Twist Targeted Methylation Sequencing Protocol

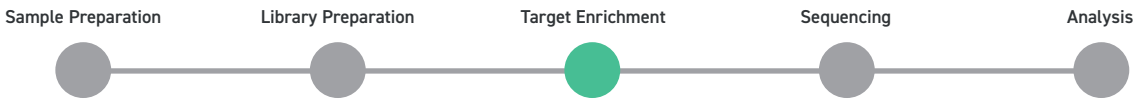
For use with the Twist NGS Methylation Detection Workflow

The Twist Targeted Methylation Sequencing Protocol uses the Fast Hybridization Target Enrichment workflow to generate target-enriched DNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems. This protocol details the steps for a high-throughput target enrichment system, designed to target specific custom sequences of interest using a hybridization time of 2-hours or less.

A component of the Twist Targeted Methylation Sequencing solution, this protocol is:

- Designed for single or multiplex hybridization reactions using either Twist fixed or custom methylation panels; optional secondary methylation panels (spike-ins) can be added for additional content
- Optimized for use with the Twist-supplied NEBNext Enzymatic Methyl-seq Methylation Library Preparation Kit

  NOTE: Alternatively modified in Appendix B in order to be used with the Twist Human Methylome Panel



Twist NGS workflow. The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis. A component of this workflow, the Twist Targeted Methylation Sequencing Protocol works in conjunction with the other components' protocols.

*This product is for **research use only**.*

PUBLISHED: February 2021

PROTOCOL COMPONENTS

Read the product packaging and storage recommendations carefully for each component, and store components as recommended below immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
TWIST FAST HYBRIDIZATION AND WASH KIT WITH AMP MIX (For target enrichment with Twist Fast hybridization)			
104180: 12 rxn 104181: 96 rxn	Twist Fast Hybridization Reagents (Box 1)	• Fast Hybridization Mix • Hybridization Enhancer • Amplification Primers	–25 to –15°C
	Twist Fast Wash Buffers (Box 2)	• Fast Binding Buffer • Fast Wash Buffer 1 • Wash Buffer 2	2 to 8°C
	Equinox Library Amp Mix (Box 3)	• Equinox Library Amp Mix (2x)	–25 to –15°C
TWIST PROBE PANELS (Ordered separately)			
Choice of panel type and reaction size	Twist Custom or Fixed Methylation Panel	E.g. Specific CpG Regions, Genes of Interest, etc.	–25 to –15°C
TWIST BLOCKERS & BEADS FOR TARGET ENRICHMENT			
100856: 2 rxn 100578:12 rxn 100767: 96 rxn	Twist Universal Blockers	For the prevention of nonspecific capture: • Universal Blockers • Blocking Solution	–25 to –15°C
101262: 2 rxn 100983: 12 rxn 100984: 96 rxn	Twist Binding and Purification Beads	For target enrichment and purification: • Streptavidin Binding Beads • DNA Purification Beads	2 to 8°C
103556: 2 rxn 103557:12 rxn 103558: 96 rxn	Twist Methylation Enhancer	For the prevention of methylation related nonspecific capture: • Methylation Enhancer	–25 to –15°C

LEGAL

This document may contain references to other third-party resources such as sources of information, hardware or software, products, or services and/or web sites owned or licensed by third parties. Twist Bioscience does not control or take responsibility for any third-party resources, including, without limitation, the accuracy, reliability, copyright compliance, compatibility, performance, legality, or any other aspect of third-party resources. The inclusion of such resources in this document does not imply endorsement by Twist Bioscience of any third-party resources.

Certain processes described in this document may be subject to patent rights or licenses in local jurisdictions, including those owned or licensed by parties other than Twist Bioscience. Purchase of this product does not include a license to perform any such processes. Users of this product may, therefore, be required to obtain a patent license depending upon the particular application and country in which the product is used before performing such processes.

