



duet evoC

User guide

Laboratory instructions

For the preparation of NGS-ready duet construct for sequencing
on Illumina NGS Platforms.

Suitable for use with 8 and 24 reaction kits.



This user guide is specifically compatible with **Product Numbers: 6205-1 / 6205-2 (8 reactions) and 6206-1 / 6206-2 (24 reactions)**.
If you have a different product number, please refer to the appropriate guide or contact biomodal support for assistance.

Version 5.3, February 2026

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



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A. Introduction

The duet evoC multiomics solution can provide complete genetic information whilst also discriminating between the epigenetic modifications 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC). The duet evoC 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 genomic DNA and cell-free DNA into a single workflow using a standard Illumina NGS platform.

See *Reagents Provided* on page 5 and 6.

B. Kit Method Overview

The duet evoC solution comprises 9 key steps, shown in Figure 1. End repair and A-tailing, hairpin adapter ligation, hairpin digestion (HD), strand copying (SC) and Forkhead adapter ligation resulting in a duet construct. This duet construct then undergoes DNA modification with copy methylation, oxidation, and deamination steps, followed by a PCR amplification step to add unique dual indexes (UDIs) and NGS sequencing adapters, producing a final duet construct ready for NGS on Illumina Platforms.

The protocol can be performed as a 2-day or 3-day process, with recommended stopping points for each version noted below.

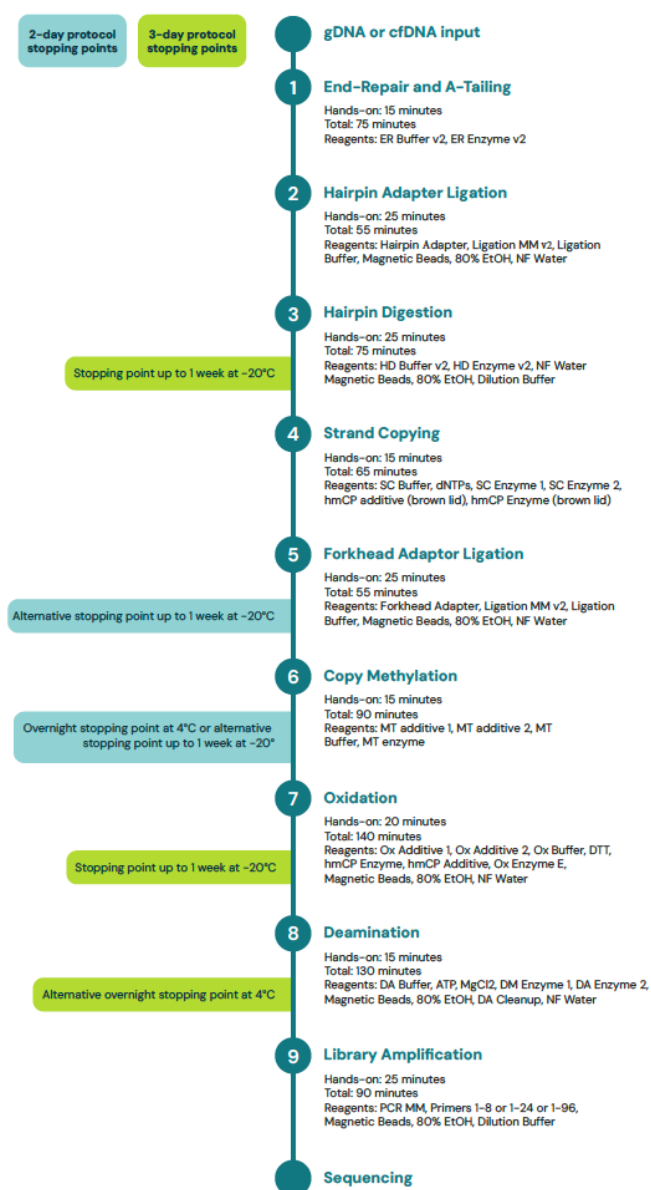


Figure 1. duet evoC

C. Kit Information

1. Reagents Provided

duet evoC 8RXN and 24RXN Room temperature kit box shipped at ambient temperature (Qty 1)

duet evoC 6205-1 (8 RXN), 6206-1 (24 RXN)

Product Name	8RXN Volume (ml)	24RXN Volume (ml)	Lid colour	Store at
NF Water	5.0	5.0	White	15°C to 30°C
Magnetic Beads	3.5	10.0	White	15°C to 30°C
Dilution Buffer	1.0	1.35	White	15°C to 30°C

duet evoC 8RXN and 24RXN Frozen kit box shipped on dry ice (Qty1)

duet evoC 6205-2 (8 RXN), 6206-2 (24 RXN)

Product Name	8RXN Volume (µl)	24RXN Volume (µl)	Lid colour	Store at
Control DNA (CTRL)	8.0	24.0	White	-20°C
ER Buffer v2 (ERB2)	24.0	72.0	Yellow	-20°C
ER Enzyme v2 (ERE2)	24.0	72.0	Yellow	-20°C
Hairpin Adapter (HA)	33.0	100.0	Green	-20°C
Ligation Buffer (LB)	211.0	632.0	Green	-20°C
Ligation MM v2 (LMM2)	76.0	228.0	Green	-20°C
HD Enzyme v2 (HDE2)	32.0	96.0	Orange	-20°C
HD Buffer v2 (HDB2)	24.0	72.0	Orange	-20°C
SC Buffer (SCB)	20.0	60.0	Blue	-20°C
dNTPs (dNTP)	16.0	48.0	Blue	-20°C
SC Enzyme 1 (SCE1)	16.0	48.0	Blue	-20°C
SC Enzyme 2 (SCE2)	16.0	48.0	Blue	-20°C
Forkhead Adapter (FA)	20.0	60.0	Purple	-20°C
MT Additive 1 (MTA1)	10.0	10.0	Pink	-20°C
MT Additive 2 (MTA2)	12.0	24.0	Pink	-20°C
MT Buffer (MTB)	100.0	280.0	Pink	-20°C

