethanol at the end of the day.
- Avoid transferring magnetic beads to the next reaction.
- Bead clean-up steps are critical for yield. Loss of beads at any stage will result in loss of material and reduced yield. Users unfamiliar with bead handling may benefit from practicing this step or contacting support for guidance.

2. DNA Sample Input Preparation

This product is designed and validated exclusively for use with human cell-free DNA (cfDNA).

Prepare your cfDNA sample as indicated below and by adding spike-in control DNA as shown in step 2.3.

2.1 cfDNA does not need fragmenting before the assay.

2.2 Recommended input for cfDNA is 5–30 ng.

 **IMPORTANT:** Ensure there is no gDNA contamination by verifying the fragment lengths on a Bioanalyzer, TapeStation or similar.

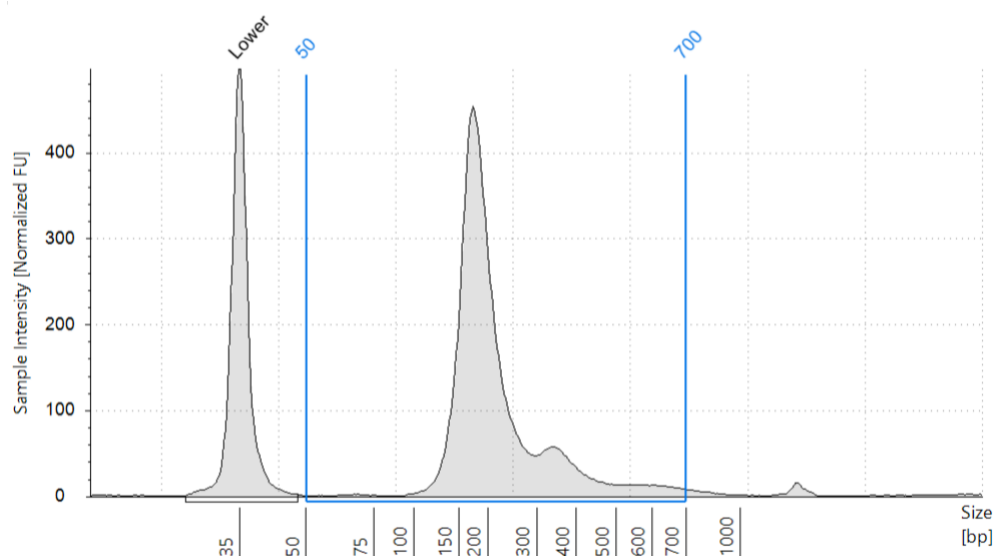


Figure 2. A typical electropherogram of a cfDNA ScreenTape. cfDNA profiles present a prominent peak at approximately 170 bp (mononucleosome) and potential subsequent peaks (nucleosome multimers).

Spike-in Control DNA Preparation

- 2.3 Prepare spike-in Control DNA according to DNA input mass and planned sequencing depth.



Note: Shallow sequencing is < 10x coverage. Deep sequencing is ≥ 10x coverage. See *Sequencing* on page 33.

Guidance for spike-in Control DNA (white lid)*		
DNA input mass	Shallow sequencing	Deep sequencing
5–19 ng	Dilute 1:10 in low TE–buffer, use 1.5 µL of diluted spike-in Control DNA	Dilute 1:10 in low TE–buffer, use 1.5 µL of diluted spike-in Control DNA
20–30 ng	Use 1.5 µL of undiluted spike-in Control DNA	

*See *Sequencing* on page 33.

Sample Preparation

IMPORTANT: cfDNA is diluted in low TE–buffer, using a different buffer or water might affect yield.

- 2.4 Adjust appropriate volume of cfDNA with low TE–buffer to a final volume of 60 µL and add 1.5 µL spike-in Control DNA according to guidance in 2.3.
- 2.5 Mix well and pulse spin to collect all liquid from the sides of the tubes.

3. DNA Denaturation Step

- 3.1 Incubate the DNA prepared in step 2.4 in a thermal cycler programmed as below:

Temp	Time	Heated Lid	Volume
95°C	3 mins	105°C	61.5 µL
Cool down to 4°C	at -1.5°C/s.		
4°C	Hold		

- 3.2 Proceed immediately to the next step.

4. Tailing and Hairpin Adapter Ligation

Reagents

- TT Buffer (yellow lid) (TTB)
- TT Enzyme 1 (yellow lid) (TTE1)
- TT Enzyme 2 (yellow lid) (TTE2)
- TT Hairpin adapter (yellow lid) (TTH)

4.1 Prepare TT Buffer (yellow lid), TT Enzyme 1 (yellow lid), TT Enzyme 2 (yellow lid) and TT Hairpin adapter (yellow lid) by thawing and placing on ice just before use.

