Aug 12, 2021

Whole Genome Bi-sulphite Sequencing (WGBS)

Klaus H. Kaestner Lab[1], Suzanne Shapira[1]

[1]University of Pennsylvania Perelman School of Medicine

Suzanne Shapira: HIRN-HPAP correspondence: sshapira@pennmedicine.upenn.edu

dx.doi.org/10.17504/protocols.io.bwaupaew

Human Islet Research Network

Lili Liang

ABSTRACT

The molecular mechanisms that underlie islet dysfunction in Type 1 (autoimmune) and Type 2 diabetes are not fully understood, and genetic risk factors are of limited clinical and predictive value. Epigenetic modifications to histones or DNA provide a potential readout of environmental influence from sources such as diet and the microbiome. DNA methylation is an epigenetic modification of cytosine residues that alters DNA accessibility and downstream gene expression patterns. Whole genome bisulphite sequencing (WGBS) is a technique that interrogates such genome-wide DNA methylation patterns. Treatment with sodium bisulfite converts unmethylated cytosine residues to uracil, which then appear as thymine bases after sequencing; thus, this technique allows for identification of methylated and unmethylated DNA with single base pair resolution. WGBS can be used to identify differentially methylated regions between samples from non-diabetic and diabetic pancreatic tissue.

Note:
Date Revised: July 15, 2020

DOI

dx.doi.org/10.17504/protocols.io.bwaupaew

EXTERNAL LINK

https://hpap.pmacs.upenn.edu/explore/workflow/islet-molecular-phenotyping-studies?protocol=3

PROTOCOL CITATION

Klaus H. Kaestner Lab, Suzanne Shapira 2021. Whole Genome Bi-sulphite Sequencing (WGBS). **protocols.io** https://dx.doi.org/10.17504/protocols.io.bwaupaew

KEYWORDS

WGBS, HPAP, HIRN

CREATED

Jul 02, 2021

LAST MODIFIED

Aug 12, 2021

PROTOCOL INTEGER ID

51252

Fragmentation

1 **1.** Covaris Fragmented gDNA: 55ng. Fragmentation program: 200bp as the Covaris recommended in **130 µl** in the low TE buffer by using snap-cap microtube (Covaris, cat#520045)

Bisulfite by using Ultralow Methylation-seq with TrueMethl oxBS (NuGEN, CAT#0541) Only use the indicated mock-oxidation steps in the following

2 **1.** Book thermo cycler and qPCR macine

Table 3. Thermal Cycler Programming

END REPAIR		VOLUME
Program 1 End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	16 µL
ADAPTOR LIGATION		VOLUME
Program 2 Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	31 µL
FINAL REPAIR		VOLUME
Program 3 Final Repair	60 °C – 10 min, hold at 4 °C	40 µL
OXIDATION		VOLUME
Program 4 Denaturation	37 °C – 5 min, hold at 25 °C	10 µL
BISULFITE CONVERSION		VOLUME
Program 5 Bisulfite Conversion	95 °C – 5 min, 60 °C – 20 min, 95 °C – 5 min, 60 °C – 40 min, 95 °C – 5 min, 60 °C – 45 min, hold at 20 °C	40 µL
AMPLIFICATION		VOLUME
Program 6 Library Amplification	95 °C – 2 min, N(95 °C – 15 s, 60 °C – 1 min, 72 °C – 30 s), hold at 10 °C	

Note:
Extra order: Evagreen
Check every box, make sure things are ready
Protocol-Day0: prepare all the material, check all the material, fragment DNA, run bioanalyzer, put sample in -20C
Protocol-Day1: until bisulfate, hold on +20C

Fragmented DNA Purification

3 **1.** Choose a nucleic acid column-based purification system that allows small volume elution, such as the MinElute® Reaction Cleanup Kit (QIAGEN, Cat. #28204). Alternatively, you may use the Agencourt bead- based purification protocol detailed below, which is provided for your convenience. This protocol is designed to work with a starting volume of **50 µl** of fragmented DNA. If your starting volume is different, change the volume of beads added in Step 5 to 1.8 times the volume of your fragmented DNA.

2. Ensure the Agencourt beads and Nuclease-free Water (D1) have completely reached room tempera- ture before proceeding.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

3. Resuspend beads by vortexing the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads.

4. Prepare a fresh 70% ethanol wash solution.

5. Briefly spin down samples.

6. Add **90 µl** (1.8 volumes) of bead suspension to each reaction. Mix thoroughly by pipetting 10 times.

7. Incubate at room temperature for 10 minutes.

8. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.

9. Carefully remove the binding buffer and discard it

Note:
The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of purified DNA, so ensure beads are not removed with the binding buffer or the wash.

10. With the tubes still on the magnet, add **200 µl** of freshly prepared 70% ethanol and allow to stand for 30 seconds.