MT Enzyme (MTE)	1 lyophilised bead/tube (8-strip tubes – 1 pouch)	1 lyophilised bead/tube (3x 8-strip tubes – 3 pouches)	Clear	-20°C
Ox Buffer (OxB)	450.0	1600.0	Brown	-20°C
Ox Additive 1 (OxA1)	Powder (0.584 mg) – 1 tube	Powder (0.584 mg) – 3 tubes	Brown	-20°C
DTT (DTT)	8.0	24.0	Brown	-20°C
hmCP Additive (hmCPA)	28.0	84.0	Brown	-20°C
hmCP Enzyme (hmCPE)	16.0	48.0	Brown	-20°C
Ox Enzyme E (OxEE)	16.0	48.0	Brown	-20°C
Ox Additive 2 (OxA2)	8.0	24.0	Brown	-20°C
DA Buffer (DAB)	140.0	420.0	Red	-20°C
ATP (ATP)	15.0	44.0	Red	-20°C
MgCl ₂ (Mg)	28.0	84.0	Red	-20°C
DM Enzyme 1 (DME1)	20.0	48.0	Red	-20°C
DA Enzyme 2 (DAE2)	20.0	60.0	Red	-20°C
DA Cleanup (DAC)	52.0	156.0	Red	-20°C
PCR MM (PCR)	200.0	600.0	Clear	-20°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 1C–8C (4103) are unique and differ from duet UDIs 1–24/1B–24B (4102). duet UDIs 1–8 (4101) are identical to the first 8 UDIs in the duet 1–24/1B–24B kit (4102). duet 1–24 and 1B–24B (4102) are identical.

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](#) if you are planning to combine UDI kits.

2. Required Equipment and Reagents Not Provided

In addition to the reagents supplied in the duet evoC 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
*M220 Covaris Focused-ultrasonicator (for DNA genomic input only) with associated reagents and consumables or similar fragmentation instrument with associated reagents and consumables	Covaris, cat # 500295 General lab supplier
Thermal cycler with heated lid suitable for use with 70 µl 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
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	General lab supplier
100% Absolute Ethanol (EtOH), molecular biology grade	General lab supplier
Low TE Buffer that contains: 10 mM Tris HCl (pH 8.0), 0.1 mM EDTA	Thermo Fisher, cat # 12090015

*Covaris M220 is recommended.

**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 or 3 days, and safe stopping points are described in the protocol for both options.

Please read and follow these recommendations.

Mixing Advice

- Assemble 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.
- Vortex buffers 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.
- Invert and flick mix master mixes containing enzymes 5–10 times. Pulse spin afterwards to collect all liquid at the bottom of the tube.
- Pipetting up and down 10–15 times can be used instead of flicking to mix.
- Make sure to close tube lids properly before mixing.
- **Do not** vortex enzymes and enzyme-containing master mixes. To mix enzymes, flick tubes after thawing and pulse spin to collect liquid at the bottom of the tube.
- We **do not** recommend adding adapter to a master mix in adapter ligation steps. Premix adapter and sample, and then add the other ligation reagents.
- 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 4 or 8 samples 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 – Master Mix
Reagent	Reagent volume x no. of samples x 1.1

- For the bead-mediated purification steps 80% ethanol solution should be freshly prepared with lab supplied nuclease-free water. Do not use kit supplied NF Water (white lid). 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.
- Unless at a safe stopping point, begin the next step of the protocol immediately.

2. DNA Sample Input Preparation

Prepare your DNA sample as indicated below for genomic DNA and cell-free DNA, then add spike-in control DNA as shown in step 2.8.

Genomic DNA (gDNA)

- 2.1 gDNA must be fragmented before the assay.
 -  **IMPORTANT:** Do not use enzymatic fragmentation for this protocol.
- 2.2 Recommended input range for gDNA is 10–80 ng of double-stranded DNA quantified post-sonication.
- 2.3 For fragmentation, dilute the appropriate amount of gDNA in low TE buffer (0.1 mM EDTA) and shear to an average fragment size of 250 bp (recommended range 220–280 bp). To achieve an 80 ng yield post-sonication, 120–150 ng of double stranded DNA is recommended as pre-sonication input.
- 2.4 Quantify fragmented DNA using a Qubit Fluorometer, and determine fragment size using a Bioanalyser, TapeStation, or similar.
 -  **Note:** The protocol is optimised for a 260/280 absorbance ratio value of 1.8–2.0.

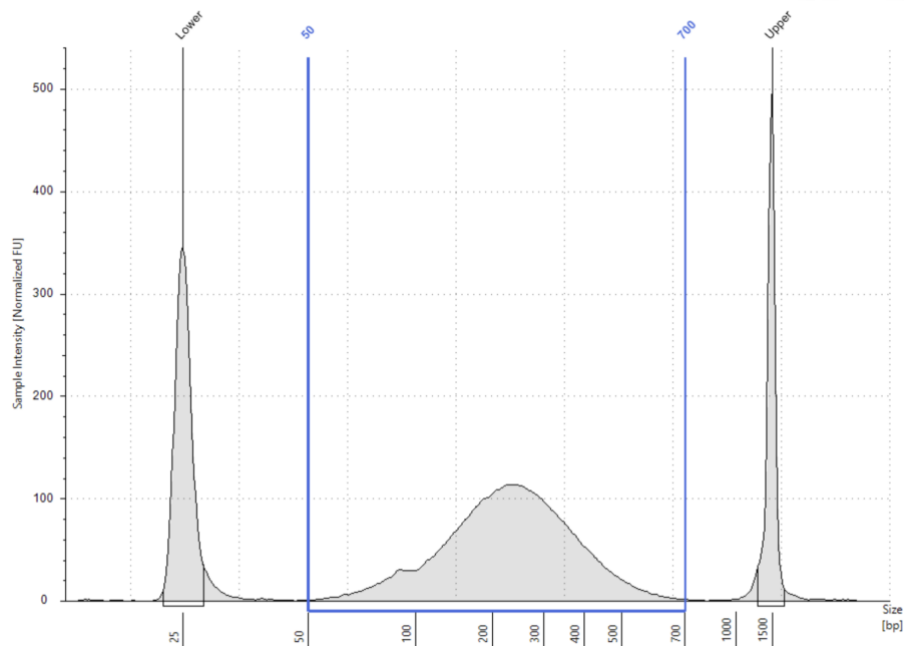


Figure 2. A typical TapeStation trace (D1000) of gDNA on a TapeStation at 250 bp.