4.2 Prepare a reaction master mix on ice in a low retention 1.5 mL tube as set out in the table below:

 **IMPORTANT:** Ensure the Master Mix is prepared **immediately** prior to use, **do not prepare** in advance.

Component	Volume per reaction (μL)	Volume 8 reactions* (μL)	Volume 24 reactions* (μL)
TT Buffer (yellow lid)	7.5	66.0	198.0
TT Enzyme 1 (yellow lid)	0.5	4.4	13.2
TT Enzyme 2 (yellow lid)	2.0	17.6	52.8
TT Hairpin Adapter (yellow lid)	0.5	4.4	13.2
Total Master Mix Volume:	10.5	92.4	277.2

*Volumes include 10% overage.

4.3 Vortex for 2–4 seconds and pulse spin to collect all liquid from the sides of the tubes.

4.4 Add **10.5 μL** of master mix to each 0.2 mL PCR tube containing 61.5 μL of denatured DNA sample from step 3.1. Mix well and pulse spin to collect all liquid from the sides of the tubes.

4.5 Incubate in a thermal cycler programmed as below:

Temp	Time	Heated Lid	Volume
37°C	60 mins	105°C	72.0 μL
95°C	10 mins		
Cool down to 10°C	at -0.1°C/s.		
10°C	Hold		

4.6 Proceed immediately to Bead-Mediated Purification 1.

5. Bead-Mediated Purification 1 (with Isopropyl Alcohol)

Reagents

- Magnetic Beads
- 100% Isopropyl alcohol
- NF Water
- Freshly prepared 80% ethanol

5.1 Bring the reaction mix from step 4.5 to room temperature.

5.2 Vortex the Magnetic Beads for 30 secs, or until fully resuspended and homogenous. Use immediately. **Do not** let the beads settle before use.

5.3 Perform a 0.7x bead-based purification at room temperature by combining the following:

 **IMPORTANT:** Addition of isopropyl alcohol for this bead-mediated purification step is essential to achieve high library yields. Omitting it will significantly reduce library yield. Note that this is the only bead-mediated purification that requires isopropyl alcohol.

 **IMPORTANT:** This purification step has a large volume. Consider transferring to larger tubes if necessary to avoid spillage and contamination. Make sure the lids are closed tightly before vortexing.

Component	Volume per reaction (µL)
Reaction mix from step 4.5	72.0
Magnetic Beads	50.0
100% Isopropyl alcohol	78.0
Total Volume:	200.0

5.4 Mix the bead + DNA mixture thoroughly by vortexing for 5–10 secs or until fully homogenous and incubate the tube at room temperature for 10 mins to bind the DNA to the beads.

5.5 Pulse spin the samples to collect the liquid from the sides of the tube and place the tube into a magnet to capture the beads for 5 mins.

5.6 Keeping the tube on the magnet, carefully remove and discard the supernatant using a P200 pipette.

- 5.7 Keeping the tube on the magnet, add 200 μL of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube on the magnet at room temperature for 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.
- 5.8 Repeat step 5.7.
- 5.9 Pulse spin the bead pellet, replace the tubes on the magnet and incubate for 1 min, remove any residual ethanol with a P10 or P20 pipette without disturbing the beads.
-  **IMPORTANT:** Ensure all ethanol is removed before proceeding to the next step.
- 5.10 Dry the beads at room temperature for ~1–3 mins or until all the ethanol evaporates.
-  **Caution:** Over/under-drying the beads may result in reduced final yield.
- 5.11 Remove the tubes from the magnet, add **15.0 μL** of NF Water and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for 2 mins to elute the DNA from the beads.
- 5.12 Pulse spin the samples to collect the liquid from the sides of the tubes. Place the tubes in a magnet to capture the beads and incubate until the liquid is clear (~2–3 mins).
- 5.13 Transfer **14.0 μL** of the clear supernatant to a new 0.2 mL PCR tube on ice.
- 5.14 Proceed immediately to Strand Copying step.

6. Strand Copying

Reagents

- CS Buffer (blue lid) (CSB)
- CS Enzyme (blue lid) (CSE)
- hmCP Additive (brown lid) (hmCPA)
- hmCP Enzyme (brown lid) (hmCPE)

 **Note:** hmCP Additive and hmCP Enzyme are used again in section 11.

- 6.1 Prepare CS Buffer (blue lid), CS Enzyme (blue lid), hmCP Additive (brown lid) and hmCP Enzyme (brown lid) by thawing and placing on ice just before use.