Twist Bioscience, the various Twist logos, and the Twist NGS logo used herein are trademarks of Twist Bioscience Corporation. All other trademarks are the property of their respective owners.

INTENDED USE

This product is for research use only. This product is not intended for the diagnosis, prevention, or treatment of a disease or condition. Twist Bioscience assumes no liability regarding use of the product for applications in which it is not intended.

TABLE OF CONTENTS

MATERIALS SUPPLIED BY USER

The following materials or their equivalents are required to generate enriched libraries using the Twist Targeted Methylation Sequencing Workflow.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
1.5-ml compatible magnetic stand	Beckman Coulter, Thermo Fisher Scientific
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA High Sensitivity Quantification Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2ml tubes	—
Thermomixer for 1.5-ml tubes	Eppendorf
Thermal cycler (96-well) with heated lid	—
Lab shaker, rocker, rotator	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies
Vacuum concentrator (if unavailable, contact technical support)	—

GENERAL NOTES AND PRECAUTIONS

Wear appropriate protective equipment (lab coat, gloves, and protective glasses or goggles) at all times when performing this protocol.

For best results, read this document before performing the protocol, and follow the provided instructions. Twist cannot guarantee the performance of the Twist Targeted Methylation Sequencing Workflow if modifications are made to the protocol.

Test the compatibility of your PCR tubes with your thermal cycler by incubating them at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

NOTE: If using the Twist Human Methylome Panel during the hybridization step, follow the protocol modifications outlined in Appendix B.

If using a non-human capture methylation panel, replace the Blocker Solution with a species-specific blocking solution (not provided).

This protocol details different methods for mixing reagents (gentle pipetting, flicking or tapping, vortexing), depending on the volume, vessel, and reagents involved.

The Fast Hybridization Mix is a viscous reagent. Pipette slowly to ensure accuracy.
For technical support, contact customersupport@twistbioscience.com

PROTOCOL OVERVIEW

This protocol uses an enzyme-based conversion process to convert unmethylated cytosines to thymines in amplified, indexed genomic DNA (gDNA) libraries. The resulting libraries are used to generate target-enriched DNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems. The protocol features flexible hybridization timing, allowing the entire workflow to be completed in less than one day. Twist recommends starting with a 2-hour hybridization time and adjusting to as low as 30 minutes or as long as 4 hours to allow for flexibility due to time constraints.

NOTE: For more information about using the Twist Human Methylome Panel during the hybridization step, follow the protocol modifications outlined in Appendix B.

TWIST TARGETED METHYLATION SEQUENCING WORKFLOW	TIME
AMPLIFIED, INDEXED, ENZYMATICALLY CONVERTED LIBRARIES	
STEP 1 **Prepare libraries for methylation hybridization** Indexed enzyme-converted library pool	**1 hour**
STOPPING POINT	
STEP 2 **Hybridize custom methylation panel with pools** Hybridized targets in solution	**0.5 hour plus 2 hours**
STEP 3 **Bind hybridized methylation targets to streptavidin beads** Captured methylation targets on beads	**1.5 hours**
STEP 4 **Post-capture PCR amplify, purify, and perform QC** Enriched enzyme-converted libraries	**1 hour**
STOPPING POINT	
STEP 5 **Sequence on an Illumina platform** Libraries ready for sequencing on Illumina platform.	**–**

STEP 1 PREPARE LIBRARIES FOR METHYLATION HYBRIDIZATION

This step involves aliquoting the appropriate amount of amplified, indexed, enzymatically converted libraries (generated previously by using the Twist-supplied NEBNext Enzymatic Methyl-seq Methylation Library Preparation Kit) and preparing the hybridization reaction solution.

- When multiplexing, follow the pooling guidelines included as Appendix B in the NEBNext Enzymatic Methyl-seq Library Preparation Protocol used.
- If using another library preparation method, use the pooling guidelines specific to that method.