Cell-Free DNA (cfDNA)

2.5 cfDNA does not need fragmenting before the assay.

2.6 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.

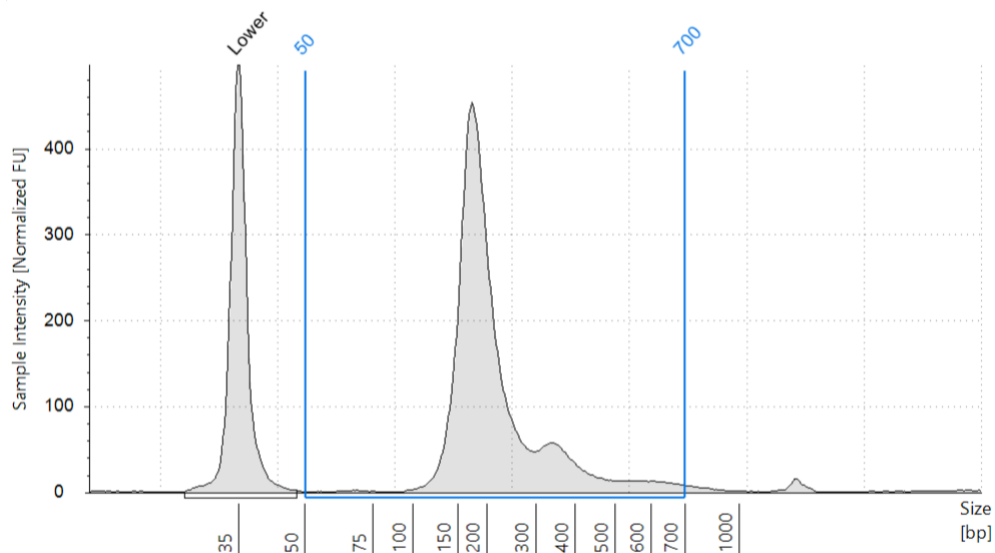


Figure 3. A typical of a cfDNA ScreenTape. cfDNA profiles present a prominent peak at approximately 170 bp (mononucleosome) and potential subsequent peaks (nucleosome multimers).

Spike-in Control DNA Preparation

- 2.7 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 38.

Guidance for spike-in Control DNA (white lid)*		
DNA input mass	Shallow sequencing	Deep sequencing
50–80 ng	Use 1 µl undiluted spike-in Control DNA	Dilute 1:10 in nuclease-free water (lab supply), use 1 µl of diluted spike-in Control DNA
< 50 ng	Dilute 1:10 in nuclease-free water (lab supply), use 1 µl of diluted spike-in Control DNA	

*See *Sequencing* on page 38.

Sample Preparation

- 2.8 Adjust appropriate volume of cfDNA or fragmented gDNA (as specified above) with NF Water (white lid) to a final volume of **24 µl** and add **1 µl spike-in Control DNA** according to guidance in 2.8.



Note: The protocol is optimised for a 260/280 absorbance ratio value of 1.8–2.0.

3. End Repair and A-Tailing

Reagents

- ER Buffer v2 (yellow lid) (ERB2)
- ER Enzyme v2 (yellow lid) (ERE2)

- 3.1 Prepare ER Buffer v2 (yellow lid) and ER Enzyme v2 (yellow lid) by thawing and placing on ice just before use.



Note:

- Vortex ER Buffer v2 (yellow lid) before use to ensure any precipitate is fully dissolved.
- Mix carefully ER Enzyme v2 (yellow lid) and spin down briefly to collect all liquid from the sides of the tubes.

3.2 Prepare a reaction master mix **on ice** as set out in the table below:

 Note:

- ER Enzyme v2 and ER Buffer v2 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 the DNA, a small amount of froth does not affect the reaction.

Component	Volume – per reaction (µl)	Volume – 4 reactions* (µl)	Volume – 8 reactions* (µl)
ER Buffer v2 (yellow lid)	3.0	13.2	26.4
ER Enzyme v2 (yellow lid)	3.0	13.2	26.4
Total Master Mix Volume:	6.0	26.4	52.8

*Volumes include 10% overage. If preparing a master mix for a different number of samples than shown, calculate the volume needed for each reagent using the formula:
Reagent volume x no. of samples x 1.1.

3.3 Mix well and pulse spin to collect all liquid from the sides of the tubes.

3.4 Add **6 µl** of master mix to each 0.2 ml PCR tube containing 25.0 µl of DNA sample from step 2.8. Mix well and pulse spin to collect all liquid from the sides of the tubes.

3.5 Incubate in a thermal cycler programmed as below:

Temp (°C)	Time	Heated Lid	Volume (µl)
20	30 min	75 °C	31.0
65	30 min		
4	Hold		

3.6 Proceed immediately to Hairpin Adapter Ligation.

4. Hairpin Adapter Ligation

Reagents

- Hairpin Adapter (green lid) (HA)
- Ligation MM v2 (green lid) (LMM2)
- Ligation Buffer (green lid) (LB)
- NF Water (white lid)

4.1 Prepare Hairpin Adapter (green lid), Ligation MM v2 (green lid) and Ligation Buffer (green lid) by thawing and placing on ice just before use.

 **Note:** Ligation MM v2 and Ligation Buffer are used again in section 9.

4.2 Add **4.1 µl** of Hairpin Adapter (green lid) to each sample from step 3.5 **on ice**.

4.3 Prepare a reaction master mix **on ice** as set out in the table below:

 **Note:**

- **Do not** add adapters to the master mix. Add individually to each reaction.
- Reagents are highly viscous. Mix carefully and pulse spin briefly to collect all liquid from the sides of the tubes. Take care when pipetting and ensure required volume ejects from the tip.