 **Note:** Ensure CS Buffer is fully resuspended. CS Buffer may have a visible precipitate on the side of the tube or in the lid. If this occurs, invert the tube and flick-mix followed by thorough vortexing.

- 6.2 Prepare a reaction master mix on ice in a low retention 1.5 mL tube as set out in the table below:

 **Note:**

- CS Enzyme and CS buffer become very frothy if mixed too vigorously. Please take care when handling before adding to samples to ensure maximum recovery of the reagents.
- Once mixed with DNA, a small amount of froth does not affect the reaction.

Component	Volume per reaction (µL)	Volume 8 reactions* (µL)	Volume 24 reactions* (µL)
CS Buffer (blue lid)	2.5	22.0	66.0
CS Enzyme (blue lid)	5.0	44.0	132.0
hmCP Additive (brown lid)	2.5	22.0	66.0
hmCP Enzyme (brown lid)	1.0	8.8	26.4
Total Master Mix Volume:	11.0	96.8	290.4

*Volumes include 10% overage.

- 6.3 Vortex for 2–4 seconds and pulse spin to collect all liquid from the sides of the tube.
- 6.4 Aliquot **11 µL** of master mix into each 0.2 mL PCR tube containing 14 µL purified DNA from step 5.13. Mix well and pulse spin to collect all liquid from the sides of the tube.
- 6.5 Incubate in a thermal cycler programmed as below:

Temp	Time	Heated Lid	Volume
20°C	30 min	85°C	25.0 µL
65°C	30 min		
4°C	Hold		

- 6.6 Proceed immediately to Forkhead Adapter Ligation.

7. Forkhead Adapter Ligation

Reagents

- Forkhead adapter (purple lid) (FA)
- Ligation buffer (purple lid) (FLB)
- Ligation MM v2 (purple lid) (FLMM)

7.1 Prepare Forkhead Adapter (purple lid), Ligation MM v2 (purple lid) and Ligation Buffer (purple lid) by thawing and placing on ice just before use.

7.2 Add **1.5 µL** of Forkhead Adapter (purple lid) to each sample from step 6.5 on ice.



Note: Do not add adapters to the master mix. Add individually to each reaction.

7.3 Prepare a reaction master mix on ice in a low retention 1.5 mL tube as set out in the table below:



Note: Ligation MM v2 is viscous, pipette slowly to ensure accurate volume.

Component	Volume per reaction (µL)	Volume 8 reactions* (µL)	Volume 24 reactions* (µL)
Ligation Buffer (purple lid)	10.0	88.0	264.0
Ligation MM v2 (purple lid)	3.5	30.8	92.4
Total Master Mix Volume:	13.5	118.8	356.4

*Volumes include 10% overage.

7.4 Vortex for 2–4 seconds and pulse spin the master mix to collect all liquid from the sides of the tube.

7.5 Aliquot **13.5 µL** of master mix into each 0.2 mL PCR tube containing 26.5 µL DNA from step 7.2 above. Mix well and pulse spin to collect all liquid from the sides of the tube.

7.6 Incubate in a thermal cycler programmed as below:

Temp	Time	Heated Lid	Volume
20°C	15 mins	OFF	40.0 µL
4°C	Hold		

7.7 Proceed immediately to Bead-Mediated Purification 2.

8. Bead-Mediated Purification 2

Reagents

- Magnetic Beads
- NF Water
- Freshly prepared 80% ethanol

- 8.1 Bring the reaction mix from step 7.6 to room temperature.
- 8.2 Vortex the Magnetic Beads for 30 secs, or until fully resuspended and homogenous. Use immediately. **Do not** let the beads settle before use.
- 8.3 Perform a 1.2x bead-based purification at room temperature by combining the following:

Component	Volume per reaction (µL)
Reaction mix from step 7.6	40.0
Magnetic Beads	48.0
Total Volume:	88.0

- 8.4 Mix the bead + DNA mixture thoroughly by vortexing for 5–10 secs or until fully homogenous and incubate the tube at room temperature for 10 mins to bind the DNA to the beads.
- 8.5 Pulse spin the samples to collect the liquid from the sides of the tube and place the tube into a magnet to capture the beads for 5 mins.
- 8.6 Keeping the tube on the magnet, carefully remove and discard the supernatant using a P200 pipette.
- 8.7 Keeping the tube on the magnet, add 200 µL of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube on the magnet at room temperature for 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.
- 8.8 Repeat step 8.7.
- 8.9 Pulse spin the bead pellet, replace the tubes on the magnet and incubate for 1 min. Remove any residual ethanol with a P10 or P20 pipette without disturbing the beads.
-  **IMPORTANT:** Ensure all ethanol is removed before proceeding to the next step.
- 8.10 Dry the beads at room temperature for ~1–3 mins or until all the ethanol evaporates.
-  **Caution:** Over/under-drying the beads may result in reduced final yield.