Reagents Required

- Amplified, indexed, enzymatically converted library
- Twist fixed or custom methylation panel
- Twist custom methylation secondary (spike-in) panel(s) (optional)
- Twist Universal Blockers:
  - Universal Blockers
  - Blocker Solution (If using a non-human capture methylation panel, replace with a species-specific blocking solution, not provided)
- Twist Methylation Enhancer (optional)

NOTE: The on-target benefit of using the Methylation Enhancer will depend on the methylation levels of your gDNA and the target regions of your custom methylation panel.

Before You Begin

- Thaw all required reagents on ice, then pulse-vortex for 2 seconds to mix and pulse-spin.
- In preparation for Step 2 (Hybridize custom methylation panel with pools), also thaw at room temperature:
  - From the Twist Fast Hybridization Reagents:
    - Fast Hybridization Mix
    - Hybridization Enhancer

ALIQUOT THE LIBRARIES

This protocol supports a singleplex or multiplex (up to 8-plex) hybridization capture. The amount of indexed library used depends on the number of indexed samples per pool.

1.1

Use the concentration of each amplified, indexed, enzymatically converted library to calculate the volume (in µl) of each library needed for hybridization:

- Determine the amount of each indexed library per pool from the table below.
- Divide the amount of each indexed library per pool by the concentrations measured in ng/µl from the library preparation QC. For example: If multiplexing eight libraries per hybridization reaction, the amount of each library will be 187.5 ng and the total mass of the pool will be 1,500 ng.

1.1 (continued)

NUMBER OF INDEXED SAMPLES PER POOL	AMOUNT OF EACH INDEXED SAMPLE PER POOL	TOTAL MASS PER POOL
1	200 ng	200 ng
2	200 ng	400 ng
3	200 ng	600 ng
4	200 ng	800 ng
8	187.5 ng	1,500 ng

NOTES:

- If the amount of library you have is insufficient, you can use a smaller amount; however, using less may result in decreased library complexity.
- More than 1,500 ng (1.5 µg) total DNA can be used; however, do not use more than 4 µg total DNA, as this might lead to incomplete enrichment.

1.2

- Transfer the calculated volumes from each amplified indexed library to a hybridization reaction tube (either a 0.2-ml thin-walled PCR strip-tube or 96-well plate) for each hybridization reaction to be performed.

PREPARE THE METHYLATION PRE-HYBRIDIZATION SOLUTION

1.3

Add the following volumes of reagents to each amplified indexed library to create a methylation pre-hybridization solution. Mix by flicking the tube(s).

REAGENT	VOLUME
Twist Custom Methylation or Fixed Methylome Panel	4 µl
Optional: Secondary Methylation Panel (If a secondary panel is not used, do not add water as the entire solution will be dried down)	4 µl
Universal Blockers	8 µl
Blocker Solution	5 µl
Methylation Enhancer	2 µl

NOTE: Depending on the methylation level of the starting gDNA, the target regions of your custom methylation panel, and the GC content of your panel, more Methylation Enhancer (up to 5 µl) may be added to reduce the off-target metric in the final sequencing metrics. Likewise, if there is low methylation accompaniment in the starting gDNA, little to no Methylation Enhancer may be needed to decrease the off-target metric. Further optimization may be needed.

NOTE: If using the Twist Human Methylome Panel while preparing the methlation pre-hybridization solution, follow the protocol modifications outlined in Appendix B.

1.4

Pulse-spin the tube(s) and ensure there are minimal bubbles present.

1.5

Dry the methylation pre-hybridization solution (library, panel, blockers) in the tube(s) used for the hybridization reaction using a SpeedVac system (or a similar evaporator device) with low or no heat.

SAFE STOPPING POINT: The dried pre-hybridization solution may be stored at -20°C for up to 24 hours before proceeding to Step 2.