Component	Volume – per reaction (µl)	Volume – 4 reactions* (µl)	Volume – 8 reactions* (µl)
Ligation Buffer (green lid)	13.8	60.7	121.4
Ligation MM v2 (green lid)	5.0	22.0	44.0
NF Water (white lid)	1.1	4.8	9.7
Total Master Mix Volume:	19.9	87.5	175.1

*Volumes include 10% overage. If preparing a master mix for a different number of samples than shown, calculate the volume needed for each reagent using the formula: **Reagent volume x no. of samples x 1.1**.

4.4 Mix well and pulse spin to collect all liquid from the sides of the tubes.

4.5 Aliquot **19.9 µl** of master mix into each 0.2 ml PCR tube containing 35.1 µl DNA from step 4.2 above. Mix well and pulse spin to collect all liquid from the sides of the tubes.

4.6 Incubate in a thermal cycler programmed as below:

Temp (°C)	Time	Heated Lid	Volume (µl)
20	15 mins	OFF	55.0
4	Hold		

4.7 Proceed immediately to Bead-Mediated Purification 1.

5. Bead-Mediated Purification 1

Reagents

- Magnetic Beads (white lid)
- NF Water (white lid)
- Freshly prepared 80% ethanol

5.1 Bring the DNA from step 4.6 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 bead-based purification at room temperature by combining the following:

Component	Volume – per reaction (µl)
DNA from step 4.6	55.0
Magnetic Beads (white lid)	38.5
Total Volume:	93.5

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 liquid from the sides of the tube and place the tube into a magnet to capture the beads. Leave until the liquid is clear (~3–5 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 in the magnet at room temperature for minimum of 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, re-place 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 from the magnet, add **24.7 µl** of NF Water (white lid) and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for a minimum of 2 mins to elute the DNA from the beads.
- 5.12 Pulse spin the samples to collect 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 **23.7 µl** of the clear supernatant to a new 0.2 ml PCR tube on ice.
- 5.14 Proceed immediately to Hairpin Digestion.

6. Hairpin Digestion

Reagents

- HD Buffer v2 (orange lid) (HDB2)
 - HD Enzyme v2 (orange lid) (HDE2)
 - NF Water (white lid)
- 6.1 Prepare HD Buffer v2 (orange lid) and HD Enzyme v2 (orange lid) by thawing and placing on ice just before use.

6.2 Prepare a reaction master mix **on ice** as set out in the table below:

 **Note:** HD Enzyme v2 and HD Buffer v2 become very frothy if mixed too vigorously. Please take care when handling before adding to samples to ensure maximum recovery of the reagents.

 **Note:** the master mix can be frothy, but it does not impact performance.

Component	Volume – per reaction (µl)	Volume – 4 reactions* (µl)	Volume – 8 reactions* (µl)
HD Buffer v2 (orange lid)	3.0	13.2	26.4
HD Enzyme v2 (orange lid)	4.0	17.6	35.2
NF Water (white lid)	0.3	1.3	2.6
Total Master Mix Volume:	7.3	32.1	64.2

*Volumes include 10% overage. If preparing a master mix for a different number of samples than shown, calculate the volume needed for each reagent using the formula: **Reagent volume x no. of samples x 1.1**.

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

6.4 Aliquot **7.3 µl** of master mix into each 0.2 ml PCR tube containing 23.7 µl DNA from step 5.13 above. 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 (°C)	Time	Heated Lid	Volume (µl)
37	30 mins	47°C	31.0
4	Hold		

6.6 Proceed immediately to Bead-Mediated Purification 2.

7. Bead-Mediated Purification 2

Reagents

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

- 7.1 Bring the DNA from step 6.5 to room temperature.
- 7.2 Vortex the Magnetic Beads for 30 secs, or until fully resuspended and homogenous. Use immediately. Do not let the beads settle before use.
- 7.3 Perform bead-based purification at room temperature by combining the following:

Component	Volume – per reaction (µl)
DNA from step 6.5	31.0
Magnetic Beads (white lid)	55.8
Total Volume:	86.8

- 7.4 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.
- 7.5 Pulse spin the samples to collect liquid from the sides of the tube and place the tube into a magnet to capture the beads. Leave until the liquid is clear (~3–5 mins).
- 7.6 Keeping the tube on the magnet, carefully remove and discard the supernatant using a P200 pipette.
- 7.7 Keeping the tube on the magnet, add 200 µl of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube in the magnet at room temperature for minimum of 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.
- 7.8 Repeat step 7.7.
- 7.9 Pulse spin the bead pellet, re-place 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.

- 7.10 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.

- 7.11 Remove the tubes from the magnet. Add in **14 µl** of Dilution Buffer (white lid) and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for a minimum of 2 mins to elute the DNA from the beads.

- 7.12 Pulse spin the samples to collect 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).
- 7.13 Transfer **13 µl** of the clear supernatant to a new 0.2 ml PCR tube on ice using a P20 pipette.


SAFE STOP POINT:

For a 3-day protocol, samples can be stored for up to 7 days at -20°C.

8. Strand Copying

Reagents

- SC Buffer (blue lid) (SCB)
- dNTPs (blue lid) (dNTP)
- SC Enzyme 1 (blue lid) (SCE1)
- SC Enzyme 2 (blue lid) (SCE2)
- hmCP Additive (brown lid) (hmCPA)
- hmCP Enzyme (brown lid) (hmCPE)

- 8.1 Prepare SC Buffer (blue lid), dNTPs (blue lid), SC Enzyme 1 (blue lid), SC Enzyme 2 (blue lid), hmCP Additive (brown lid) and hmCP Enzyme (brown lid) by thawing and placing on ice just before use.