- 8.11 Remove the tubes from the magnet. Add **17 µL** of NF Water and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous. Incubate at room temperature for 2 mins to elute the DNA from the beads.
- 8.12 Pulse spin the samples to collect the liquid from the sides of the tubes. Place the tubes in a magnet to capture the beads and incubate until the liquid is clear (~2–3 mins).
- 8.13 Transfer **15 µL** of the clear supernatant to a new 0.2 mL PCR tube on ice using a P20 pipette.

**SAFE STOP POINT:**

Samples can be stored for up to 7 days at –20°C.

9. Copy Methylation

Reagents

- MF Enzyme (pink lid) (MFE)
- MF Additive 1 (pink lid) (MFA1)
- MF Additive 2 (pink lid) (MFA2)
- MF Buffer (pink lid) (MFB)

- 9.1 Prepare MF Buffer (pink lid), MF Additive 1 (pink lid), MF Additive 2 (pink lid), and MF Enzyme (pink lid) by thawing and placing on ice just before use.
- 9.2 Prepare a reaction master mix on ice in a low retention 1.5 mL tube as set out in the table below:

 **IMPORTANT:** Ensure the Master Mix is prepared **immediately** prior to use, **do not prepare** in advance. **Mix thoroughly by vortexing.**

Component	Volume per reaction (μL)	Volume 8 reactions* (μL)	Volume 24 reactions* (μL)
MF Buffer (pink lid)	11.0	96.8	290.0
MF Additive 1 (pink lid)	0.5	4.4	13.2
MF Additive 2 (pink lid)	1.5	13.2	39.6
MF Enzyme (pink lid)	2.0	17.6	52.8
Total Master Mix Volume:	15.0	132.0	396.0

*Volumes include 10% overage.

- 9.3 Vortex for 2–4 seconds and pulse spin the master mix to collect all liquid from the sides of the tube.
- 9.4 Aliquot **15.0 μL** of master mix into each 0.2 mL PCR tube containing 15.0 μL DNA from step 8.13 above.
- 9.5 Mix well and pulse spin to collect all liquid from the sides of the tube.

9.6 Incubate in a thermal cycler programmed as below:

Temp	Time	Heated Lid	Volume
27°C	30 mins	75°C	30.0 µL
65°C	15 mins		
4°C	Hold		

9.7 Proceed to Bead mediated purification 3.



SAFE STOP POINT:

Samples can be stored overnight at 4°C.

10. Bead-Mediated Purification 3

Reagents

- Magnetic Beads
- NF Water
- Freshly prepared 80% ethanol

10.1 Bring the reaction mix from step 9.5 to room temperature.

10.2 Vortex the Magnetic Beads for 30 secs, or until fully resuspended and homogenous. Use immediately. **Do not** let the beads settle before use.

10.3 Perform a 1.4x bead-based purification at room temperature by combining the following:

Component	Volume per reaction (µL)
Reaction mix from step 9.5	30.0
Magnetic Beads	42.0
Total Volume:	72.0

10.4 Mix the bead + DNA mixture thoroughly by vortexing for 5–10 secs or until fully homogenous and incubate the tube at room temperature for 10 mins to bind the DNA to the beads.

10.5 Pulse spin the samples to collect the liquid from the sides of the tube and place the tube into a magnet to capture the beads for 5 mins.

10.6 Keeping the tube on the magnet, carefully remove and discard the

supernatant using a P200 pipette.