11. Remove the 70% ethanol wash using a pipette.

12. Repeat steps 9 and 10 for a total of two 70% ethanol washes.

13. Air dry the beads on the magnet for 5–10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated.

14. Remove the tubes from the magnet.

15. Add **14 µl** room temperature of 1X low-EDTA TE buffer or Nuclease-free Water (green: D1) to the dried beads. Mix thoroughly to ensure all the beads are resuspended.

16. Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.

17. Carefully remove **13 µl** of the eluate, ensuring as few beads as possible are carried over; transfer to a fresh set of PCR tubes and place on ice.

Note:
Optional stopping point: Store samples at −20 °C.

End Repair

4 **Table 5. End Repair Master Mix**

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

REAGENT	END REPAIR BUFFER MIX (BLUE: ER1 ver 8)	END REPAIR ENZYME MIX (BLUE: ER2 ver 4)	END REPAIR ENHANCER (BLUE: ER3 ver 2)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	2.0 µL	0.5 µL	0.5 µL

1. Thaw ER1 at room temperature. Mix by vortexing, spin down and place on ice.

2. Spin down ER2 and ER3 and place on ice.

3. Obtain **13 µl** of fragmented DNA sample (10–300 ng) from the DNA Purification protocol.

Note:
To interrogate 5hmC, split the sample into two 13 µL aliquots to perform parallel library preparations with oxBS and MOCK oxBS workflows. The aliquots can be diluted using Nuclease-free Water or 1X low-EDTA TE buffer.

4. Prepare a master mix by combining ER1, ER2 and ER3 in a **0.5 mL** capped tube, according to the volumes shown in **Table 5**.

5. Add **3 µl** of End Repair Master Mix to each sample tube for a total of **16 µl** .

6. Mix by pipetting, cap and spin tubes and place on ice.

7. Place tubes in a pre-warmed thermal cycler programmed to run Program 1 (End Repair; see **Table 3**):

a. **25 °C** – 30 min

b. **70 °C** – 10 min

c. hold at **4 °C**

8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.

Adaptor Ligation

5 **1.** Remove Agencourt beads from **4 °C** . Place on the bench top to reach room temperature for use in the next step.

Table 6. Ligation Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	LIGATION BUFFER MIX (YELLOW: L1 ver 4)	LIGATION ENZYME MIX (YELLOW: L3 ver 4)
STORAGE	---	-20 °C	-20 °C
1X REACTION VOLUME	4.5 µL	6 µL	1.5µL

2. Spin down L3 and place on ice.

3. Thaw adaptor plate on ice, spin down, and return to ice.

4. Thaw L1 at room temperature. Mix by vortexing, spin down and place on ice. Add adaptors to each sample as follows:

a. If using adaptors from 32-plex Adaptor Plate (S02694), add **3 µl** of the appropriate Ligation Adaptor Mix to each sample. Mix thoroughly by pipetting.

b. If using adaptors from 96-plex Adaptor Plate (S02169), add the entire **16 µl** of sample to the appropriate adaptor well, mix well by pipetting, then transfer the entire sample to a PCR tube

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

Note:
In order to multiplex the oxBS and MOCK oxBS processed samples, unique indexes are required.

5. Prepare a master mix by combining D1, L1 and L3 in a **0.5 mL** capped tube, according to the vol- umes shown in **Table 6**. Mix by pipetting slowly, without introducing bubbles, spin and place on ice. Use the master mix immediately.

Note:
The L1 Ligation Buffer Mix is very viscous. Pipet this reagent slowly and mix thoroughly.

6. Add **12 µl** Ligation Master Mix to each reaction for a total of **31 µl** . Mix thoroughly by pipetting slowly and gently, spin down and place on ice. Proceed immediately with the incubation.

7. Place tubes in a pre-warmed thermal cycler programmed to run Program 2 (Ligation; see **Table 3**):

a. **25 °C** – 30 min

b. **70 °C** – 10 min

c. hold at **4 °C**

8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

Post-Ligation Purification

6 **1.** Ensure the Agencourt beads and D1 have completely reached room temperature before proceeding.

2. Resuspend the beads by vortexing the tube. Ensure the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.

3. At room temperature, add **45 µl** (1.5 volumes) of the bead suspension to each reaction. Mix thor- oughly by pipetting 10 times.

4. Incubate at room temperature for 10 minutes.

5. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.

6. Carefully remove the binding buffer and discard it.

Note:
The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of purified DNA, so ensure beads are not removed with the binding buffer or the wash.

7. With the tubes still on the magnet, add **200 µl** of freshly prepared 70% ethanol and allow to stand for 30 seconds.

8. Remove the 70% ethanol wash using a pipette.

9. Repeat steps 7 and 8 for a total of two 70% ethanol washes.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

Note:
With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

10. Air dry the beads on the magnet for 5–10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.

11. Remove the tubes from the magnet.

12. Add **16 µl** room temperature of 1X low-EDTA TE buffer or D1 to the dried beads. Mix thoroughly to ensure all the beads are resuspended.

13. Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.

14. Carefully remove **15 µl** of the eluate, ensuring as few beads as possible are carried over; transfer to a fresh set of PCR tubes and place on ice.

Final Repair

7 **1.** Remove the TrueMethyl oxBS Module Magnetic Bead Solution, Binding Buffer 1 and Binding Buffer 2 from **4 °C** and place at room temperature for use in the next step.

Table 7. Final Repair Master Mix

REAGENT	FINAL REPAIR BUFFER MIX (PURPLE: FR1 ver 4)	FINAL REPAIR ENZYME MIX (PURPLE: FR2)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	4.5 µL	0.5 µL

2. Spin down FR2 and place on ice.

3. Thaw FR1 at room temperature. Mix by vortexing, spin down and place on ice.

4. Prepare a master mix by combining FR1 and FR2 in a **0.5 mL** capped tube, according to the volumes shown in **Table 7**.

5. Add **5 µl** of the Final Repair Master Mix to each sample tube for a total of **20 µl** .

6. Mix by pipetting, cap and spin tubes and place on ice.

7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (Final Repair; see **Table 3**).

8. **60 °C** – 10 min, hold at **4 °C**

9. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

Note:
Optional stopping point: Store samples at –20 °C.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

10. If using the True Methyl oxBS Module, continue with section H. DNA Purification and Denaturation. For alternative bisulfite conversion methods, perform bisulfite conversion according to the manufac- turer recommendations and proceed to protocol section **L. Library Amplification Optimization with qPCR**.

Note:
Bisulfite conversion is critical for successful library amplification

DNA Purification and Denaturation

8 1. Remove the TrueMethyl oxBS Module Oxidant Solution from **-20 °C** and thaw on ice for use in the next step.

Note:
The Oxidant Solution is light-sensitive. Keep protected from light.

Table 8. Magnetic Bead Binding Solution 1 Master Mix

REAGENT	BINDING BUFFER 1	MAGNETIC BEAD SOLUTION
STORAGE	4 °C	4 °C
1X REACTION VOLUME	120 µL	2.4 µL

2. Remove Acetonitrile, Ultra Pure water, and Denaturing Solution from storage and place on bench top.

3. Prepare a fresh stock of 80% acetonitrile, using the Ultra Pure water provided with the kit. Mix by vortexing or inversion and place at room temperature.

4. Ensure Magnetic Bead Solution and Binding Buffer 1 have reached room temperature before proceeding.

5. Mix Binding Buffer 1 by inversion until homogenized.

6. Vortex Magnetic Bead Solution until homogenized.

7. Prepare a master mix of Magnetic Bead Binding Solution 1 (MBBS1) as directed in **Table 8**.

Note:
MBBS1 should be prepared fresh on the day of use. Do not store for longer than 1

8. Vortex MBBS1 master mix thoroughly to ensure the beads are homogenized in solution.

9. At room temperature, add **30 µl** water of Ultra Pure water to each sample for a total of **50 µl** .

10. Add **100 µl** of MBBS1 master mix to each **0.2 mL** tube containing **50 µl** NGS library for a total of **150 µl** . Mix by pipetting and centrifuge briefly.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

11. Incubate at room temperature for 20 min.

12. Transfer tubes to a magnetic separation plate and incubate at room temperature for 5 minutes to completely clear the solution of beads.

13. Keeping the tubes on the magnet, carefully remove the supernatant and discard it.

14. With the tubes still on the magnet, carefully add **200 µl** of 80% Acetonitrile wash to the tubes with- out disturbing the bead pellet.

15. Remove and discard the **200 µl** 80% Acetonitrile wash, carefully avoiding aspiration of the bead pellet.

16. Repeat Steps 13 and 14 twice to perform 3 x 200 µL 80% Acetonitrile washes in total. Remove as much of the final wash as possible.

Note:
Ensure the tubes are dry before continuing the protocol. If the tubes aren't dry after 5 minutes, incubate for a longer period of time.

17. Air dry the bead pellets for 5 minutes at room temperature, leaving the lids of the tubes open.

18. Remove the tubes from the magnet.

19. Add **10 µl** of Denaturing Solution directly onto the bead pellet. Mix thoroughly to ensure all beads are resuspended.

20. Centrifuge briefly to collect solution at bottom of the tubes.

21. Place the tubes in a pre-warmed thermal cycler programmed to run Program 4 (Denaturation; see **Table 3**):
a. **37 °C** – 5 min
b. hold at **25 °C**

22. Remove the tubes from the thermal cycler, spin to collect condensation and transfer to the magnet.

23. Incubate at room temperature for 2 minutes.

24. Carefully remove **9 µl** of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh **1.5 mL** microcentrifuge tube and place at room temperature.

