# 164mer Libraries: Testing cfDNA with 164mer Model

Tanner Baldwin – 08 August 2024

**Background**

Previously, we optimized the R8 recipe to achieve very satisfactory conversion efficiency while causing minimal DNA damage to lambda DNA with pUC19 and 164mer spike-ins. We sent the reagents for this optimized recipe to Dr. Yuval, our Israeli collaborator and they tested the method on cfDNA. Surprisingly, the results showed that our method performed less well than the Zymo DNA Methylation Gold kit when assessed with digital droplet PCR (ddPCR). We do not have a ddPCR machine in Chicago, but we will test our method more rigorously with cfDNA provided by Yushuai as well as a methylated ds164mer spike-in as a control. We will also test our method starting from 5, 0.5, and 0.05 ng cfDNA with dsDNA 164mer as spike in and compare with EM-seq and Zymo gold kit with the same DNA input.

The final round of R8 optimization showed that R8-4 is the optimal recipe in both 55 C for 90 min and 75 C for 20 min. We also found that the reaction with 10% DME achieves best conversion efficiency (although perhaps at the cost of some additional damage). Since R8-4 at 55 C for 90 min showed better performance in terms of both the DNA damage and BS efficiency. We will try this condition. In addition, since our sequencing data in last round showed that adding 10% DME can further improve the BS efficiency, we will also test R8-4 (40 ul) + 5 ul denatured DNA + 5 ul DME to make comparison.

Tanner did the dsDNA ligation for both cfDNA and 164 bp DNA. The QC of the ligated DNA showed the expected pattern, suggesting that the mistake caused by adding ligation did cause serious self-ligation of cfDNA. However, the OCC purification to try to recovery the cfDNA did cause significant cfDNA loss. Based on the QC results, the concentration of the ligated cfDNA is 4 ng/µl and based on calculation the original cfDNA retained is ~3 ng/µl. We decided to do four group of libraries, with each group containing 3 samples starting from 5, 0.5, and 0.05 ng cfDNA.

**Materials**

| Name | Ref/Cat Number |
|---|---|
| ~~NEBNext UltraShear~~ | ~~#E6655~~ |
| ~~Zymo Methylated and Non-methylated pUC19 DNA Set~~ | ~~D5017~~ |
| 164mer dsDNA oligo (79 ng/µl) | Yiding's Box |
| KAPA Evo Prep Kit | 10096039001 |
| Zymo EZ DNA Methylation Gold Kit (For Desulphonation) | # D5005/6 |
| KAPA HiFi HotStart Uracil+ ReadyMix (For qPCR/PCR) | KK2801/2 |
| AMPure XP Magnetic Beads (For all beads purifications) | Lab Aliquots (Multiple Vendors?) |
| NEB EM-seq Kit | |
| Zymo L-Desulphonation Buffer | |

**Design**

Material Prep Phase

1. Start first with 474 ng of double stranded 164mer oligo
2. Use KAPA EvoPrep Kit to do End Prep and 5mC adaptor ligation, then beads purification eluting with water
3. Separately, start with ~100 ng of Yushuai's cfDNA (4.98 ng/µl)
4. Use KAPA EvoPrep Kit to do End Prep and 5mC adaptor ligation, then beads purification eluting with water
5. Mix the ligated cfDNA and the ligated 164mer spike-in so that the ration is 99.5:0.5. Take out the calculated amount to do denature and then split into 4 groups. The rest is stored at -80 C containing 10 mM Tris buffer 7.5 for future use.

Treatment Phase

6. Make 10-fold dilutions in series to prepare stock tubes at 5, 0.5, and 0.05 ng/µl concentrations
7. Before doing BS treatment, add 0.5 M KOH to a final conc. of 0.3 M and incubate at 42 C for 20 min to denature all material
8. Split into 5 µl samples for treatments adding 45 µl of BS recipe
9. Treatments (19 total) – See the sample information table below
10. Using L-desulphonation buffer to conduct desulphonation for all BS treated samples (X total)
11. qPCR and PCR with unique indexes
12. Give to Chang for sequencing

**Sample Information (19 total)**

| Sample ID | Recipe ID | Starting Material | Recipe | Time | Temperature |
|---|---|---|---|---|---|
| ST | - | 0.5 ng | Only denaturing - no BS | - | - |
| EA | - | 5 ng | EM-seq Kit Reagents | ~4.5 hours | Varied |
| EB | - | 0.5 ng | EM-seq Kit Reagents | ~4.5 hours | Varied |
| EC | - | 0.05 ng | EM-seq Kit Reagents | ~4.5 hours | Varied |
| ZA | - | 5 ng | Zymo DNA Methylation Gold Kit | 10min/2.5hr | 98 C / 64 C |
| ZB | - | 0.5 ng | Zymo DNA Methylation Gold Kit | 10min/2.5hr | 98 C / 64 C |
| ZC | - | 0.05 ng | Zymo DNA Methylation Gold Kit | 10min/2.5hr | 98 C / 64 C |
| 5A | R8-4 | 5 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C |
| 5B | R8-4 | 0.5 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C |
| 5C | R8-4 | 0.05 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C |
| 5DA | R8-4 | 5 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C |
| 5DB | R8-4 | 0.5 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C |
| 5DC | R8-4 | 0.05 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C |
| 7A | R8-4 | 5 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C |
| 7B | R8-4 | 0.5 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C |
| 7C | R8-4 | 0.05 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C |
| 7DA | R8-4 | 5 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C |
| 7DB | R8-4 | 0.5 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C |
| 7DC | R8-4 | 0.05 ng | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C |