- 10.7 Keeping the tube on the magnet, add 200 μ L of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube on the magnet at room temperature for 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.
- 10.8 Repeat step 10.7.
- 10.9 Pulse spin the bead pellet, replace the tubes on the magnet and incubate for 1 min. Remove any residual ethanol with a P10 or P20 pipette without disturbing the beads.
 **IMPORTANT:** Ensure all ethanol is removed before proceeding to the next step.
- 10.10 Dry the beads at room temperature for ~1–3 mins or until all the ethanol evaporates.
 **Caution:** Over/under-drying the beads may result in reduced final yield.
- 10.11 Remove the tubes from the magnet. Add **31 μ L** of NF Water and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous. Incubate at room temperature for 2 mins to elute the DNA from the beads.
- 10.12 Pulse spin the samples to collect the liquid from the sides of the tubes. Place the tubes in a magnet to capture the beads and incubate until the liquid is clear (~2–3 mins).
- 10.13 Transfer **30 μ L** of the clear supernatant to a new 0.2 mL PCR tube on ice using a P200 pipette.

**SAFE STOP POINT:**

Samples can be stored for up to 7 days at -20°C .

11. Oxidation – Protection of Modified Cytosines

Reagents

- Ox Buffer (brown lid) (OxB)
- Ox Additive 1 (brown lid) (OxA1)



Note: Centrifuge Ox Additive 1 before use to collect reagent at the bottom of the tube.

- DTT (brown lid) (DTT)
- Ox Additive 2 (brown lid) (OxA2)



Note: Only remove Ox Additive 2 from freezer immediately before use at step 11.7.

- hmCP Additive (brown lid) (hmCPA)
- hmCP Enzyme (brown lid) (hmCPE)
- Ox Enzyme E (brown lid) (OxEE)

11.1 Prepare Ox Buffer (brown lid), Ox Additive 1 (brown lid), DTT (brown lid), hmCP Additive (brown lid), hmCP Enzyme (brown lid), and Ox Enzyme E (brown lid) by thawing and placing on ice just before use.

11.2 Add **400 µL** Ox Buffer to Ox Additive 1 tube to form an Ox Additive 1 solution.

11.3 Mix well by vortexing until the reagent is fully dissolved. Write date on tube.



Note: The Ox Additive 1 solution, once prepared, should be stored at -20°C in the dark for up to 4 months once reconstituted.

- 11.4 Prepare a reaction master mix on ice in a low retention 1.5 mL tube as set out in the table below:

Component	Volume per reaction (μL)	Volume 8 reactions* (μL)	Volume 24 reactions* (μL)
Ox Additive 1 solution from step 11.2 (brown lid)	10.0	88.0	264.0
DTT (brown lid)	1.0	8.8	26.4
hmCP Additive (brown lid)	1.0	8.8	26.4
hmCP Enzyme (brown lid)	1.0	8.8	26.4
Ox Enzyme E (brown lid)	2.0	17.6	52.8
Total Master Mix Volume:	15.0	132.0	396.0

*Volumes include 10% overage.

- 11.5 Vortex for 2–4 seconds and pulse spin to collect all liquid from the sides of the tube.
- 11.6 Aliquot **15 μL** of master mix into each 0.2 mL PCR tube containing 30 μL DNA from step 10.13. Mix well and pulse spin to collect all liquid from the sides of the tube.
- 11.7 Remove Ox Additive 2 from the freezer directly before use. Prepare Ox Additive 2 by thawing, then vortex thoroughly, pulse spin, and keep on ice.
-  **Note:** Ensure Ox Additive 2 is fully resuspended. Ox Additive 2 may have a visible precipitate on the side of the tube or in the lid. If this occurs, invert the tube and flick-mix followed by thorough vortexing.

- 11.8 Prepare diluted Ox Additive 2 in a 2.0 mL microcentrifuge tube as follows:

 **Note:** The complete mixing of Ox Additive 2 (brown lid) with nuclease-free water is a critical step. Improper mixing adversely impacts final yield.

 **IMPORTANT:** Use freshly diluted Ox Additive 2 immediately and discard after use. Return remaining undiluted Ox Additive 2 to **-20°C** immediately after use. Storage of Ox Additive 2 above -20°C for extended periods can cause oxidation.

Component	Volume (μL)
Ox Additive 2* (brown lid)	1.5
Nuclease-free water	1873.5
Total Volume:	1875.0

*Return to **-20°C** immediately after use.

- 11.9 Mix well by vortexing for 30 seconds and pulse spin to collect all liquid from the sides of the tube.

 **IMPORTANT:** proper mixing is essential.

- 11.10 Add **5 μL** of diluted Ox Additive 2 solution to each 0.2 mL PCR tube containing 45 μL DNA from step 11.6.

 **Note:** Do not add diluted Ox Additive 2 to the master mix. Instead, add directly to each 0.2 mL PCR tube containing DNA.

- 11.11 Mix well and pulse spin to collect all liquid from the sides of the tube.