 **Note:** hmCP Additive and hmC Enzyme are used again in section 13.

- 8.2 Prepare a reaction master mix **on ice** as set out in the table below:

Component	Volume – per reaction (µl)	Volume – 4 reactions* (µl)	Volume – 8 reactions* (µl)
SC Buffer (blue lid)	2.5	11.0	22.0
dNTPs (blue lid)	2.0	8.8	17.6
SC Enzyme 1 (blue lid)	2.0	8.8	17.6
SC Enzyme 2 (blue lid)	2.0	8.8	17.6
hmCP Additive (brown lid)	2.5	11.0	22.0
hmCP Enzyme (brown lid)	1.0	4.4	8.8
Total Master Mix Volume:	12.0	52.8	105.6

*Volumes include 10% overage. If preparing a master mix for a different number of samples than shown, calculate the volume needed for each reagent using the formula: **Reagent volume x no. of samples x 1.1.**

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

8.4 Aliquot **12 µl** of master mix into each 0.2 ml PCR tube containing 13 µl purified DNA from step 7.13. Mix well and pulse spin to collect all liquid from the sides of the tube.

8.5 Incubate in a thermal cycler programmed as below:

Temp (°C)	Time	Heated Lid	Volume (µl)
37	30 mins	105°C	25.0
95	2 mins		
Cool down to 10°C	at -0.1°C/s.		
10	Hold		

8.6 Proceed immediately to Forkhead Adapter Ligation.

9. Forkhead Adapter Ligation

Reagents

- Forkhead Adapter (purple lid) (FA)
- Ligation MM v2 (green lid) (LMM2)
- Ligation Buffer (green lid) (LB)
- NF Water (white lid)

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

9.2 Add **2.5 µl** of Forkhead Adapter (purple lid) to each sample from step 8.5 **on ice**.

9.3 Prepare a reaction master mix on ice as set out in the table below:



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

Component	Volume – per reaction (µl)	Volume – 4 reactions* (µl)	Volume – 8 reactions* (µl)
Ligation Buffer (green lid)	12.5	55.0	110.0
Ligation MM v2 (green lid)	4.5	19.8	39.6
NF Water (white lid)	5.5	24.2	48.4
Total Master Mix Volume:	22.5	99.0	198.0

*Volumes include 10% overage. If preparing a master mix for a different number of samples than shown, calculate the volume needed for each reagent using the formula:
Reagent volume x no. of samples x 1.1.

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

9.5 Aliquot **22.5 µl** of master mix into each 0.2 ml PCR tube containing 27.5 µl DNA from step 9.2 above. 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 (°C)	Time	Heated Lid	Volume (µl)
20	15 mins	OFF	50.0
4	Hold		

9.7 Proceed immediately to Bead-Mediated Purification 3.

10. Bead-Mediated Purification 3

Reagents

- Magnetic Beads (white lid)
- NF Water (white lid)
- Freshly prepared 80% ethanol

- 10.1 Bring the DNA from step 9.6 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 bead-based purification at room temperature by combining the following:

Component	Volume – per reaction (µl)
DNA from step 9.6	50.0
Magnetic Beads (white lid)	60.0
Total Volume:	110.0

- 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 liquid from the sides of the tube and place the tube into a magnet to capture the beads. Leave until the liquid is clear (~3–5 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 µl of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube in the magnet at room temperature for minimum of 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, re-place 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 **16 µl** of NF Water (white lid) and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for a minimum of 2 mins to elute the DNA from the beads.
- 10.12 Pulse spin the samples to collect 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 **15 µl** of the clear supernatant to a new 0.2 ml PCR tube on ice using a P20 pipette.

**SAFE STOP POINT:**

For a 2-day protocol, samples can be stored for up to 7 days at -20°C.

11. Copy Methylation

Reagents

- MT Enzyme (1x 8-strip tubes per pouch) (MTE)
- MT Additive 1 (pink lid) (MTA1)
- MT Additive 2 (pink lid) (MTA2)
- MT Buffer (pink lid) (MTB)



Note: Each pouch contains 1x 8-strip tubes, where each strip contains 1 lyophilised bead. Each bead is for one reaction.

- 11.1 Prepare MT Buffer (pink lid), MT Additive 1 (pink lid), MT Additive 2 (pink lid), MT Enzyme by thawing and placing on ice just before use.



Note: Pouches containing lyophilised beads do not need thawing. Remove and place on ice until use.

11.2 Prepare a reaction master mix **on ice** 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 – 4 reactions* (µl)	Volume – 8 reactions* (µl)
MT Buffer (pink lid)	11.6	51.0	102.1
MT Additive 1 (pink lid)	0.4	1.8	3.5
MT Additive 2 (pink lid)	1.0	4.4	8.8
Total Master Mix Volume:	13	57.2	114.4

*Volumes include 10% overage. If preparing a master mix for a different number of samples than shown, calculate the volume needed for each reagent using the formula:

Reagent volume x no. of samples x 1.1.

11.3 Remove required number of strip-tubes containing MT enzyme beads (1x 8-strip tube for 8 reactions – 1 strip/sample).



Note: Only tear open the numbers of pouches required for the sample (once torn-open, use within 3 months).

11.4 Spin the tubes containing MT enzyme beads to ensure the beads are at the bottom.

11.5 Add 13 µl of the master mix to each of the MT enzyme tubes to reconstitute the lyophilized material.



Note: Flick-mix the strip tubes until the lyophilised materials are homogenously resuspended. Pulse spin to collect all volume at the bottom of the tube.

11.6 Transfer all the reconstituted material from step 11.5 to the strips containing Forkhead ligated DNA from step 10.13.

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

11.8 Incubate in a thermal cycler as below:

Temp (°C)	Time	Heated Lid	Volume (μl)
23	60 mins	75°C	30.0
65	15 mins		
4	Hold		

11.9 Proceed to Bead mediated purification 4.



SAFE STOP POINT:

For a 2-day protocol, samples can be stored overnight at 4°C.