25. Remove Ultra Pure water from storage and place on bench top.

26. Set a heat block to **40 °C** .

27. Prepare individual oxidation and mock oxidation reactions as follows:
a. **Do not do:** For each sample to be processed through the oxBS workflow, add 1 µL of oxidant solution to 9 µL of DNA for a total of 10 µL.
b. **Only do:** For each sample to be processed through the MOCK oxBS workflow, add 1 µL of Ultra Pure water to 9 µL of DNA for a total of 10 µL.

28. Mix reactions by vortexing and centrifuge briefly.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

29. Place tubes in heat block and incubate for 10 min at **40 °C** .

30. Centrifuge reactions at 14000 x g for 10 minutes at room temperature to pellet any black precipitate. MOCK treated samples will remain clear and will not have any black precipitate.

Note:
In samples treated with oxidant solution, the color of the oxidation reaction should remain orange after the 10 minute centrifugation, indicating a successful oxidation. If the solution turns any color other than orange, please see topic XVI below.

31. Transfer the orange supernatant to a fresh **0.2 mL** PCR tube and place at room temperature. Proceed immediately to the next step.

Note:
- ***Take care not to carry any black precipitate over as this could inhibit downstream steps.***
- ***Do not place the oxidized samples on ice to cool as this may cause the solution to precipitate.***

Bisulfite Conversion

9 **1.** Set a heat block or heated orbital incubator to **60 °C** .

2. Remove Bisulfite Diluent and Bisulfite Reagent aliquots from storage and place on bench top. Remove 1 aliquot of Bisulfite Reagent for every 20 reactions to be processed.

3. Prepare Bisulfite Reagent Solution by adding **700 µl** of Bisulfite Diluent to each aliquot of Bisulfite Reagent.

Note:
Each aliquot of Bisulfite Reagent Solution is sufficient for up to 20 samples. A fresh aliquot of solution should be prepared each time the kit is used and disposed of immedi- ately after use.

4. Seal the lid of each aliquot with Bisulfite Reagent Solution tightly.

5. Incubate the aliquots of Bisulfite Reagent Solution for 15 min at **60 °C** . Vortex regularly until the Bisulfite Reagent Solution is completely dissolved.

6. Spin down Bisulfite Reagent Solution briefly and place at room temperature.

7. Ensure oxidized DNA samples from previous step are at room temperature before proceeding.

8. Prepare Bisulfite Conversion Reaction mix by adding **30 µl** of Bisulfite Reagent Solution to each **10 µl** of DNA for a total of **40 µl** . Ensure that each sample pair being processed through the oxBS and MOCK oxBS workflow is treated with the same aliquot of Bisulfite Reagent Solution. Mix by pipetting, spin down and place at room temperature.

9. Mix by pipetting, spin down and place at room temperature.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

Note:
- ***If the Bisulfite Reagent Solution precipitates, return to 60 °C until dissolved.***
- ***Samples treated with the oxidant solution may turn light gray in color.***

10. Place the tubes in a pre-warmed thermal cycler programmed to run Program 5 (Bisulfite Conversion, see **Table 3**):

a. **95 °C** – 5 min, **60 °C** – 20 min

b. **95 °C** – 5 min, **60 °C** – 40 min

c. **95 °C** – 5 min, **60 °C** – 45 min

d. hold at **20 °C**

Note:
Optional stopping point: You may hold samples at room temperature (+20°C) for up to 16 hours. Do not store below +20 °C.

11. Once the bisulfite conversion is complete, centrifuge samples briefly to collect solution at bottom of the tubes.

12. Transfer samples to **1.5 mL** tubes and centrifuge for 10 min at 14000 x g.

13. Continue to K. Bisulfite-Converted DNA Desulfonation and Purification while the samples are in the centrifuge.

Bisulfite-Converted DNA Desulfonation and Purification

10 **1.** Remove Desulfonation Buffer, Binding Buffer 2, Magnetic Bead Solution and Elution Buffer from stor- age and place at room temperature for a minimum of 30 minutes before use.

Table 9. Magnetic Bead Binding Solution 2 Master Mix

REAGENT	BINDING BUFFER 2	MAGNETIC BEAD SOLUTION
STORAGE	4 °C	4 °C
1X REACTION VOLUME	200 µL	2.4 µL

2. Prepare a fresh stock of 70% Ethanol. Mix by vortexing or inversion.

3. Mix Binding Buffer 2 by inversion until homogenized.

4. Vortex Magnetic Bead Solution until homogenized.

5. Prepare a master mix of Magnetic Bead Binding Solution 2 (MBBS2) as directed in **Table 9**.

Note:
- ***MBBS2 should be prepared fresh on the day of use. Do not store for longer than 1 week.***
- ***MBBS2 is a viscous solution. Pipet this reagent slowly and mix thoroughly. Ensure that MBBS2 and the MBBS2-sample mix are well-mixed.***

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

6. Transfer **40 µl** of the supernatant to a fresh set of **0.2 mL** PCR tubes. Avoid disturbing the pellet in the oxBS-treated samples.