- 11.12 Incubate in a thermal cycler programmed as below:

Temp	Time	Heated Lid	Volume
37°C	60 mins	55°C	50.0 μL
4°C	Hold		

- 11.13 Proceed immediately to Bead-Mediated Purification 4.

12. Bead-Mediated Purification 4

Reagents

- Magnetic Beads
- NF Water
- Freshly prepared 80% ethanol

12.1 Bring the reaction mix from step 11.12 to room temperature.

12.2 Vortex the Magnetic Beads for 30 secs, or until fully resuspended and homogenous. Use immediately. **Do not** let the beads settle before use.

12.3 Perform a 1.8x bead-based purification at room temperature by combining the following in a 0.2 mL PCR tube.

 **IMPORTANT:** This purification step has a large volume. Consider transferring to larger tubes if necessary to avoid spillage and contamination.

Component	Volume per reaction (μL)
Reaction mix from step 11.12	50.0
Magnetic Beads	90.0
Total Volume:	140.0

12.4 Mix the bead + DNA mixture thoroughly by vortexing for 5–10 secs or until fully homogenous and incubate the tube at room temperature for 10 mins to bind the DNA to the beads.

12.5 Pulse spin the samples to collect the liquid from the sides of the tube and place the tube into a magnet to capture the beads for 5 mins.

12.6 Keeping the tube on the magnet, carefully remove and discard the supernatant using a P200 pipette.

12.7 Keeping the tube on the magnet, add 200 μL of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube on the magnet at room temperature for 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.

12.8 Repeat step 12.7.

12.9 Pulse spin the bead pellet, replace the tubes on the magnet and incubate for 1 min, remove any residual ethanol with a P10 or P20 pipette without disturbing the beads.

 **IMPORTANT:** Ensure all ethanol is removed before proceeding to the next step.

- 12.10 Dry the beads at room temperature for ~1–3 mins or until all the ethanol evaporates.



Caution: Over/under-drying the beads may result in reduced final yield.

- 12.11 Remove tubes from magnet. Add **16 µL** of NF Water and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous. Incubate at room temperature for 2 mins to elute the DNA from the beads.
- 12.12 Pulse spin the samples to collect the liquid from the sides of the tubes. Place the tubes in the magnet to capture the beads and incubate until the liquid is clear (~2–3 mins).
- 12.13 Transfer **15 µL** of the clear supernatant to a new 0.2 mL PCR tube on ice using a P20 pipette.



SAFE STOP POINT:

Samples can be stored for up to 7 days at –20°C.

13. Deamination of Cytosines

Reagents

- DA Buffer Mix (red lid) (DBM)
- DA Enzyme 1 (red lid) (DAE1)
- DA Enzyme 2 (red lid) (DAE2)

- 13.1 Prepare DA Buffer Mix (red lid), DA Enzyme 1 (red lid), DA Enzyme 2 (red lid) by thawing and placing on ice just before use.



Note: DA buffer Mix does not contain enzymes and can be vortexed for 2–4 seconds. Do not mix too vigorously to avoid frothing and loss of volume.

- 13.2 Prepare a reaction master mix on ice in a low retention 1.5 mL tube as set out in the table below:

Component	Volume per reaction (μL)	Volume 8 reactions* (μL)	Volume 24 reactions* (μL)
DA Buffer Mix (red lid)	10.5	92.4	277.2
DA Enzyme 1 (red lid)	2.0	17.6	52.8
DA Enzyme 2 (red lid)	2.5	22.0	66.0
Total Master Mix Volume:	15.0	132.0	396.0

*Volumes include 10% overage.

- 13.3 Vortex for 2–4 seconds and pulse spin to collect all liquid from the sides of the tube.
- 13.4 Aliquot **15 μL** of master mix into each 0.2 mL PCR tube containing 15 μL DNA from step 12.13. Mix well and pulse spin to collect all liquid from the sides of the tube. Before proceeding to step 13.5, ensure there are no bubbles in the DNA from step 13.3. If bubbles are present, spin longer or gently tap the tube and spin again.
- 13.5 Incubate in a thermal cycler programmed as below:

Temp	Time	Heated Lid	Volume
37°C	90 mins	105°C	30.0 μL
70°C	10 min		
4°C	Hold		



IMPORTANT: the heat inactivation step at 70°C for 10 mins is essential. Skipping this will result in significant impact on yield.



SAFE STOP POINT:

This reaction can be left at 4°C overnight.

