DNA and RNA Damage Assay and Library Construction of New Ammonium Bisulfite Recipe for Ultramild BS-Seq

Yushuai Liu

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Background

Since Tanner has been focusing on finishing UMBS work, I will do more optimizations for lower temperature. In this batch, we will test two different methods and different temperatures from 25 o 55 C while keeping the recipe the same. Method A is mixing protect buffer and sample first, then adding BS reagent to conducting BS treatment. Method B is mixing BS reagent and protect buffer first, then add sample DNA sample. To test the conversion efficiency under different treatment conditions, I will build UMBS-seq libraries using selected DNA and RNA samples with less damage.

Design

1. Treatment conditions:

Take 2 ng ligated lambda DNA each to do the following BS treatment:

Sample ID	Recipe	Temp (C)	Time
ST	Denaturation but without BS treatment		
1	BS China + protect buffer	55	60 mins
2	BS China + protect buffer	50	90 mins
3	BS China + protect buffer	50	120 mins
4	BS China + protect buffer	45	4 hrs
5	BS China + protect buffer	45	8 hrs
6	BS China + protect buffer	40	8 hrs
7	BS China + protect buffer	37	8 hrs
8	BS China + protect buffer	40	16 hrs
9	BS China + protect buffer	37	16 hrs
10	BS China + protect buffer	30	16 hrs
11	BS China + protect buffer	25	16 hrs

Materials

Lambda DNA	Tanner's Box in -20 C freezer, 90 ng/uL

Protection Buffer	Yushuai's Box in 4 C freezer
Kit- EZ RNA Methylation Kit	Shelf above the bench
Kit-EZ DNA Methylation-Gold kit	Shelf above the bench
New Bisulfite reagent	In cabinet under fume hood
NEBNext UltraShear	#E6655, Tanner's Box in -20 C freezer
Zymo Methylated pUC19 DNA Set	D5017, Tanner's Box in -20 C freezer
KAPA Evo Prep Kit	Tanner's Box in -20 C freezer

Recipe Preparation

1. New Recipe

New BS reagent (37.5 uL) **+ protect buffer** (7.5 uL)

How to make the recipe:

method A- Mix protect buffer and DNA sample first, then add BS reagent

method B- Mix BS reagent and DNA protect buffer first, then add DNA sample

DNA Damage Assay – prepare ligated DNA (2 Samples)

*Remove beads to warm them

Prepare 500 ng Lambda: Take **5.6 µl** Lambda DNA (90 ng/µl)
Prepare 10 ng PUC19 plasmid DNA: Take **10 µl** plasmid DNA (10 ng/µl)

1. **Enzymatic Fragmentation -** with NEBNext UltraShear (2 Samples)

For each sample, start with:

Component	**Total Volume for two samples (ul)**	**Volume per sample (ul)**
Sterile Water	10.4	18.2
Methylated pUC19 plasmid (1 ng/ul → 10 ng, ~2%)	10	5
Lambda (500 ng)	5.6	2.8
UltraShear Reaction Buffer (white)	14	14
NEBNext UltraShear (white)	4	4
Total volume	**44**	**44**

Assemble each fragmentation reaction **on ice** by adding the components in the above order, incubate in a thermocycler **with lid at 75C**

Step	**Temp**	**Time**
Fragmentation	37°C	**30 min**

Additional Incubation	65°C	15 min
HOLD	4°C	∞

Consolidate 2 samples into 1 samples of equal volume (88 µl)

2. AmPure Beads Clean-Up

a. Warm beads to RT before use!

i) 1.0x beads ratio – 88 µl for each sample (after combining!)

ii) Elute with 36 µl water to get 35 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

3. End Prep and A-Tailing

To 35 µl of fragmentated DNA (500 ng) and then add:

Reagent	**Volume per sample (ul)**
~~164bp 5mC dsDNA (1ng/ul → 5 ng, ~1.0%)~~	~~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)**

4. Adaptor Ligation

a. To 60 µl of end repaired DNA (500 ng)

Reagent	**Volume per sample (ul)**
NEBNext *Methylated* Adaptor (No Dilution) *- 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 15 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**

5. AmPure Beads Purification

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

i) 0.8x beads ratio – 62.4 µl.
Elute with 251 µl RNA water to collect 250 µl (~2 ng/µl)

Take out 30 uL ligated DNA to do KOH denaturation for this batch of experiment, and store the rest in -80 C freezer. For example, add 5 uL 20 M KOH to 195 uL H_2O, get 0.5 M KOH, then take out 45 uL 0.5 M KOH and mix with 15 uL sample and 15 uL H_2O, that is 75 uL (final concentration of KOH is 0.3 M). Then 42 C for 20 mins, after the reaction is done, place on ice, take out 5 uL denatured DNA to do BS treatment.