## Ligation of ds164mer (KAPA EvoPrep Kit – Full volume)

### 1. End Prep and A-Tailing

To 6 µl of double stranded 164mer DNA (~474 ng) add:

| Reagent | Volume per sample (ul) |
|---|---|
| RNA $H_2O$ | 29 µl |
| KAPA End Repair and A-Tailing Ready Mix | 25 µl |

Sample final volume: **60 µl**

Incubate at: **Pre-cool the block to 4C, add sample, then incubate at 35 C for 30 min followed by 65 C for 30 min (Lid @ 80C)**

### 2. Adaptor Ligation

a. To 60 µl of end repaired DNA (~474 ng)

| Reagent | Volume per sample (ul) |
|---|---|
| NEBNext *Methylated* Adaptor (No Dilution)<br>*- NOT the adaptor included in kit* | 5 µl |
| KAPA Ligation ReadyMix | 10 µl |

Sample final volume: **75 µl**

Incubate at: **Pre-cool block to 4 C, add sample, then incubate at 20 C for 5 minutes with lid at 50 C**

b. To **75 µL of sample**, add:

| Reagent | Volume per sample (ul) |
|---|---|
| USER Enzyme | 3.0 |

Sample final volume: **78**

Incubate at: **37 °C for 15 mins with lid at 50 °C**

### 3. AmPure Beads Purification

*a. Warm beads to RT before use! (~30 min)*

i) 0.8x beads ratio – 62.4 µl.

Elute with 94 µl RNA water to collect 93 µl (~5.04 ng/µl)

**AmPure Beads Purification Procedure**

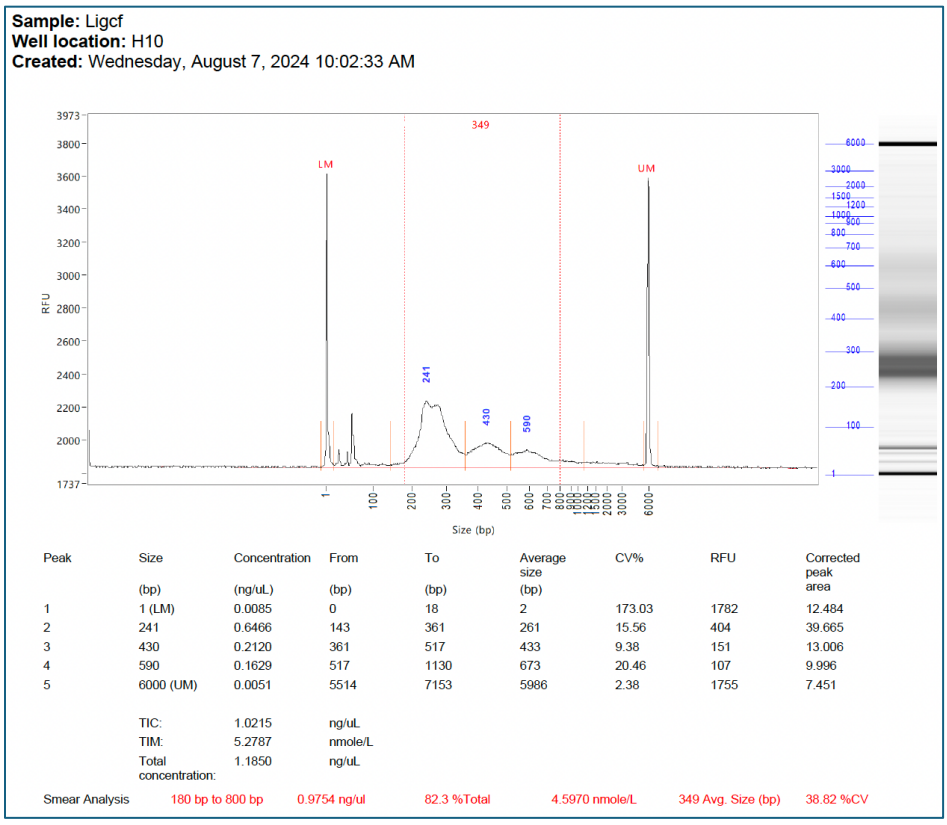
a. Vortex beads to resuspend
b. Add **X** µl beads to sample (pipette up and down to mix)
c. Incubate for up to 5 minutes at RT

d. Place on magnet and discard supernatant once clear
e. Add 200 µl of fresh 80% ethanol
f. Incubate at RT for 30 seconds and remove and discard supernatant
g. Wash again with 200 µl 80% EtOH
h. Air dry the beads for 5 minutes (DO NOT OVER DRY)
i. Elute with **X** µl RNA $H_2O$ by incubating at RT for 2 min
j. Place on magnet and take **X** µl of supernatant to next step

## Ligation of Yushuai's cfDNA (KAPA EvoPrep Kit – Full volume)

NOTE: Ligation ready mix was mistakenly added in place of ERAT ready mix. OCC was performed by adding 40 µl of water to the 60 µl of sample, splitting into two, 50 µl samples and then following zymo protocol exactly with all brand-new reagents. Elution was done with 18 µl and 17.5 was successfully recovered from each sample to give a combined 35 µl to repeat the ERAT step using the proper ready mix.