14. Library Amplification

Reagents

- PCR MM (clear lid) (PMM)
- duet UDI primers 1-8 or 1C-8C or 1-24/1B-24B (clear lid) or 1A-96A (plate)

14.1 Prepare PCR MM (clear lid) and UDIs (clear lid) by thawing and placing on ice just before use.

14.2 Assemble each amplification reaction on ice as follows.



Note:

- Reagents include up to 96 different UDIs. UDIs 1-8 or 1C-8C and 1-24/1B-24B are found in separate tubes. UDIs 1A-96A are provided in a plate.
- Please record which UDIs (1-8, 1C-8C, 1-24, 1B-24B, 1A-96A) are used for each prepared reaction, as only one individual adapter is used per reaction.

Component	Volume per reaction (μL)
Reaction mix from step 13.5	30.0
PCR MM (clear lid)	15.0
UDIs primers 1-8 or 1C-8C or 1-24/1B-24B (clear lid) or 1A-96A (plate)	5.0
Total Volume:	50.0

14.3 Mix well and pulse spin to collect all liquid from the sides of the tube.

14.4 Amplify in a thermal cycler programmed as below:

	Temp	Time	Cycles	Heated Lid	Volume
Initial Denaturation	98°C	30 secs	1	105°C	50.0 µL
Denaturation	98°C	10 secs	5–8 cycles*		
Annealing	62°C	30 secs			
Extension	65°C	60 secs			
Final extension	65°C	5 mins	1		
Hold	4°C	Hold	1		

*PCR cycle recommendations according to input amount:

5 ng to 10 ng input: 8 cycles

11 to 20 ng input: 7 cycles

21 to 30 ng input: 5 cycles

15. Bead-Mediated Purification 5

Reagents

- Magnetic Beads
- Dilution Buffer (white lid)
- Freshly prepared 80% ethanol

15.1 Bring the reaction mix from step 14.4 to room temperature.

15.2 Vortex the Magnetic Beads for 30 secs, or until fully resuspended and homogenous. Use immediately. **Do not** let the beads settle before use.

15.3 Perform a 0.9x bead-based purification at room temperature by combining the following:

Component	Volume per reaction (µL)
Reaction mix from step 14.4	50.0
Magnetic Beads	45.0
Total Volume:	95.0

15.4 Mix the bead + DNA mixture thoroughly by vortexing for 5–10 secs or until fully homogenous and incubate the tube at room temperature for 10 mins to bind the DNA to the beads.

15.5 Pulse spin the samples to collect the liquid from the sides of the tube and place the tube into a magnet to capture the beads for 5 mins.

- 15.6 Keeping the tube on the magnet, carefully remove and discard the supernatant using a P200 pipette.
- 15.7 Keeping the tube on the magnet, add 200 μ L of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube on the magnet at room temperature for 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.
- 15.8 Repeat step 15.7.
- 15.9 Pulse spin the bead pellet, the tubes on the magnet and incubate for 1 min, remove any residual ethanol with a P10 or P20 pipette without disturbing the beads.
-  **IMPORTANT:** Ensure all ethanol is removed before proceeding to the next step.
- 15.10 Dry the beads at room temperature for ~1–3 mins or until all the ethanol evaporates.
-  **Caution:** Over/under-drying the beads may result in reduced final yield.
- 15.11 Remove tubes from the magnet. Add **15 μ L of Dilution Buffer** (white lid) and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for 2 mins to elute the DNA from the beads.
- 15.12 Pulse spin the samples to collect the liquid from the sides of the tubes. Place the tubes in a magnet to capture the beads and incubate until the liquid is clear (~2–3 mins).
- 15.13 Transfer **14 μ L** of the clear supernatant to a new 0.2 mL PCR tube.

16. Library Quantification

- 16.1 Use 1 μL final library to quantify using a Qubit dsDNA HS kit. Expected final yield range is approximately 20–100 ng/ μL .



Note: dilution might be needed for Qubit quantification.

- 16.2 Using the Qubit results, dilute 1 μL of the final library to 0.1–50 ng/ μL for TapeStation D5000 or Bioanalyzer analysis.
- 16.3 Use a TapeStation or Bioanalyzer to determine the size distribution and concentration of the library in nM. Expected average fragment size should be 550–650 bp. Establish a region table that bounds the library to determine its average insert size (suggested range is 200–2000 bp).