BS Treatment – 22 Samples

Method A:

1. Transfer 7.5 ul protect buffer to the empty 200 ul PCR tube.
2. Add 5 ul denatured DNA to DNA protect buffer, pipette, vortex and quickly spin down.
3. Add 37.5 uL BS reagent to the mix in step 2, pipette, vortex and quickly spin down. The liquid is clear.
4. Incubate the reactions at different temperatures and times based on the table in the design part.
5. After the reaction is done, add 100 uL RNA H_2O required in desulphonation procedures and place on ice. Make sure there is no precipitation.

Method B:

1. Transfer 7.5 ul protect buffer to the empty 200 ul PCR tube.

2. Add 37.5 ul BS reagent to protect buffer, pipette, vortex and quickly spin down. The liquid is not clear now, like milk.
3. Add 5 uL denatured DNA to the mix in step 2, pipette, vortex and quickly spin down. The liquid is clear now.
4. Incubate the reactions at different temperatures and times based on the table in the design part.
5. After the reaction is done, add 100 uL RNA H_2O required in desulphonation procedures and place on ice. Make sure there is no precipitation.

Desulphonation (22 Samples) - EZ DNA Methylation Gold-Kit

a. For each sample→50 ul, add 100 µL H_2O.
a. M-Binding Buffer – 600 µL → Centrifuge 16,000 xg for 30s
b. M-Wash Buffer – 100 µL → Centrifuge 16,000 xg for 30s
b. M-Desulphonation Buffer – 200 µL on column
c. Incubate at Rm Temp for 15 mins → Centrifuge 16,000 xg for 30s
d. M-Wash Buffer – 200 µL → Centrifuge 16,000 xg for 30s
e. Repeat washing for another 3 times
f. Discard → Centrifuge 16,000 xg for 2 mins
Elute with H_2O – 21 µL → Centrifuge 16,000 xg for 1min, get 20 uL in total.

Commented [QD1]: Make sure you use the desulphonation buffer in DNA kit here.

Commented [YL2]: Hi Qing, I use 21 uL water to elute here, and take 1 uL to do qPCR. Also, to the no treatment sample (D-ST) is 1 uL ligated DNA + 19 uL water and take 1 uL to do qPCR, is it OK?

qPCR – all 23 samples

To **1 ul** of all 23 samples, add:

Reagent	**Volume per sample (ul)**	**Volume per 23.8 samples (ul)**
KAPA HiFi Hotstart Uracil+ 2X Ready Mix	10	238
Universal primer	1	23.8
Mixed Index primer	1	23.8
H_2O	6	142.8
SYBR Green	1	23.8
Final volume	**19**	

Sample final volume**: 20 ul** →

Pre-incubation	95 C; 600 s
X40	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

After you got qPCR data, let's discuss what libraries should proceed to PCR to build libraries for sequencing.

DNA Sample information and qPCR results

How to make the recipe:

method A- Mix DNA protect buffer and DNA first, then add BS reagent

method B- Mix BS reagent and DNA protect buffer first, then add DNA

Sample ID	Recipe	Temp (C)	Time	How to make the recipe	qPCR
ST	Denaturation but without BS treatment				8.5
A-1	BS China + DNA protect buffer	55	60 mins	Method A	12
A-2	BS China + DNA protect buffer	50	90 mins	Method A	12
A-3	BS China + DNA protect buffer	50	120 mins	Method A	12.5
A-4	BS China + DNA protect buffer	45	4 hrs	Method A	12
A-5	BS China+ DNA protect buffer	45	8 hrs	Method A	13.7
A-6	BS China + DNA protect buffer	40	8 hrs	Method A	13
A-7	BS China+ DNA protect buffer	37	8 hrs	Method A	12.6
A-8	BS China + DNA protect buffer	40	16 hrs	Method A	13.7
A-9	BS China + DNA protect buffer	37	16 hrs	Method A	13.8
A-10	BS China + DNA protect buffer	30	16 hrs	Method A	12.8
A-11	BS China + DNA protect buffer	25	16 hrs	Method A	12.1
B-1	BS China + DNA protect buffer	55	60 mins	Method B	11.3