- Zymo OCC claims 16 nt and up will be recovered with 90% efficiency (their data is with ssDNA however), DCC advertises 70-90% recovery of 50ng and up, based on Ampure Beads data recovery would be low and there could be small loss of 160mer even with 2.5x volume




| Peak | Size (bp) | Concentration (ng/uL) | From (bp) | To (bp) | Average size (bp) | CV% | RFU | Corrected peak area |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 (LM) | 0.0085 | 0 | 18 | 2 | 173.03 | 1782 | 12.484 |
| 2 | 241 | 0.6466 | 143 | 361 | 261 | 15.56 | 404 | 39.665 |
| 3 | 430 | 0.2120 | 361 | 517 | 433 | 9.38 | 151 | 13.006 |
| 4 | 590 | 0.1629 | 517 | 1130 | 673 | 20.46 | 107 | 9.996 |
| 5 | 6000 (UM) | 0.0051 | 5514 | 7153 | 5986 | 2.38 | 1755 | 7.451 |

| | | |
|---|---|---|
| TIC: | 1.0215 | ng/uL |
| TIM: | 5.2787 | nmole/L |
| Total concentration: | 1.1850 | ng/uL |

| Smear Analysis | 180 bp to 800 bp | 0.9754 ng/ul | 82.3 %Total | 4.5970 nmole/L | 349 Avg. Size (bp) | 38.82 %CV |
|---|---|---|---|---|---|---|

Factoring in adapter dimer, peaks are at 110-130, 300, and 460bp.
Total material with adapter dimer is 1.1850*4 (dilution factor) = 4.74*14.5ul (total volume remaining) = 68.73ng of material total
If we assume the ~130bp adapter dimer comprises ~37% of material (130bp/avg size) then we have 43.299ng of cfDNA after the OCC, ligation, and beads purification.

## 4. End Prep and A-Tailing

To 35 µl of Yushuia's cfDNA (~160 ng?) add:

| Reagent | Volume per sample (ul) |
|---|---|
| RNA $H_2O$ | 1.5 µl |
| KAPA End Repair and A-Tailing Ready Mix | 25 µl |

Sample final volume: **60 µl**

Incubate at: **Pre-cool the block to 4C, add sample, then incubate at 35 C for 30 min followed by 65 C for 30 min (Lid @ 80C)**

## 5. Adaptor Ligation

a. To 60 µl of end repaired DNA (~160 ng?)

| Reagent | Volume per sample (ul) |
|---|---|
| NEBNext *Methylated* Adaptor (No Dilution)<br>- *NOT the adaptor included in kit* | 5 µl |
| KAPA Ligation ReadyMix | 10 µl |

Sample final volume: **75 µl**

Incubate at: **Pre-cool block to 4 C, add sample, then incubate at 20 C for 5 minutes with lid at 50 C**

b. To **75 µL of sample**, add:

| Reagent | Volume per sample (ul) |
|---|---|
| USER Enzyme | 3.0 |

Sample final volume: **78**

Incubate at: **37 °C for 15 mins with lid at 50 °C**

**6. AmPure Beads Purification**

*a. Warm beads to RT before use! (~30 min)*

i) 0.8x beads ratio – 62.4 µl.

Elute with 16 µl RNA water to collect 15 µl (~4 ng/µl)

Factoring out the adapter dimer, peaks are at 110-130, 300, and 460bp.
Total material with adapter dimer is 1.1850*4 (dilution factor) = 4.74*14.5ul (total volume remaining) = 68.73ng of material total (including adapter!)
If we assume the ~130bp adapter dimer comprises ~37% of material (130bp/avg size) then we have 43.299ng of cfDNA after the OCC, ligation, and beads purification.

**cfDNA conc. = ~3 ng/µl**

**AmPure Beads Purification Procedure**

k. Vortex beads to resuspend
l. Add **X** µl beads to sample (pipette up and down to mix)
m. Incubate for up to 5 minutes at RT
n. Place on magnet and discard supernatant once clear
o. Add 200 µl of fresh 80% ethanol
p. Incubate at RT for 30 seconds and remove and discard supernatant
q. Wash again with 200 µl 80% EtOH
r. Air dry the beads for 5 minutes (DO NOT OVER DRY)
s. Elute with **X** µl RNA $H_2O$ by incubating at RT for 2 min
t. Place on magnet and take **X** µl of supernatant to next step

7. **Split Ligated material into aliquots of 5, 0.5, and 0.05 ng/µl by making a series dilution**

   a. Dilute the ligated ds164mer (~5 ng/µl) by taking 1 µl of ds164mer and adding 22 µl water → final conc. ~0.217 ng/µl (not including the adapter)