12. Bead-Mediated Purification 4

Reagents

- Magnetic Beads (white lid)
- NF Water (white lid)
- Freshly prepared 80% ethanol

12.1 Bring the DNA from step 11.8 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 bead-based purification at room temperature by combining the following:

Component	Volume – per reaction (μl)
DNA from step 11.8	30.0
Magnetic Beads (white lid)	36.0
Total Volume:	66.0

- 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 liquid from the sides of the tube and place the tube into a magnet to capture the beads. Leave until the liquid is clear (~3–5 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 in the magnet at room temperature for minimum of 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, re-place 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 the tubes from the magnet. Add **31 µl** of NF Water (white lid) and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for a minimum of 2 mins to elute the DNA from the beads.
- 12.12 Pulse spin the samples to collect 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).
- 12.13 Transfer **30 µl** of the clear supernatant to a new 0.2 ml PCR tube on ice using a P20 pipette.

**SAFE STOP POINT:**

For a 2-day protocol, samples can be stored for up to 7 days at -20°C.

13. 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.
- hmCP Additive (brown lid) (hmCPA)
- hmCP Enzyme (brown lid) (hmCPE)
- Ox Enzyme E (brown lid) (OxEE)

- 13.1 Prepare Ox Buffer (brown lid), Ox Additive 1 (brown lid), DTT (brown lid), hmCP Additive (brown lid), hmCP Enzyme (brown lid), Ox Enzyme E (brown lid) and by thawing and placing on ice just before use.
- 13.2 Add **400 µl** Ox Buffer to Ox Additive 1 tube to form an Ox Additive 1 solution.
- 13.3 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.

13.4 Prepare a reaction master mix **on ice** as set out in the table below:

Component	Volume – per reaction* (μl)	Volume – 4 reactions* (μl)	Volume – 8 reactions* (μl)
Ox Additive 1 solution from step 13.2 (brown lid)	10.0	44.0	88.0
DTT (brown lid)	1.0	4.4	8.8
hmCP Additive (brown lid)	1.0	4.4	8.8
hmCP Enzyme (brown lid)	1.0	4.4	8.8
Ox Enzyme E (brown lid)	2.0	8.8	17.6
Total Master Mix Volume:	15.0	66.0	132.0

*Volumes include 10% overage. If preparing a master mix for a different number of samples than shown, calculate the volume needed for each reagent using the formula:
Reagent volume x no. of samples x 1.1.

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

13.6 Aliquot **15 μl** of master mix into each 0.2 ml PCR tube containing 30 μl DNA from step 12.13. Mix well and pulse spin to collect all liquid from the sides of the tube.

13.7 Remove Ox Additive 2 from the freezer directly before use. Vortex thoroughly and keep on ice.

 **Note:** Ensure Ox Additive 2 is fully resuspended. Ox Additive 2 may be visible as a precipitate on the side of the tube or in the lid. If this occurs, invert the tube and flick-mix followed by thorough vortexing.

13.8 Prepare diluted Ox Additive 2 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)	1.0
Nuclease-free water (lab supply)	1249.0
Total Volume:	1250.0

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

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

13.10 Add **5 μl** of diluted Ox Additive 2 solution to each 0.2 ml PCR tube containing 45 μl DNA.



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

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

13.12 Incubate in a thermal cycler programmed as below with a heated lid set to **47°C**.

Temp (°C)	Time	Heated Lid	Volume (μl)
37	60 mins	47°C	50.0
4	Hold		

13.13 Proceed immediately to Bead-Mediated Purification 5.

14. Bead-Mediated Purification 5

Reagents

- Magnetic Beads (white lid)
- NF Water (white lid)
- Freshly prepared 80% ethanol

14.1 Bring the DNA from step 13.12 to room temperature.

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

14.3 Perform 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)
DNA from step 13.12	50.0
Magnetic Beads (white lid)	90.0
Total Volume:	140.0

- 14.4 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.
- 14.5 Pulse spin the samples to collect liquid from the sides of the tube and place the tube into a magnet to capture the beads. Leave until the liquid is clear (~3–5 mins).
- 14.6 Keeping the tube on the magnet, carefully remove and discard the supernatant using a P200 pipette.
- 14.7 Keeping the tube on the magnet, add 200 µl of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube in the magnet at room temperature for minimum of 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.
- 14.8 Repeat step 14.7.
- 14.9 Pulse spin the bead pellet, re-place 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.

- 14.10 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.

- 14.11 Remove tubes from magnet. Add **32 µl** of NF Water (white lid) and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for a minimum of 2 mins to elute the DNA from the beads.
- 14.12 Pulse spin the samples to collect 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).

- 14.13 Transfer **31 µl** of the clear supernatant to a new 0.2 ml PCR tube on ice using a P200 pipette.
- 14.14 Set aside 1 µl of supernatant for Qubit analysis. The remaining **30 µl** is taken forward to the next step.

 **Note:** the 1 µL of supernatant can be used for Qubit analysis in the event of low yields after the final library quantification stage at step 19.

**SAFE STOP POINT:**

For 3-day protocol, samples can be stored for up to 7 days at -20°C.

15. Deamination of Cytosines

Reagents

- DA Buffer (red lid) (DAB)
- NF Water (white lid)
- ATP (red lid) (ATP)
- MgCl₂ (red lid) (Mg)
- DM Enzyme 1 (red lid) (DME1)
- DA Enzyme 2 (red lid) (DAE2)

- 15.1 Prepare DA Buffer (red lid), ATP (red lid), MgCl₂ (red lid), DM Enzyme 1 (red lid), DA Enzyme 2 (red lid) by thawing and placing on ice just before use.

15.2 Prepare a reaction master mix **on ice** as set out in the table below:

Component	Volume – per reaction (µl)	Volume – 4 reactions* (µl)	Volume – 8 reactions* (µl)
NF Water (white lid)	12.75	56.1	112.2
DA Buffer (red lid)	17.5	77.0	154.0
ATP (red lid)	1.75	7.7	15.4
MgCl ₂ (red lid)	3.5	15.4	30.8
DM Enzyme 1 (red lid)	2.0	8.8	17.6
DA Enzyme 2 (red lid)	2.5	11.0	22.0
Total Master Mix Volume:	40.0	176.0	352.0

*Volumes include 10% overage. If preparing a master mix for a different number of samples than shown, calculate the volume needed for each reagent using the formula:
Reagent volume x no. of samples x 1.1.