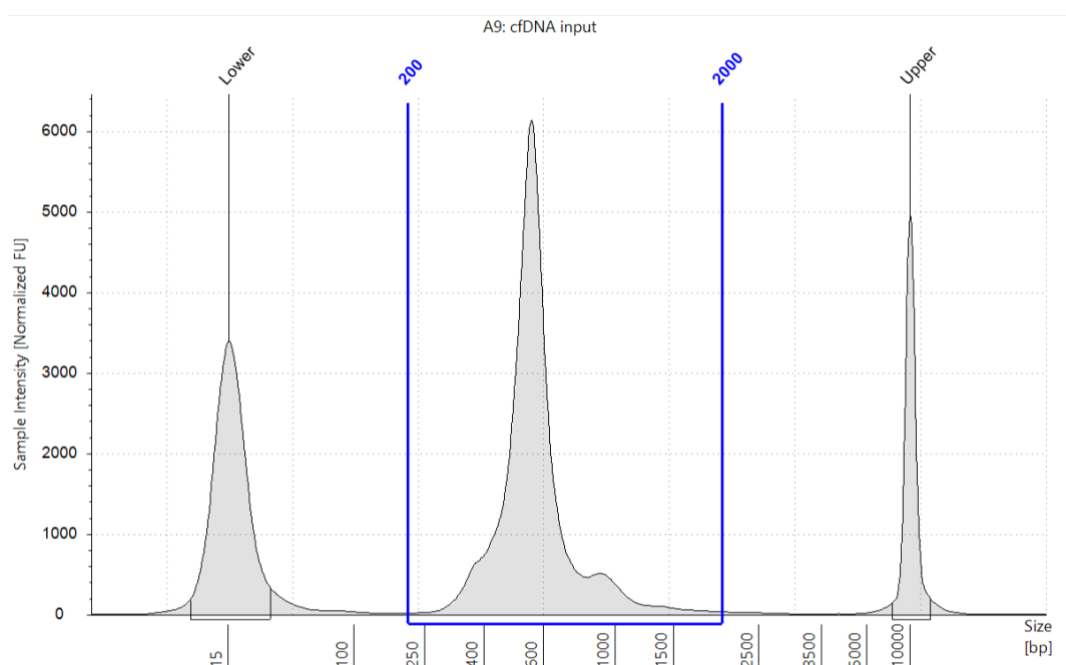


Figure 3. A typical TapeStation trace (D5000) of a duet 6-base mosaic library.

16.4 Determine the concentration in ng/μL of the final libraries using either method described below:

A. Fluorometric assay

- Assess concentration in ng/μL using a fluorometric assay (Qubit dsDNA HS kit).
- Determine the average library size in bp using the TapeStation (D5000 ScreenTape) or Bioanalyzer.

B. qPCR assay

- Perform a qPCR assay such as KAPA Library Quantification Kit (Illumina/Universal).
- Determine the average library size in bp using the TapeStation (D5000 ScreenTape) or Bioanalyzer.

16.5 Determine the molar concentration in nM of the final libraries using the calculation below:

$$\text{concentration in nM} = \frac{\text{concentration } \left(\frac{\text{ng}}{\mu\text{l}} \right)}{660 \text{ mol} \times \text{average library size (bp)}} \times 10^6$$



Note: Once the libraries have been prepared, they can be stored up to 1 month at -20°C.

E. Sequencing

1. Set-up

Libraries must be sequenced on NovaSeq6000 or NovaSeqX platforms as paired-end Sequencing Runs.

-  **IMPORTANT:** Spike-in of a base balanced library, such as PhiX, is required due to the C to T conversion during the deamination step.
 - For NovaSeq6000 sequencing, 5–15% of PhiX is recommended to pooled libraries (8% is a good starting point).
 - For NovaSeqX sequencing, 5% PhiX is recommended for best results with the NovaSeqX Series v1.4.0 software.
 - For best results, we recommend quantifying the PhiX before calculating the amount needed to spike-in.
- Perform a 2 x 151 bp paired-end sequencing analysis. Include 2 x 8 bp UDI index reads for sample pooling and demultiplexing.
 - Optimise loading concentration for the sequencer used.
 - For NovaSeq6000 sequencing 300 pM (1.5 nM pool) should be used as a starting point for optimization.
 - For NovaSeqX sequencing 150 pM should be used as a starting point for optimization.

 **IMPORTANT:** we recommend 4 or more samples with unique UDIs multiplexed to avoid potential demultiplexing issues known on Illumina sequencers.

2. Index Sequences

Index sequences are provided separately and can be found in the sequences for UDIs document in the customer documentation portal.

F. Revision History

Version	Date	Description
1	August 2026	Protocol release
1.1	September 2026	Implemented minor typographical and formatting updates, and terminology throughout to improve clarity and reduce the risk of user error.