**Our Samples**

| | |
|---|---|
| **Tube 1**<br>5 ng samples desired<br>(~4.69 ng) | 14 µl of ligated sample (~42 ng cfDNA)<br>+ 3 µl water<br>+ 1 µl diluted ds164mer<br><br>TOTAL: 18 µl, cfDNA = 42 ng (2.33 ng/µl), ligated ds164mer = 0.217 ng (0.012 ng/µl = ~0.5%)<br><br>**Take 10 µl for Denaturing, save 8 µl in 10 mM Tris-HCl Buffer pH 7.5 @ -80 C**<br>Add 6 µl 20 M KOH to 234 µl RNA water to make 0.5 M KOH<br>Add 15 µl of 0.5 M KOH to the 10 µl of material<br>Incubate in LARGE thermocycler (which has volume >50 µl) at:<br>**42 C for 20 minutes with lid at 62 C**<br><br>TOTAL: 25 µl |
| **Tube 2**<br>0.5 ng samples desired<br>(~0.52 ng) | 3 µl Tube 1 + 24 µl water (0.094 ng/µl – 5 µl per sample)<br><br>*Take ST sample from this stock* |
| **Tube 3**<br>0.05 ng samples desired<br>(~0.052 ng) | 3 µl Tube 2 + 27 µl water (0.0094 ng/µl – 5 µl per sample) |

**EM-seq and Zymo Kit Samples**

| | |
|---|---|
| **Tube 1**<br>5 ng samples desired<br>(~4.67 ng) | Take 10 µl of the stored material at -80 C (~1.87 ng/µl)<br>- Add 10 µl water<br>- Take 12 µl to be the stock for these "5 ng" samples |
| **Tube 2** | Take 2 µl of Tube 1 and add 16 µl water (9-fold dilution)<br>- |

| | |
|---|---|
| 0.5 ng samples desired (~ ng) | |
| **Tube 3** 0.05 ng samples desired (~ ng) | Take 2 µl of Tube 2 and add 18 µl water (10-fold dilution) |

## Homemade BS treatments

1. **Prepare Reagents**

**20 M KOH**

| Reagent | Volume per sample (ul) |
|---|---|
| KOH | 1119 mg |
| $H_2O$ | 997 ul |

Sample final volume: **~997 µl @ ~20 M**

**R8-4 Recipe**

| Reagent | Volume per sample (ul) |
|---|---|
| NEW 68% ammonium bisulfite | 300 |
| 20 M potassium hydroxide | 6 |

Sample final volume: **~306 µl**

2. **Treatments**
   a. For all other treatments, take two tube strips of *easy cut* 200 µl tubes and label them with the sample IDs
   b. Add 5 µl of denatured material to each sample
      i) Note: For DME samples add 5 µl DME here BEFORE adding bisulfite
      ii) Then in the next step, add only 40 µl of BS recipe (total recipe volume = 45 µl)
   c. Fill sample tubes with 45 µl of the proper BS recipe
   d. Treat according to the sample information table
   ~~e. Preheat the recipes at the proper treatment temperature (as indicated by the sample information table) for up to 2 minutes.~~

f. ~~Pipette 2.0 µl of starting material (ligated lambda DNA) into each preheated recipe and let the reaction proceed for time specified in the sample information table~~

g. When the reaction finishes, remove and add the 100 µl of $H_2O$ that is required for desulphonation. Proceed to desulphonation.

Notes:

- DME smells putrid after treating the DNA, worse smell in 75 C than in 55 C
- DME samples are slightly cloudy after adding the BS recipe, after treatment they become clear
- EM-seq DTT reagent had precipitates that cannot be vortexed away

3. **Desulphonation** (BS treated samples only) **USE DNA KIT** – Note any precipitates!
   a. Prepare X, 1.5 ml tubes and fill them with M-Binding Buffer – 600 µL
   b. Add the 150 µl sample (from treatment step) to the binding buffer and mix well.
   c. Once M-binding buffer and sample have been mixed → Centrifuge Max. speed for 30 s
   d. M-Wash Buffer – 100 µL → Centrifuge Max. speed for 30 s
   e. ~~**L-Desulphonation Buffer**~~ – 200 µL on column
      M-Desulphonation Buffer was used because L-desulph did not arrive!
   f. Incubate at Rm Temp for 20 mins → Centrifuge Max. speed for 30s
   g. M-Wash Buffer – 200 µL → Centrifuge Max. speed for 30 s
   h. Repeat washing for another 3 times
   i. **Discard → Centrifuge Max. speed for 2 mins**
   j. Elute with $H_2O$ with 24 µl to get 23 µl for each sample.

## qPCR (19 Samples)

1. **qPCR**
   a. To **1 µl** of each sample (<1.8 ng), add

| Reagent | Volume per 1 sample (ul) | Volume per 20 samples (ul) |
|---|---|---|
| KAPA HiFI U+ MM | 10 | 200 |
| Universal primer | 1 | 20 |

| | | |
|---|---|---|
| Mixed Index primer | 1 | 20 |
| $H_2O$ | 6 | 120 |
| *20x* SYBR Green | 1 | 20 |
| **Final volume** | **19** | **380** |