PROCEED TO STEP 2: HYBRIDIZE CAPTURE PROBES WITH POOLS

STEP 2 HYBRIDIZE CUSTOM METHYLATION PANEL WITH POOLS

Use the methyation pre-hybridization solution from Step 1, as well as the Fast Hybridization Mix and Hybridization Enhancer you thawed in Step 1.

⚠ IMPORTANT: Before proceeding with this step, test the compatibility of your thermal cycler and PCR tubes or plates by incubating them at 95°C for up to 5 minutes to ensure they do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

Reagents Required

- Dried methylation pre-hybridization material (from Step 1.5)
- Reagents thawed in Step 1:
  - Fast Hybridization Mix
  - Hybridization Enhancer

Before You Begin

Program a 96-well thermal cycler with the following conditions and set the heated lid to 85°C or on:

TEMPERATURE	TIME
95°C	HOLD
95°C	5 minutes
60°C	2 hours[1,2]

[1]Set this hybridization time as needed for your application (anywhere from 30 minutes to 4 hours).
[2]If using the Twist Human Methylome Panel, hybridization time should be extended to over night. Please see Appendix B.

RESUSPEND THE METHYLATION PRE-HYBRIDIZATION SOLUTION

2.1 Heat the Fast Hybridization Mix at 65°C for 10 minutes, or until all precipitate is dissolved. Vortex and use immediately.

⚠ IMPORTANT: Do not allow the Fast Hybridization Mix to cool to room temperature.

2.2 Resuspend the dried methylation pre-hybridization material from Step 1.5 in 20 µl Fast Hybridization Mix.

NOTES:

- If this resuspended solution requires transfer into a secondary vessel for hybridization, mix by flicking and please wait an additional 5 minutes for resuspension.
- Fast Hybridization Mix is viscous. Pipette slowly to ensure accuracy.
- Small white particles may be present in the custom methylation panel. They do not affect the final captured product.

2.3 Pulse-spin the tube(s) and ensure there are no bubbles present.

PERFORM THE HYBRIDIZATION REACTION

2.4 Add 30 µl Hybridization Enhancer to the top of the methylation pre-hybridization solution.

2.5 Pulse-spin the tube(s) to ensure the entire solution is at the bottom of the tube(s).

NOTE: Hybridization Enhancer settles on top of the hybridization reaction after the pulse-spin. This does not affect the final captured product.

2.6 Transfer the tube(s) to the preheated thermal cycler and move to Steps 2 and 3 of the thermocycler program.

NOTE: We recommend starting with a 2-hour hybridization time, but hybridization times between 30 minutes and 4 hours may also be used depending on your application. Further optimization may be needed.

⚠ IMPORTANT: Make sure the tube is sealed tightly to prevent evaporation during the incubation.

PROCEED TO STEP 3: BIND HYBRIDIZED METHYLATION TARGETS TO STREPTAVIDIN BEADS

STEP 3 BIND HYBRIDIZED METHYLATION TARGETS TO STREPTAVIDIN BEADS

Reagents Required

- Fast Hybridization reactions (from Step 2.6)
- From the Twist Fast Wash Buffers:
  - Fast Binding Buffer
  - Fast Wash Buffer 1
  - Wash Buffer 2
- From Twist Binding and Purification Beads:
  - Streptavidin Binding Beads

Before You Begin

- Inspect the following for precipitate, and if a precipitate is observed, heat at 48°C until it is dissolved:
  - Fast Binding Buffer
  - Fast Wash Buffer 1
  - Wash Buffer 2
- For each hybridization reaction:
  - Equilibrate 800 µl Binding Buffer to room temperature
  - Preheat 450 µl Fast Wash Buffer 1 to 65°C
  - Preheat 700 µl Wash Buffer 2 to 48°C
- Equilibrate the Streptavidin Binding Beads to room temperature for at least 30 minutes
- In preparation for Step 4 (Post-Capture PCR Amplify, Purify, and Perform QC):
  - DNA Purification Beads (from the Twist Binding and Purification Beads) — equilibrate to room temperature for at least 30 minutes
  - Equinox Amp Mix (2x)*—thaw on ice
  - Amplification Primers (from the Twist Fast Hybridization and Wash Kit) — thaw on ice

NOTE: If using the Twist Human Methylome Panel during hybridization, follow the protocol modifications outlined in Appendix B.