7. Vortex MBBS2 thoroughly to ensure the solution is homogenous before aliquoting.

8. Carefully add **160 µl** of MBBS2 to each tube containing **40 µl** bisulfite converted sample for a total of **200 µl** . Mix thoroughly by pipetting slowly and gently, spin down and place at room temperature.

9. Incubate at room temperature for 5 minutes.

10. Centrifuge briefly to collect solution at bottom of the tubes.

11. Place the tubes onto the magnet and incubate at room temperature for at least 5 minutes to com- pletely clear the solution of beads.

12. Carefully remove the supernatant and discard it.

13. Remove the tubes from the magnet.

14. Add **200 µl** of 70% Ethanol to each sample tube. Resuspend the beads completely by pipetting.

13. Place the tubes on the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.

14. Carefully remove the 70% Ethanol wash and discard it. Remove as much of the wash as possible.

15. Remove samples from the magnet.

16. Add **200 µl** of Desulfonation Buffer with EtOH added directly onto the bead pellet. Resuspend the beads completely by pipetting.

Note:
Be sure that the ethanol has been added to the desulfonation buffer, as described in Section III B. Preparation of Desulfonation Buffer for the TrueMethyl oxBS Module.

17. Close lids of sample tubes securely and place the tubes into the magnetic separation rack. Incubate at room temperature for 5 minutes to completely clear the solution of beads.

18. Remove the tubes from the magnet, open the tubes, and return to the magnet.

19. Carefully remove **200 µl** of the Desulfonation Buffer and discard it. Remove as much of the Desulfonation Buffer as possible without disturbing the bead pellet.

20. Remove the tubes from the magnet.

21. Add **200 µl** of 70% Ethanol to each sample tube. Resuspend the beads completely by pipetting.

22. Place the tubes onto the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.

23. Remove the **200 µl** 70% Ethanol wash and discard it.

24. Repeat Steps 23-25 to perform 2 x 200 µL 70% Ethanol washes in total. Remove as much of the final wash as

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

possible.

25. Air-dry the the beads on the magnet for 15 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated.

26. Remove the tubes from the magnet.

27. Add Elution Buffer directly onto the bead pellet and resuspend completely by pipetting:
a. For Library Amplification Optimzation with qPCR (recommended), resuspend beads in **23 µl** Elution Buffer.
b. If qPCR optimization is not required, resuspend beads in **21 µl** Elution Buffer.

28. Incubate at room temperature for 5 minutes to elute the TrueMethyl converted DNA from the beads.

29. Centrifuge briefly to collect sample at bottom of the tubes.

30. Place the tubes onto the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.

31. Carefully transfer eluate into a fresh **0.2 mL** tube:
a. For Library Amplification Optimzation with qPCR (recommended), transfer **22 µl** eluate.
b. If qPCR optimization is not required, transfer **20 µl** eluate.

Library Amplification Optimization with qPCR

11

Table 10. Library Amplification qPCR Master Mix

REAGENT	AMPLIFICATION PRIMER MIX (RED: P2 ver 8)	AMPLIFICATION ENZYME MIX (RED: P3 ver 3)	20 x EvaGreen
STORAGE	–20 °C	–20 °C	4 °C
1X REACTION VOLUME	1.0 µL	4.75 µL	0.5 µL

1. Aliquot **2 µl** of each sample into a fresh set of **0.2 mL** tubes. Reserve the remaining **20 µl** of sample on ice.

2. Add **14 µl** of Low-EDTA TE Buffer to each sample for a total of **16 µl** . Mix well by pipetting, spin down and place on the bench top.

3. Aliquot **3.75 µl** of each sample into an appropriate PCR plate or optically clear strip tubes, in tripli- cate. Mix well by pipetting, spin down and place on ice.

4. Prepare a master mix by combining P2, P3 and 20x EvaGreen in an appropriately sized capped tube according to the volumes shown in **Table 10**. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place on ice.

5. On ice, add **6.25 µl** of Library Amplification qPCR Master Mix to each **3.75 µl** of sample, in triplicate, for a total of **10 µl** per replicate.

6. Mix well by pipetting, spin down and place on ice.

7. Perform qPCR with the following cycling conditions:
a. **95 °C** – 2 min, 35x(**95 °C** – 15s
b. **60 °C** – 1 min, **72 °C** – 30s)

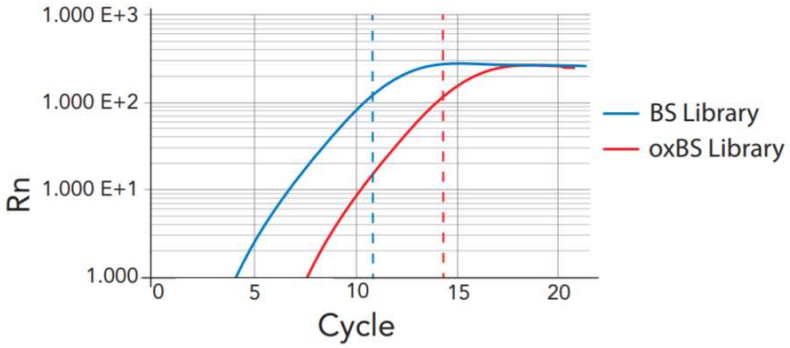
Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