Sample final volume: **20 µl →**

| | |
|---|---|
| Pre-incubation | 95 C; 600 s |
| X25 | 95 C; 20 s |
| | 60 C; 20 s |
| | 72 C; 20 s |
| Melting curves | 95 C; 10 s |
| | 65 C; 60 s |
| | 97 C; 1 s |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | |
| | | ST | 5A | 5B | 5C | 5DA | 5DB | 5DC | | | |
| | | | | | | | | | | | |
| | | | 7A | 7B | 7C | 7DA | 7DB | 7DC | | | |
| | | | | | | | | | | | |
| | | | ZA | ZB | ZC | EA | EB | EC | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |

2. **PCR**
   **a.** To **10 ul (< 18 ng)** of sample, add:

| **Reagent** | **Volume per sample (ul)** | **Volume per 24 samples (ul)** |
|---|---|---|

| | | |
|---|---|---|
| KAPA HiFI U+ MM | 12.5 | 250 |
| Universal primer | 1.25 | 25 |
| **Final Volume** | **13.75** | **275** |
| Index primer | 1.25 | *Must be added individually* |

Sample final volume: **25 ul →**

| | |
|---|---|
| x cycles # (Calculate based on the qPCR Ct value) | 98 C; 45s |
| | 98C; 15 s |
| | 60 C; 30 s |
| | 72 C; 30 s |
| | 72 C; 1 min |
| | 4C |

3. **TWO rounds of AmPure beads purification**
   a. 0.6x Beads ratio – 15 ul
   b. $H_2O$ – 21 ul

   c. 0.6x Beads ratio – 12 ul
   d. $H_2O$ – 12 ul
      i. < 2ng/ul: 1ul+2ul H2O to QC
      ii. > 2ng/ul: 0.5+2.5ul H2O to QC