B-2	BS China + DNA protect buffer	50	90 mins	Method B	11.4
B-3	BS China + DNA protect buffer	50	120 mins	Method B	11.2
B-4	BS China + DNA protect buffer	45	4 hrs	Method B	12.3
B-5	BS China + DNA protect buffer	45	8 hrs	Method B	13
B-6	BS China + DNA protect buffer	40	8 hrs	Method B	13.4
B-7	BS China + DNA protect buffer	37	8 hrs	Method B	11.9
B-8	BS China + DNA protect buffer	40	16 hrs	Method B	13.2
B-9	BS China + DNA protect buffer	37	16 hrs	Method B	12.6
B-10	BS China + DNA protect buffer	30	16 hrs	Method B	12.2
B-11	BS China + DNA protect buffer	25	16 hrs	Method B	11.5

Based on the qPCR results, we chose ST, A-1, A-2, A-3, A-4, B-1, B-2, B-3, B-4, B-7, B-8, B-9, B-10 and B-11 to build UMBS-seq libraries since the Ct values are relatively smaller suggesting lower DNA damage.

PCR – 14 samples for DNA assay

To 9 uL sample, add:

Reagent	**Volume per sample (ul)**	**Volume per 14.6 samples (ul)**
KAPA Hifi U+ 2X Master Mix	12.5	
Universal primer	0.625	
Index primer	0.625	
H_2O	2.25	
Final volume	**16**	

Sample final total volume: **25 ul** →

	98 C; 45 s
x cycles	98C; 15 s
	60 C; 30 s

(Calculate based on the qPCR Ct value)	72 C; 30 s
	72 C; 1 min
	4C

AmPure beads purification

a. 0.6x beads ratio – 15 µL

b. Elute with H_2O – 12.5 µL

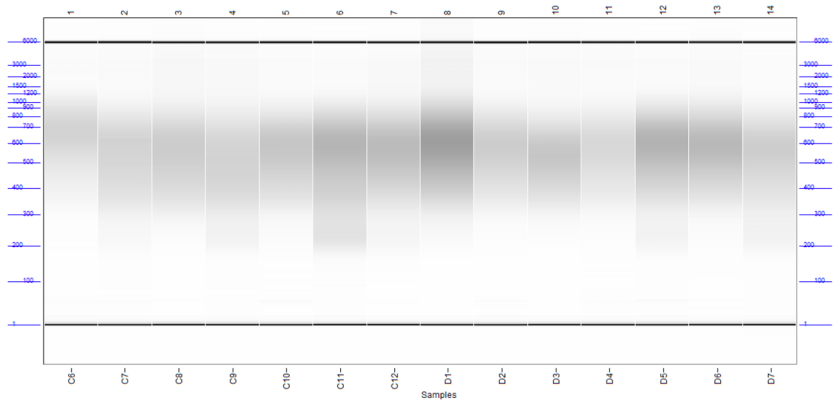
- 10 µL libraries
- 1 µL QC
- 1 µL Qubit

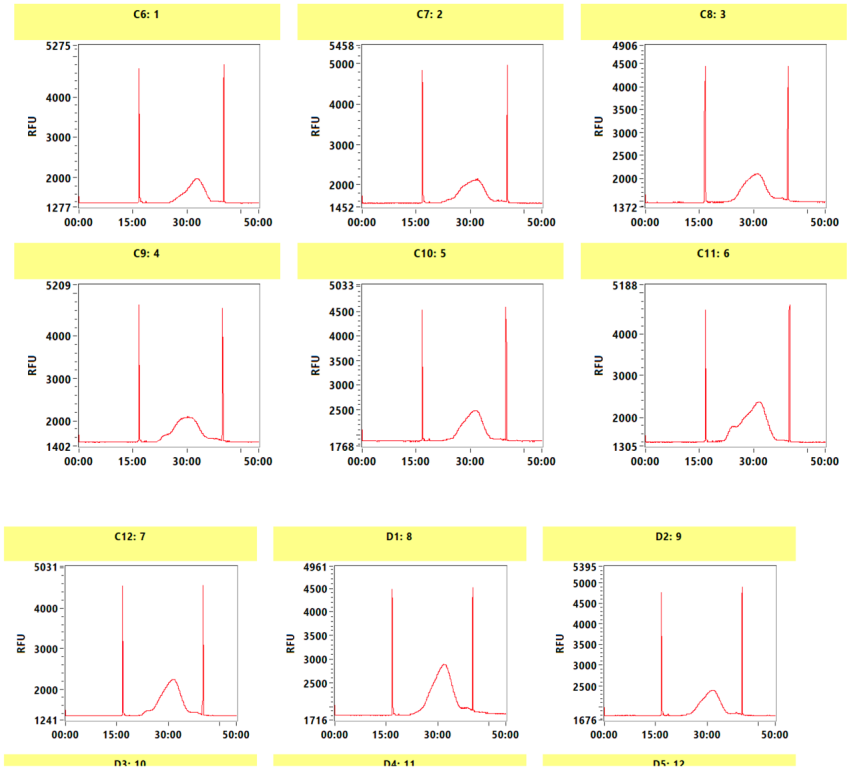
Library information – samples are from DNA damage assay (14 samples)