8. Examine the log fluorescence vs. cycle number plot from the qPCR system to determine the appro- priate number of library amplification cycles.

a. First, select a cycle number within the late exponential phase of the amplification plot. In the example in **Figure 5**, this is 11 cycles the 'BS Library' (blue dotted vertical line, left) and 14 cycles in the 'oxBS Library' (red dotted vertical line, right).

b. Next, subtract 3 from the selected cycle to determine the number of PCR cycles to perform in the next step (M. Library Amplification). This compensates for the 1:8 dilution of sample used in the qPCR reaction.

Figure 5. Stylized qPCR amplification plot



Library Amplification

12

Table 11. Library Amplification Master Mix

REAGENT	AMPLIFICATION PRIMER MIX (RED: P2 ver 8)	AMPLIFICATION ENZYME MIX (RED: P3 ver 3)
STORAGE	−20 °C	−20 °C
1X REACTION VOLUME	5.0 µL	25 L

1. Remove Agencourt beads from **4 °C** . Place on the bench top to reach room temperature for use in the next step.

2. Thaw P3 on ice if needed. Spin down and place on ice.

3. Thaw P2 at room temperature. Mix by vortexing, spin and place on ice.

4. Prepare a master mix by combining P2 and P3 in an appropriately sized capped tube according to the volumes shown in **Table 11**. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place on ice.

5. On ice, add **30 µl** of Library Amplification Master Mix to each **20 µl** of reserved sample for a total of **50 µl** .

6. Mix well by pipetting, spin down and place on ice.

7. Place tubes in a pre-warmed thermal cycler programmed to run Program 6 (Library Amplification; see **Table 3**):

a. **95 °C** – 2 min, N (**95 °C** – 15 s, **60 °C** – 1 min **72 °C** – 30 s)

b. hold at **10 °C**

8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

Amplified Library Purification

13 **1.** Ensure the Agencourt beads have completely reached room temperature before proceeding.

2. Resuspend the beads by inverting and tapping the tube. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.

3. Add **50 µl** (1 volume) of the bead suspension to each reaction. Mix thoroughly by pipetting 10 times.

4. Incubate at room temperature for 10 minutes.

5. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.

6. Carefully remove the binding buffer and discard it.

> ***Note:***
> ***The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the final yield, so ensure beads are not removed with the binding buffer or the wash.***

7. With samples still on the magnet, add **200 µl** of freshly prepared 70% ethanol.

8. Remove the 70% ethanol wash using a pipette.

9. Repeat steps 7 and 8 for a total of two washes.

> ***Note:***
> ***With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step***

10. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.

11. Remove the tubes from the magnet.

12. Add **20 µl** Low-EDTA TE Buffer to the dried beads. Mix thoroughly to ensure all beads are resuspended.

13. Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.

14. Carefully remove **18 µl** of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of PCR tubes.

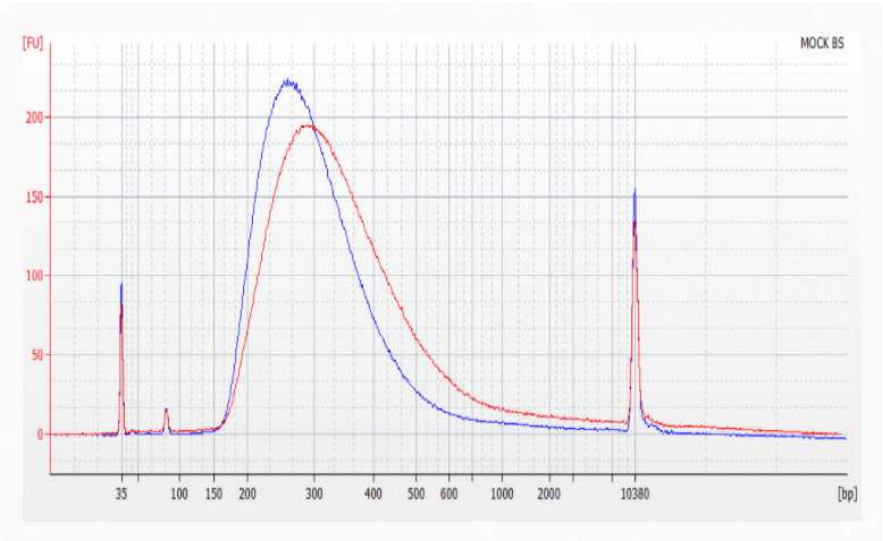
Quantitative and Qualitative Assessment of the Library

14 **1.** Quantify the libraries using a fluorometric assay and/or qPCR.