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

15.4 Aliquot **40 µl** of master mix into each 0.2 ml PCR tube containing 30 µl DNA from step 14.13. Mix well and pulse spin to collect all liquid from the sides of the tube.

15.5 Incubate in a thermal cycler programmed as below with a heated lid set to **47°C**.

Temp (°C)	Time	Heated Lid	Volume (µl)
37	90 mins	47°C	70.0
4	Hold		



SAFE STOP POINT:

For a 3-day protocol, this reaction can be left at 4°C overnight.

16. Bead-Mediated Purification 6

Reagents

- DA Cleanup (red lid)
- Magnetic Beads (white lid)
- NF Water (white lid)
- Freshly prepared 80% ethanol

16.1 Bring the DNA from step 15.5 to room temperature.

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



Note: DA Cleanup should be used at room temperature. If there is precipitate, vortex until the precipitate fully dissolves. **Make sure solution is homogenous and free of any precipitate before use.**

16.3 Add **6.5 µl** of DA Cleanup to each sample and mix the tubes by briefly vortexing. Overmixing may result in abundant foam.

Component	Volume – per reaction (µl)
DNA from step 15.5	70.0
DA Cleanup (red lid)	6.5
Total Volume:	76.5

16.4 Perform 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)
DNA from step 16.3	76.5
Magnetic Beads (white lid)	91.8
Total Volume:	168.3

16.5 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.

- 16.6 Pulse spin the samples to collect liquid from the sides of the tube and place the tube into a magnet to capture the beads. Leave until the liquid is clear (~3–5 mins).
- 16.7 Keeping the tube on the magnet, carefully remove and discard the supernatant using a P200 pipette.
- 16.8 Keeping the tube on the magnet, add 200 µl of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube in the magnet at room temperature for minimum of 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.
- 16.9 Repeat step 16.8.
- 16.10 Pulse spin the bead pellet, re-place 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.
- 16.11 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.
- 16.12 Remove tubes from magnet. Add **21 µl** of NF Water (white lid) and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for a minimum of 2 mins to elute the DNA from the beads.
- 16.13 Pulse spin the samples to collect 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).
- 16.14 Transfer **20 µl** of the clear supernatant to a new 0.2 ml PCR tube on ice.

17. Library Amplification

Reagents

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

- 17.1 Prepare PCR MM (clear lid) and UDI primers (clear lid) by thawing and placing on ice just before use.
- 17.2 Assemble each amplification reaction **on ice** as follows.

 Note:

- Reagents include up to 96 different UDI primers. UDI primers 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 UDI primers (1-8, 1C-8C, 1-24, 1B-24B, 1A-96A) are used for each prepared reaction, as only one individual primer is used per reaction.

Component	Volume – per reaction (µl)
Purified DNA from step 16.14	20.0
PCR MM (clear lid)	25.0
UDI primers 1-8 or 1C-8C or 1-24/1B-24B (clear lid) or 1A-96A (plate)	5.0
Total Volume:	50.0

- 17.3 Mix well and pulse spin to collect all liquid from the sides of the tube.
- 17.4 Amplify in a thermal cycler programmed as below with a heated lid set to **105°C**:

	Temp (°C)	Time	Cycles	Heated Lid	Volume (µl)
Initial Denaturation	98	30 secs	1	105°C	50.0
Denaturation	98	10 secs	6-8 cycles*		
Annealing	62	30 secs			
Extension	65	60 secs			
Final extension	65	5 mins	1		
Hold	4	Hold	1		

*PCR cycle recommendations according to sample type and input concentration:

if using gDNA:

10 ng to <20 ng input amount: 8 cycles
 20 ng to <40 ng input amount: 7 cycles
 40 ng to ≤80 ng input amount: 6 cycles

if using cfDNA:

5 ng to <10 ng input amount: 8 cycles
 10 ng to <30 ng input amount: 7 cycles
 30 ng input amount: 6 cycles

18. Bead-Mediated Purification 7

Reagents

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

- 18.1 Bring the DNA from step 17.4 to room temperature.
- 18.2 Vortex the Magnetic Beads for 30 secs, or until fully resuspended and homogenous. Use immediately. **Do not** let the beads settle before use.
- 18.3 Perform bead-based purification at room temperature by combining the following:

Component	Volume – per reaction (µl)
DNA from step 17.4	50.0
Magnetic Beads (white lid)	45.0
Total Volume:	95.0

- 18.4 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.
- 18.5 Pulse spin the samples to collect liquid from the sides of the tube and place the tube into a magnet to capture the beads. Leave until the liquid is clear (~3–5 mins).
- 18.6 Keeping the tube on the magnet, carefully remove and discard the supernatant using a P200 pipette.
- 18.7 Keeping the tube on the magnet, add 200 µl of freshly prepared 80% ethanol (be careful not to disturb the pellet). Incubate the tube in the magnet at room temperature for minimum of 30 secs or until the supernatant is clear. Carefully remove and discard the ethanol.
- 18.8 Repeat step 18.7.
- 18.9 Pulse spin the bead pellet, re-place 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.

- 18.10 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.
- 18.11 Remove tubes from the magnet. Add **15 µl of Dilution Buffer** (white lid) and thoroughly resuspend by vortexing for 5–10 secs or until fully homogenous and incubate at room temperature for a minimum of 2 mins to elute the DNA from the beads.
- 18.12 Pulse spin the samples to collect 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).
- 18.13 Transfer **14 µl** of the clear supernatant to a new 0.2 ml PCR tube.

19. Library Quantification

- 19.1 Use 1 µl final library to quantify using a Qubit dsDNA HS kit. Expected final yield range is approximately 4–60 ng/µl.
- 19.2 Using the Qubit results, dilute 1 µl of the final library to 20–50 ng/µl for TapeStation D5000 or Bioanalyzer analysis.
- 19.3 Use a TapeStation or Bioanalyzer to determine the size distribution and concentration of the library in nM. Expected average fragment size should be 550–750 bp. Establish a region table that bounds the library to determine its median insert size (suggested range is 250–2500 bp).