PREPARE THE BEADS

3.1 Vortex the pre-equilibrated Streptavidin Binding Beads until mixed.

3.2 Add 100 µl Streptavidin Binding Beads to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.

3.3 Add 200 µl Fast Binding Buffer to the tube(s) and mix by pipetting.

3.4 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

BIND THE TARGETS

3.5 Repeat the wash (Steps 3.3 and 3.4) two more times for a total of three washes.

3.6 After removing the clear supernatant from the third wash, add a final 200 µl Fast Binding Buffer and resuspend the beads by vortexing until homogenized.

3.7 After the hybridization (Step 2.6) is complete, open the thermal cycler lid and quickly transfer the volume of each hybridization reaction including Hybridization Enhancer into a corresponding tube of washed Streptavidin Binding Beads from Step 3.6. Mix by pipetting and flicking.

NOTE: Rapid transfer directly from the thermal cycler at 60°C is a critical step for minimizing off-target binding. Do not remove the tube(s) of hybridization reaction from the thermal cycler or otherwise allow it to cool to less than 60°C before transferring the solution to the washed Streptavidin Binding Beads.

3.8 Mix the tube(s) of the hybridization reaction with the Streptavidin Binding Beads for 30 minutes at room temperature on a shaker, rocker, or rotator at a speed sufficient to keep the solution mixed.

NOTE: Do not vortex. Aggressive mixing is not required.

3.9 Remove the tube(s) containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tube(s).

3.10 Place the tube(s) on a magnetic stand for 1 minute.

3.11 Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.

NOTE: A trace amount of Hybridization Enhancer may be visible after supernatant removal and throughout each wash step. It will not affect the final capture product.

3.12 Remove the tube(s) from the magnetic stand and add 200 µl preheated (65 C) Fast Wash Buffer 1. Mix by pipetting.

3.13 Incubate the tube(s) for 5 minutes at 65 C.

3.14 Place the tube(s) on a magnetic stand for 1 minute.

3.15 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

3.16 Remove the tube(s) from the magnetic stand and add an additional 200 µl of preheated (65°C) Fast Wash Buffer 1. Mix by pipetting.

3.17 Incubate the tube(s) for 5 minutes at 65°C.

NOTE: The temperature of the preheated Fast Wash Buffer 1 can be altered to tune off-target and uniformity in a use-case-specific manner. Please see Appendix A for more information.

3.18 Pulse-spin to ensure the entire solution is at the bottom of the tube(s).

3.19 Transfer the entire volume from Step 3.18 (~200 µl) into a new 1.5-ml microcentrifuge tube, one per hybridization reaction. Place the tube(s) on a magnetic stand for 1 minute.

⚠ IMPORTANT: This step reduces background resulting from non-specific binding to the surface of the tube.

3.20 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

3.21 Remove the tube(s) from the magnetic stand and add 200 µl of 48°C Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure the entire solution is at the bottom of the tube(s).

3.22 Incubate the tube(s) for 5 minutes at 48°C.

3.23 Place the tube(s) on a magnetic stand for 1 minute.

3.24 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

3.25 Repeat the wash (Steps 3.21–3.24) two more times, for a total of three washes.

3.26 After the final wash, use a 10 µl pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.

NOTE: Before removing supernatant, the bead pellet may be briefly spun to collect supernatant at the bottom of the tube or plate and returned to the magnetic plate.

3.27 Remove the tube(s) from the magnetic stand and add 45 µl water. Mix by pipetting until homogenized, then incubate this solution, hereafter referred to as the Streptavidin Binding Bead slurry, on ice.