2. Validate libraries using the hsDNA kit for BioAnalyzer or an equivalent assay. An example trace using **1 µl** of 5 ng/uL library on the hsDNA chip is shown in **Figure 6** below.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

Figure 6. Fragment distribution when 1 µL of 5 ng/µL library is loaded into a High Sensitivity DNA assay from 200 ng human DNA input.



3. Normalize and pool libraries following the Illumina guidelines “Best practices for manually nor- malizing library concentrations” and the “Low-Diversity Sequencing” guidelines for your specific sequencer. See **Appendix A**. of this guide for guidelines on color balancing and multiplexing of Tecan libraries.

Appendix A: Barcode Sequences and Guidelines for Multiplexing

15 **1.** Barcode sequences and multiplexing guidelines for adaptors used in the Ultralow Methyl-Seq library prep kit can be found in **Tables 12 and 13**.

2. All barcode sequences are separated by an edit distance of three. For further details on the barcode design strategy, please refer to Faircloth BC, Glenn TC (2012) Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. PLoS ONE 7(8): e42543. doi:10.1371/journal.pone.0042543.

Table 12. Barcode sequences for adaptors used in the Ultralow Methyl-Seq 32-plex Adaptor Plate (S02694, Part No. 0366)

Note:
Barcodes in the Ultralow Methyl-Seq 32-plex Adaptor Plate are color balanced in pairs (i.e. A01 + B01, C01 + D01, etc.) and in sets of 8 by column.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CGCTACAT	A03	GTACACCT
B01	AATCCAGC	B03	ACGAGAAC
C01	CGTCTAAC	C03	CGACCTAA
D01	AACTCGGA	D03	TACATCGG
E01	GTCGAGAA	E03	ATCGTCTC
F01	ACAACAGC	F03	CCAACACT
G01	ATGACAGG	G03	TCTAGGAG
H01	GCACACAA	H03	CTCGAACA
A02	CTCCTAGT	A04	ACGGACTT
B02	TCTTCGAC	B04	CTAAGACC
C02	GACTACGA	C04	AACCGAAC
D02	ACTCCTAC	D04	CCTTAGGT
E02	CTTCCTTC	E04	CCTATACC
F02	ACCATCCT	F04	AACGCCTT
G02	CGTCCATT	G04	TCCATTGC
H02	AACTTGCC	H04	CAAGCCAA

Table 13. Barcode sequences for adaptors used in the Ultralow Methyl-Seq 96-Plex Adaptor Plate (S02169, Part No. 9113)

Barcodes in the Ultralow Methyl-Seq 96-plex Adaptor Plate are color balanced in sets of 8 by column.

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CACGTCTA	A05	ATGGCGAT	A09	CAAGAAGC
B01	AGCTAGTG	B05	AACGCCTT	B09	CTGATGAG
C01	ACTATCGC	C05	GTAAGGTG	C09	CTCGAACA
D01	GCGTATCA	D05	TGTCGACT	D09	TCGACAAG
E01	ACTCTCCA	E05	ACTCTGAG	E09	AGTGCATC
F01	CGTCCATT	F05	GATGGAGT	F09	TGGCTACA
G01	AGCCGTAA	G05	CTAGCTCA	G09	GCACACAA
H01	GAGTAGAG	H05	CTGTACCA	H09	GCATAGTC
A02	ACGTCGTT	A06	CCTGTCAA	A10	AACACGCT
B02	GTCCTGTT	B06	GGTCGTAT	B10	TTCACGGA
C02	AGAAGCCT	C06	CGCTGATA	C10	TGCTGTGA
D02	GAAGATCC	D06	TAGCTTCC	D10	CCTCGAAT
E02	TAGCTGAG	E06	CAAGTCGT	E10	TGTGGCTT
F02	ACGTCCAA	F06	GTCTCATC	F10	CTGTGGTA
G02	CACACATC	G06	ACCAAGCA	G10	TCACTCGA
H02	CGGATCAA	H06	AGTCAGGT	H10	ACTCCTAC
A03	TCAGCCTT	A07	TATCGCGA	A11	CCACAACA
B03	AAGGCTCT	B07	TAGCAGGA	B11	CCGCTTAA
C03	TGTTCCGT	C07	AGAAGGAC	C11	GTGGTATG
D03	GGAATGTC	D07	TGAGCTGT	D11	GGTGTACA
E03	CATCCAAG	E07	CAGAGTGA	E11	TCTAGGAG
F03	GTCAACAG	F07	AGGTTCCT	F11	TGGAAGCA
G03	TCGCTATC	G07	AGACCTTG	G11	AACACCAC
H03	AGCCTATC	H07	CTTCCTTC	H11	CATACGGA
A04	TCGGATTC	A08	CAGGTTCA	A12	CTCTCAGA
B04	CGGAGTAT	B08	ACTGGTGT	B12	AAGCTGGT
C04	GAACCTTC	C08	GGATTCAC	C12	GTGTCCTT
D04	AGAGGATG	D08	CACGATTC	D12	AGGTCTGT
E04	ACGCTTCT	E08	AGACATGC	E12	CATTCGTC
F04	CACAGGAA	F08	GACACAGT	F12	CTCACCAA
G04	ACGAATCC	G08	CCAGTTGA	G12	GACTACGA
H04	CCTTCCAT	H08	CATGGATC	H12	ATACGCAG