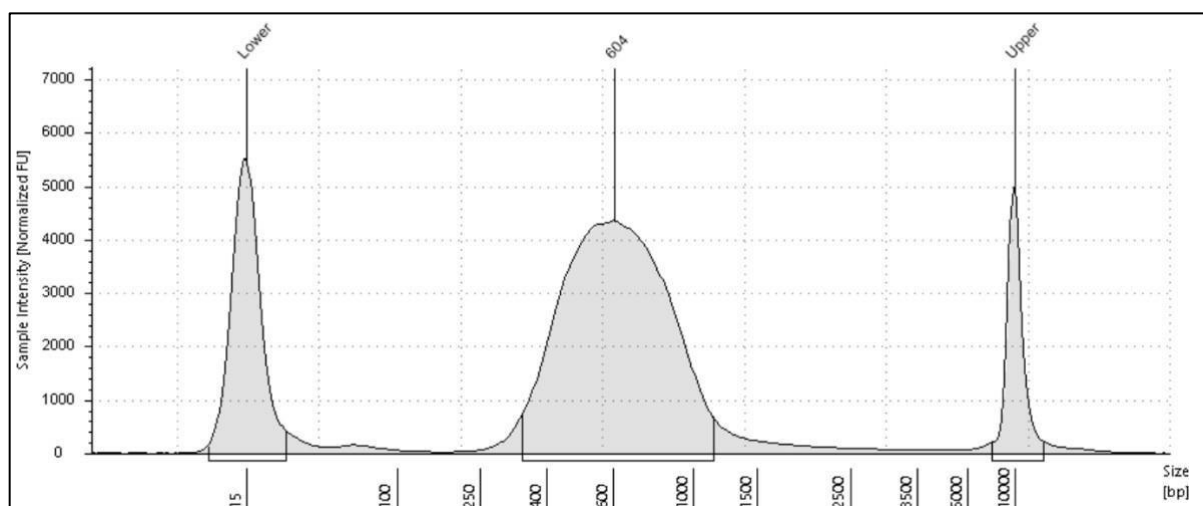


Figure 4. A typical TapeStation trace (D5000) of a duet evoC library using gDNA.

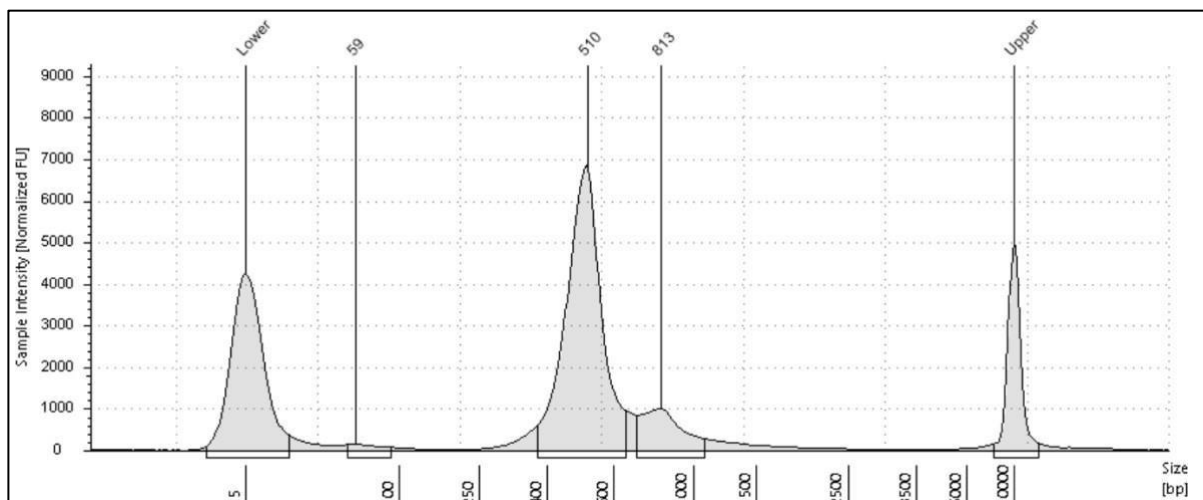


Figure 5. A typical TapeStation trace (D5000) of a duet evoC library using cfDNA.

19.4 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).

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).

19.5 Determine the molar concentration in nM of the final libraries using the following calculation:

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



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 any Illumina sequencer 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. **Add 5–15% PhiX** to pooled libraries depending on sequencer type (8% is a good starting point). 10% PhiX is recommended for best results on NovaSeqX with software 1.2 and below. 5% PhiX for NovaSeqX software 1.3 and above.
 - 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 deconvolution.
 - 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 NextSeq2000 sequencing 650 pM should be used as a starting point for optimization.
 - For NovaSeqX sequencing 150 pM should be used as a starting point for optimization.
-
-  **IMPORTANT:** if you are using another sequencing platform, reach out to biomodal support for further recommendation.
 -  **IMPORTANT:** we recommend 4 or more samples 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.

F. Revision History

Version	Date	Description
2	March 2024	Protocol release
3	July 2024	Change in MT enzyme format: 8-strip tubes instead of 8 individual tubes. Kit available in 24 reactions format in addition to 8 reactions.
5	December 2024	Updated for new kit configuration, including minor workflow adjustments. Compatible with Product Numbers 6205-1 / 6205-2 (8 reactions) and 6206-1 / 6206-2 (24 reactions).
5.1	March 2025	Updated sequencing recommendations page 38 with NovaseqX guidance. Updated for general minor consistency throughout.
5.2	December 2025	Corrected product code for 24RXN kit in the kit information section. Added reduced PhiX requirements for NovaSeq v1.3 software in the sequencing recommendations page 34. Other minor typos, figures, and wording updates for accuracy.
5.3	February 2026	Added product code for new duet UDIs 1C-8C (4103). Updated product description for duet UDIs 1B-24B (4102).