9, 13, 15, 17

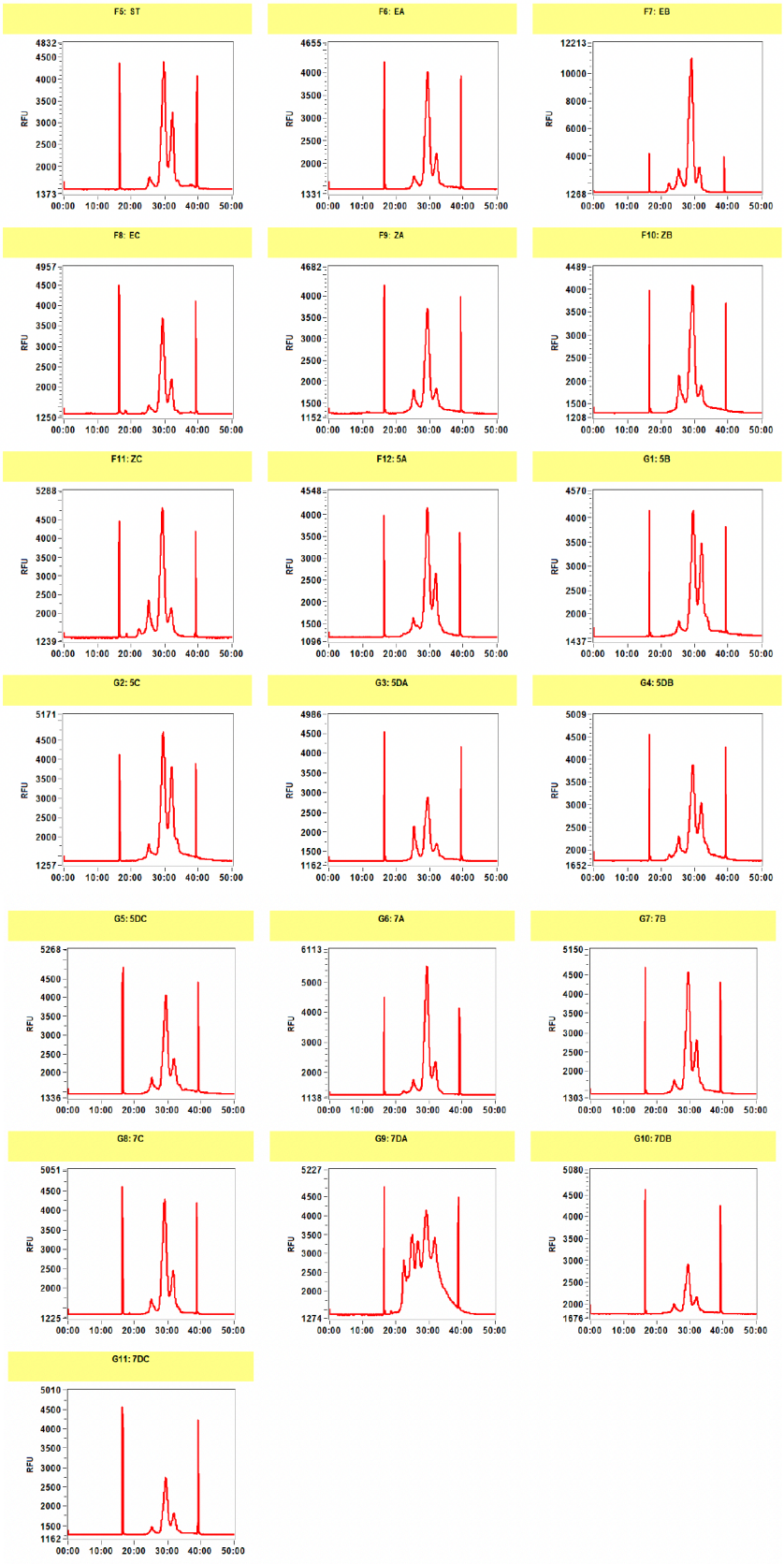
**Library information**

| ID | Recipe ID | Recipe | Time | Temperature | qPCR | PCR | Index | Qubit [ng/uL] | QC Conc [ng/uL] |
|---|---|---|---|---|---|---|---|---|---|
| **ST** | - | Only denaturing - no BS | - | - | 8.6 | 9 | **B12** | **3.44** | |
| **EA** | - | Only denaturing - no BS | ~4.5 hours | Varied | 9.7 | 9 | **C12** | **2.82** | |
| **EB** | - | EM-seq Kit Reagents | ~4.5 hours | Varied | 14.5 | 16 | **D12** | **10.2** | |
| **EC** | - | EM-seq Kit Reagents | ~4.5 hours | Varied | 18.4 | 16* | **E12** | **2.16** | |
| **ZA** | - | EM-seq Kit Reagents | 10min /2.5hr | 98 C / 64 C | 8.8 | 9 | **F12** | **2.98** | |
| **ZB** | - | Zymo DNA Methylation Gold Kit | 10min /2.5hr | 98 C / 64 C | 12.5 | 13 | **G12** | **3.8** | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **ZC** | - | Zymo DNA Methylation Gold Kit | 10min /2.5hr | 98 C / 64 C | 16.2 | 16* | **H12** | **4.54** | |
| **5A** | R8-4 | Zymo DNA Methylation Gold Kit | 90 min | 55 C | 8.4 | 9 | **C11** | **3.5** | |
| **5B** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C | 12.5 | 13 | **D11** | **3.9** | |
| **5C** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C | 15.3 | 16 | **E11** | **5.18** | |
| **5DA** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C | 9.3 | 9 | **F11** | **1.99** | |
| **5DB** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C | 12.2 | 13 | **G11** | **3.42** | |
| **5DC** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 90 min | 55 C | 15.0 | 16 | **H11** | **2.66** | |
| **7A** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C | 9.0 | 9 | **C10** | **3.7** | |
| **7B** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C | 12.8 | 13 | **D10** | **3.42** | |
| **7C** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C | 17.2 | 16* | **E10** | **3.16** | |
| **7DA** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C | 10.4 | 13 | **F10** | **6.38** | |
| **7DB** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C | 13.0 | 13 | **G10** | **1.56** | |
| **7DC** | R8-4 | R8-4 (300 µl 68% NH4BS + 6 µl 20M KOH) | 20 min | 75 C | 16.8 | 16* | **A10** | **1.91** | |

All samples that received 9 cycles were incubated too long in the initial denaturing step at 98 C. All samples incubated for