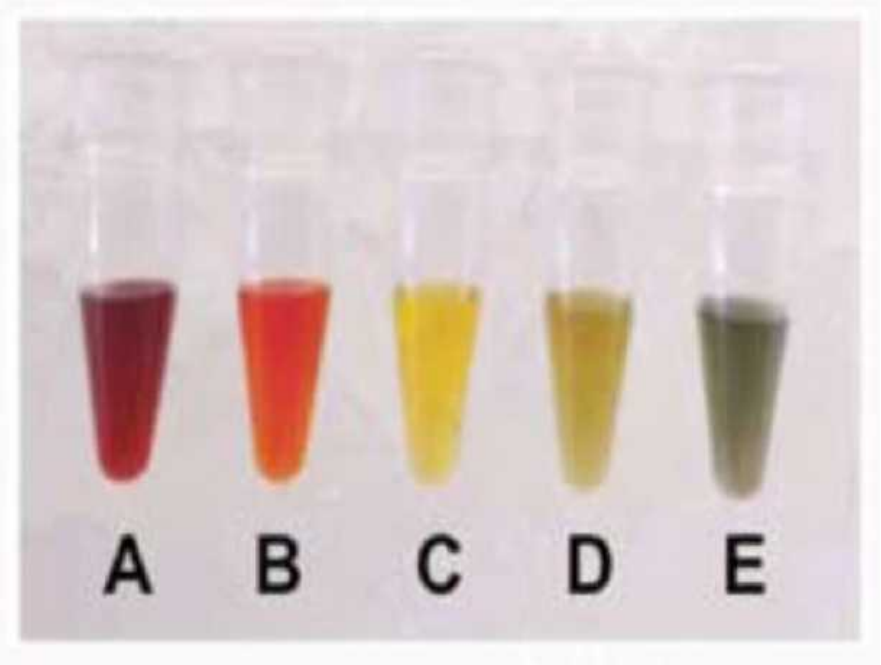
Oxidant Color Changes

16 **1.** Upon receipt of the kit, the color of the thawed Oxidant Solution should resemble Solution A in **Figure7**.

2. If the Oxidant Solution looks dark yellow or green with considerable amounts of black precipitate (Solution D or E), it is a sign that the reagent has become exposed to contaminants or CO2. If you suspect that this has occurred, please do not use the Oxidant Solution and contact Tecan NGS technical support for advice.

Figure 7. Expected Oxidant Solution Color Changes

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew



3. Figure description-
A. Oxidant Solution stock concentration supplied in the TrueMethyl oxBS Module.
B. 10-fold dilution of the Oxidant Solution in alkaline solution.
C. Working oxidant concentration (WOC).
D. 5:1 molar excess of WOC:ethanol.
E. 20:1 molar excess of ethanol:WOC.

4. During oxidation (section V. I.), the color of the oxidation reaction should be similar to Solution B or C in **Figure 7**. It is normal for a small amount of black precipitate to form during the oxidation reaction. The purpose of the strong centrifugation step following oxidation is to pellet the dark precipitate and enable removal of the clear orange/yellow solution without this contaminant. After the oxidation reaction and subsequent 10 minute centrifugation are complete, the solution should remain orange indicating a successful oxidation.

5. If the post-oxidation color appears as Solution D, it suggests partial decomposition of the oxidant, indi- cating that oxidation of the DNA samples was successful. However, if the post-oxidation color resembles Solution E, significant decomposition of the oxidant has likely occurred, resulting in incomplete con- version of 5-hmC --> U. In this instance, it is recommended to re-purify the sample in order to remove contaminants from the starting DNA sample solution. To avoid contaminants, ensure all guidance regarding oxidation solution sensitivity in section III. D. is followed, including the use of only Ultra Pure Water provided with the TrueMethyl oxBS Module in steps containing the oxidant.

6. As a final note on color changes, samples that have been processed using the Oxidant Solution may also take on a light grey color after addition of the Bisulfite Conversion Solution (section V. J.). This is normal and will not impact downstream processing.
a. Next steps
b. Qubit and BioAnalyzer to do the concentration
c. Kapa concentration using Kapa kit (KK4808)
d. Me-seq for balance
e. Nova-seq 6000 for final sequencing

Citation: Klaus H. Kaestner Lab, Suzanne Shapira (08/12/2021). Whole Genome Bi-sulphite Sequencing (WGBS). https://dx.doi.org/10.17504/protocols.io.bwaupaew