NOTE: When starting with cfDNA, the Streptavidin Binding Bead pellet can be resuspended in 22.5 µl and the entire slurry can be used going into the next step.

PROCEED TO STEP 4: POST-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

STEP 4 POST-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

Reagents Required

- Streptavidin Binding Bead slurry (from Step 3.27)
- Ethanol
- Molecular biology grade water
- Reagents thawed and equilibrated in Step 3:
  - DNA Purification Beads
  - Equinox Library Amp Mix (2x)
  - Amplification Primers
- Agilent Bioanalyzer High Sensitivity DNA Kit (or equivalent)
- Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay

Before You Begin

Prepare 500 µl 80% ethanol for each Streptavidin Binding Bead slurry to be processed.

PREPARE THE BEADS, THERMAL CYCLER, AND PCR MIX

4.1

Program a thermal cycler with the following conditions. Set the heated lid to 105°C.

STEP	TEMPERATURE	TIME	NUMBER CYCLES
1 Initialization	98°C	45 seconds	1
2 Denaturation	98°C	15 seconds	Varies
Annealing	60°C	30 seconds	
Extension	72°C	30 seconds	
3 Final Extension	72°C	1 minute	1
4 Final Hold	4°C	HOLD	–

CUSTOM PANEL SIZE	NUMBER CYCLES
>100 Mb	8
50–100 Mb	9
25–50 Mb	10
10–25 Mb	11
2.5–10 Mb	12
1–2.5 Mb	13
500–1,000 kb	14
100–500 kb	15
50–100 kb	16
<50 kb	17

NOTE: The number of amplification cycles may vary depending on custom methylation panel target size.

4.2

If the Streptavidin Binding Bead slurry has settled, mix by pipetting.

4.3

Transfer 22.5 µl of the Streptavidin Binding Bead slurry to a 0.2-ml thin-walled PCR strip-tube(s). Keep on ice until ready to use in the next step.

NOTE: Store the remaining 22.5 µl water/Streptavidin Binding Bead slurry at –20°C for future use.

4.4 Prepare a PCR mixture by adding the following reagents to the tube(s) containing the Streptavidin Binding Bead slurry. Mix by pipetting.

REAGENT	VOLUME PER REACTION
Streptavidin Binding Bead Slurry	22.5 µl
Amplification Primers, ILMN	2.5 µl
Equinox Library Amp Mix (2x)	25 µl
Total	50 µl

PCR AMPLIFY

4.5 Pulse-spin the tubes, transfer them to the thermal cycler and start the cycling program.

4.6 When the thermal cycler program is complete, remove the tube(s) from the block and immediately proceed to the Purify step.

PURIFY

4.7 Vortex the pre-equilibrated DNA Purification Beads until well mixed.

4.8 Add 90 µl (1.8x) homogenized DNA Purification Beads to the tube(s) from Step 4.6. Mix well by vortexing.

NOTE: It is not necessary to recover supernatant or remove Streptavidin Binding Beads from the amplified PCR product.

4.9 Incubate for 5 minutes at room temperature.

4.10 Place the tube(s) on a magnetic plate for 1 minute or until the supernatant is clear.

4.11 Without removing the tube(s) from the magnetic plate, remove and discard the clear supernatant.

4.12 Wash the DNA Purification Bead pellet with 200 µl freshly prepared 80% ethanol for 1 minute, then remove and discard the ethanol. Repeat this wash once, for a total of two washes, while keeping the tube on the magnetic plate.

4.13 Using a 10 µl pipet, remove all residual ethanol, making sure to not disturb the bead pellet.

NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

4.14 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

4.15 Remove the tube(s) from the magnetic plate and add 32 µl water. Mix by pipetting until homogenized and incubate at room temperature for 2 minutes.