**Post Library QC Results**


F5: ST
F6: EA
F7: EB
F8: EC
F9: 2A
F10: ZB
F11: ZC
F12: 5A
G1: 5B
G2: 5C
G3: 5DA
G4: 5DB
G5: 5DC
G6: 7A
G7: 7B
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G11: 7DC
RFU
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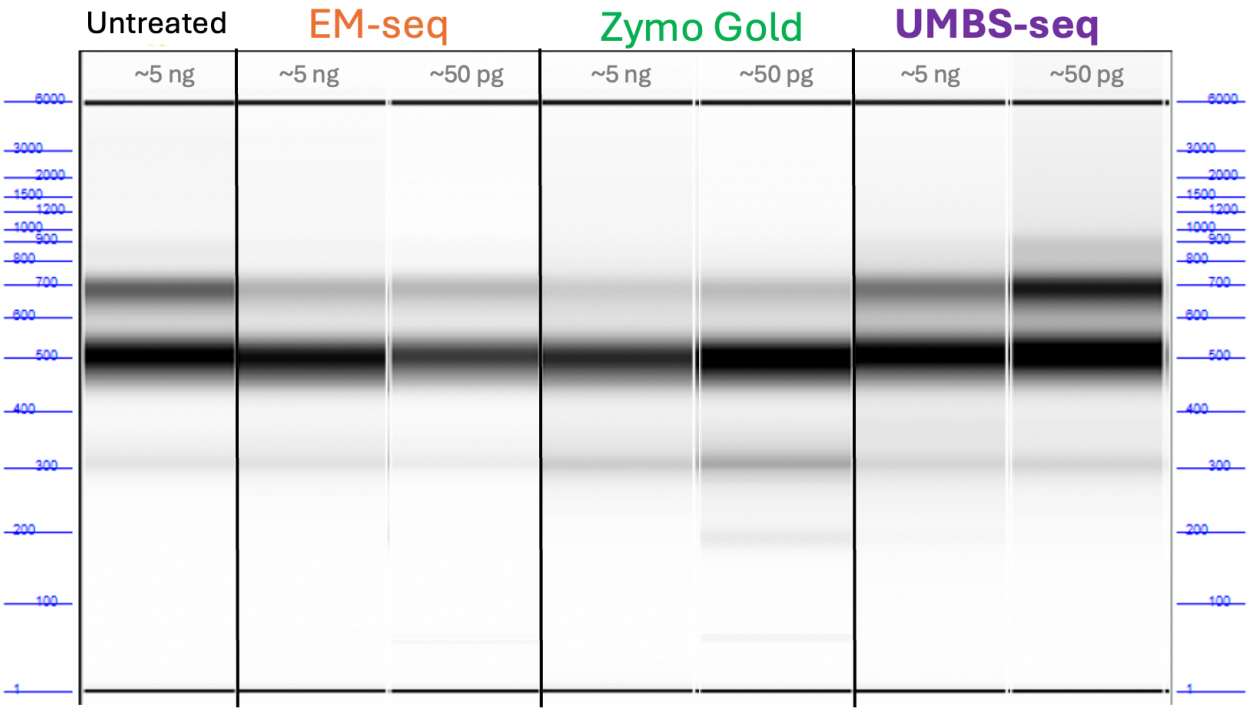
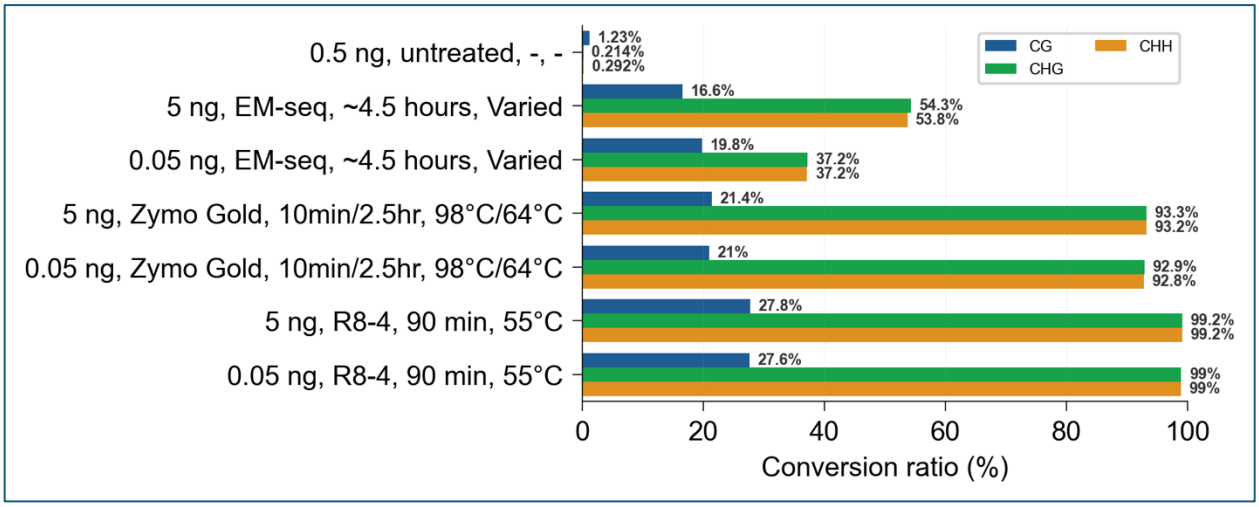
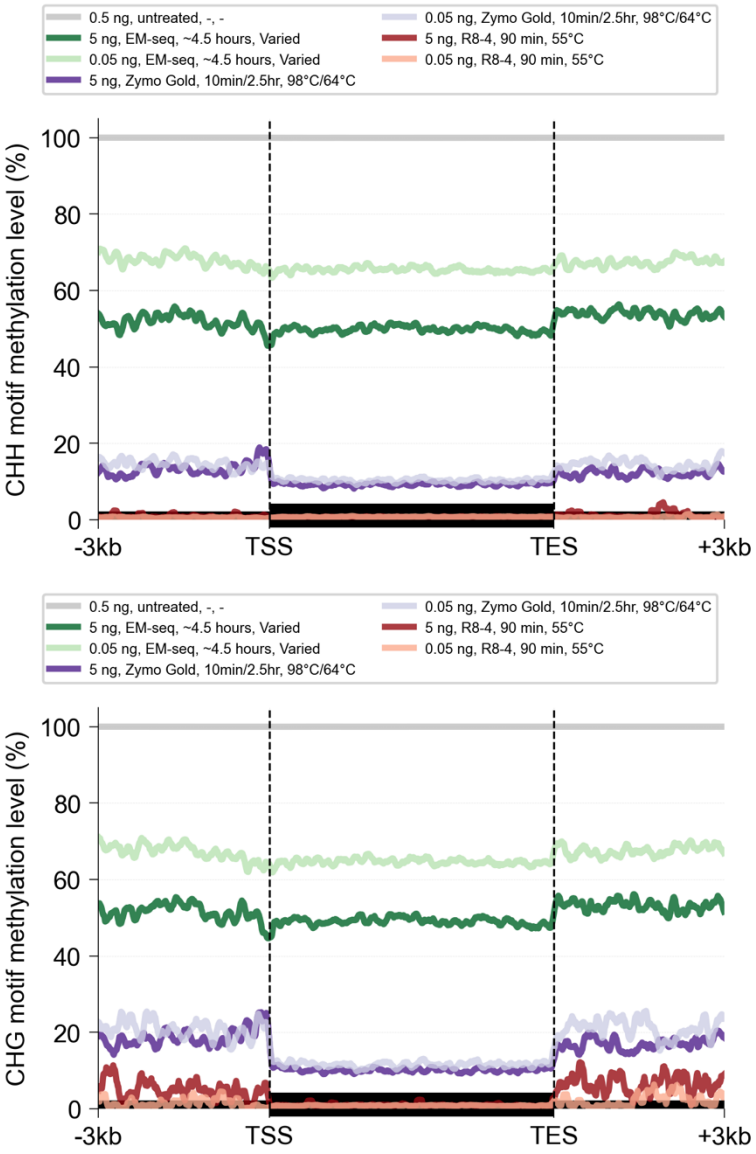
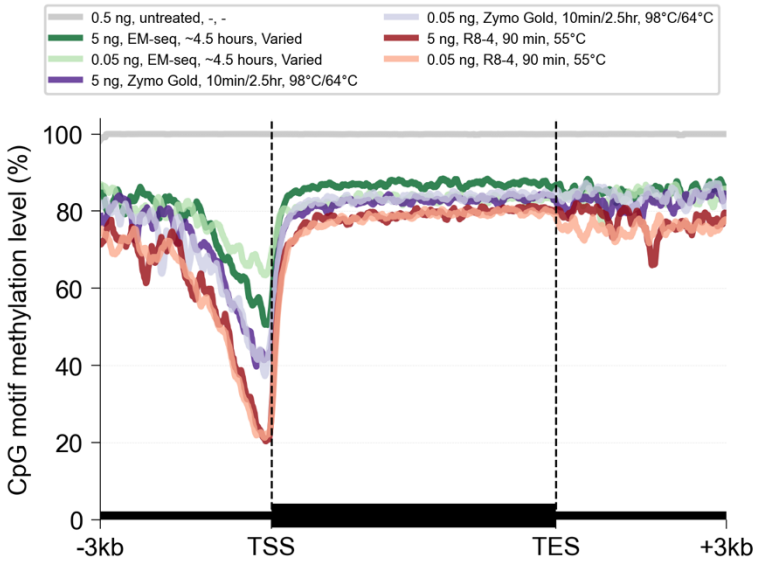