ID	Content	qPCR	PCR 9 uL sample	Index#	Kit	Qubit (ng/ul)	QC (ng/ul)	Dilution fold for QC	i7
ST	2 ng ligated DNA, Denaturation but without BS treatment	10.4	9	1	NEBNext Set # 1 and 2	5.02	1.43	5	ATCACG
A-1	2 ng ligated DNA, BS + protect buffer, 55C 60 mins, method A	12.1	12	2		7.46	1.91	5	CGATGT
A-2	2 ng ligated DNA, BS + protect buffer, 50C 90 mins, method A	15.1	12	4		8.58	2.12	5	TGACCA
A-3	2 ng ligated DNA, BS + protect buffer, 50C 120 mins, method A	8.6	13	5		8.34	2.18	5	ACAGTG
A-4	2 ng ligated DNA, BS + protect buffer, 45C 4 hrs, method A	12	12	6		8.26	2.18	5	GCCAAT
B-1	2 ng ligated DNA, BS + protect buffer, 55C 60 mins, method B	15.3	12	7		13.3	3.41	5	CAGATC
B-2	2 ng ligated DNA, BS + protect buffer, 50C 90 mins, method B	9	12	8		12.3	2.73	5	ACTTGA

B-3	2 ng ligated DNA, BS + protect buffer, 50C 120 mins, method B	12.2	12	9		14.7	3.83	5	GATCAG
B-4	2 ng ligated DNA, BS + protect buffer, 45C 4 hrs, method B	15.6	12	10		7.64	1.83	5	TAGCTT
B-7	2 ng ligated DNA, BS + protect buffer, 37C 8 hrs, method B	9.7	12	11		8.5	2.09	5	GGCTAC
B-8	2 ng ligated DNA, BS + protect buffer, 40C 16 hrs, method B	12	13	12		6.12	1.47	5	CTTGTA
B-9	2 ng ligated DNA, BS + protect buffer, 37C 16 hrs, method B	17	13	13		9.6	2.87	5	AGTCAA
B-10	2 ng ligated DNA, BS + protect buffer, 30C 16 hrs, method B	8.7	12	14		9.28	2.57	5	AGTTCC
B-11	2 ng ligated DNA, BS + protect buffer, 25C 16 hrs, method B	15.6	12	15		8.38	1.99	5	ATGTCA

Gel Image

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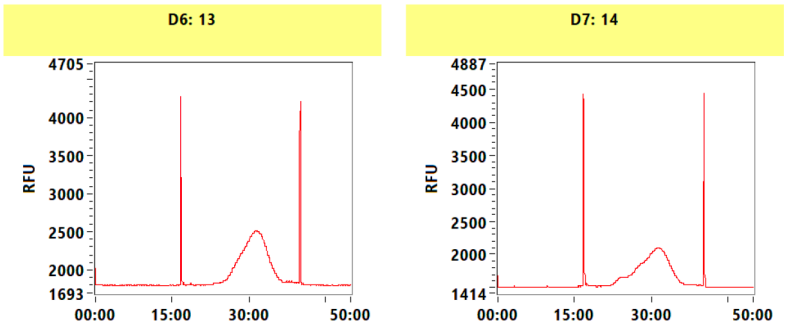

Discussion

Based on the qPCR results, we confirmed the trend that lower temperature (from 55°C and 37°C to 25°C) and longer treatment time (from 1 hrs to 16hrs) can cause less DNA damage.
To test the conversion efficiency under different treatment conditions, I built 14 UMBS-seq libraries using selected DNA samples with less damage. Now we are waiting for the sequencing data analysis.

Based on the QC results, 50 C for 90 min or 120 min gave the most library yields and the longest inserts, suggesting lower DNA damage.