Untreated
EM-seq
Zymo Gold
UMBS-seq
~5 ng
~5 ng
~50 pg
~5 ng
~50 pg
~5 ng
~50 pg
6000
3000
2000
1500
1200
1000
900
800
700
600
500
400
300
200
100
1


0.5 ng, untreated, -, -
5 ng, EM-seq, ~4.5 hours, Varied
0.05 ng, EM-seq, ~4.5 hours, Varied
5 ng, Zymo Gold, 10min/2.5hr, 98°C/64°C
0.05 ng, Zymo Gold, 10min/2.5hr, 98°C/64°C
5 ng, R8-4, 90 min, 55°C
0.05 ng, R8-4, 90 min, 55°C
CG
CHH
CHG
1.23%
0.214%
0.292%
16.6%
54.3%
53.8%
19.8%
37.2%
37.2%
21.4%
93.3%
93.2%
21%
92.9%
92.8%
27.8%
99.2%
99.2%
27.6%
99%
99%
0
20
40
60
80
100
Conversion ratio (%)


0.5 ng, untreated, -, -
5 ng, EM-seq, ~4.5 hours, Varied
0.05 ng, EM-seq, ~4.5 hours, Varied
5 ng, Zymo Gold, 10min/2.5hr, 98°C/64°C
0.05 ng, Zymo Gold, 10min/2.5hr, 98°C/64°C
5 ng, R8-4, 90 min, 55°C
0.05 ng, R8-4, 90 min, 55°C
CHH motif methylation level (%)
100
80
60
40
20
0
-3kb
TSS
TES
+3kb
0.5 ng, untreated, -, -
5 ng, EM-seq, ~4.5 hours, Varied
0.05 ng, EM-seq, ~4.5 hours, Varied
5 ng, Zymo Gold, 10min/2.5hr, 98°C/64°C
0.05 ng, Zymo Gold, 10min/2.5hr, 98°C/64°C
5 ng, R8-4, 90 min, 55°C
0.05 ng, R8-4, 90 min, 55°C
CHG motif methylation level (%)
100
80
60
40
20
0
-3kb
TSS
TES
+3kb




**Discussion**

Note: in human cfDNA CHG and CHH motifs are only lightly methylated (~0.5%?)

The post library QC is very encouraging as it reveals the relative peak heights of the three major cfDNA peaks. Our UMBS-seq method shows that longest sized peak (~700) is mostly intact whereas Zymo Gold and EM-seq have depleted this peak size (which is largest and therefor most sensitive to damage).

Sequencing results reveal that the trends are mostly as expected. We see high methylation in promoter regions, and the trend is most prominently revealed by our UMBS-seq method. Also, Em-seq showed a notable drop off in efficiency when moving from 5 ng of input to 0.05 ng. UMBS dropped off only very minimally. This is exactly what we hoped to see as EM-seq is likely less damaging than us, but our chemical method will allow us to stay efficient even at ultra-low inputs.

HOWEVER, something went wrong with the EM-seq kit (maybe the enzymes are expired) because the conversion efficiency was much less than expected. Therefore, we will have to repeat this experiment.