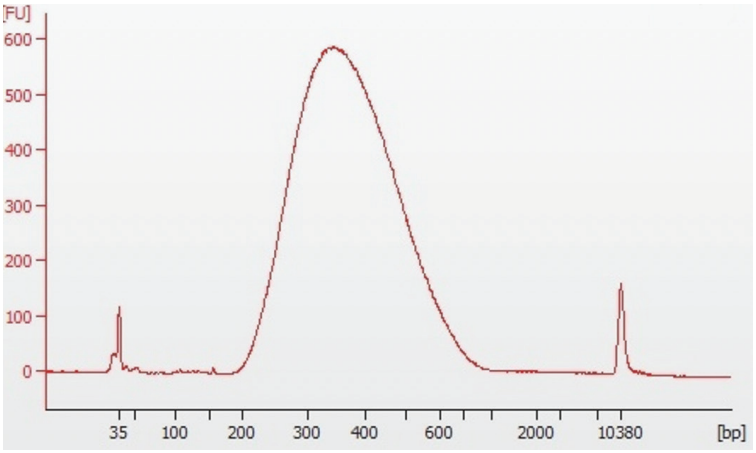
4.16 Place the tube(s) on a magnetic plate and let stand for 3 minutes or until the beads fully pellet.

4.17 Transfer 30 µl of the clear supernatant containing the enriched library to a clean thin-walled PCR 0.2-ml strip-tube, making sure not to disturb the bead pellet.

PERFORM QC

4.18 Validate and quantify each enriched library using an Agilent Bioanalyzer High Sensitivity DNA Kit and a Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay.

NOTE: When using the Agilent Bioanalyzer High Sensitivity DNA Kit, load 0.5 µl of the final sample. Average fragment length should be 350–450 bp using a range setting of 150–1,000 bp. Final concentration should be ≥5-15 ng/µl depending on the custom methylation panel target size and the number of PCR cycles used.



Electropherogram generated by an Agilent High Sensitivity DNA analysis of the enriched gDNA library samples that were prepared as described. Note the single prominent peak.

STOPPING POINT: If not proceeding immediately to the next step, store the Enriched Library sample at –20°C for up to 24 hours.

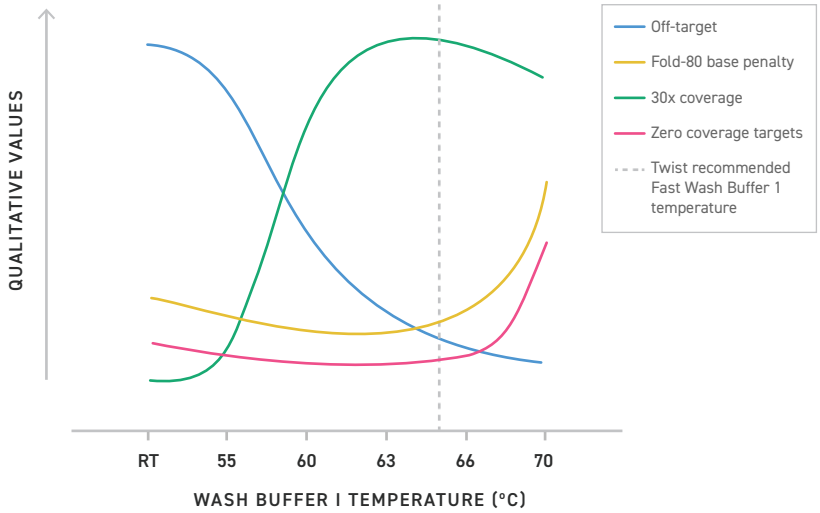
STEP 5 SEQUENCING ON AN ILLUMINA PLATFORM

Sequence the Enriched Libraries on an Illumina platform. Sequencing protocols and settings will depend on the application and instrumentation used. Please contact customersupport@twistbioscience.com for recommendations.

END OF WORKFLOW

APPENDIX A: TEMPERATURE ADJUSTMENT OF FAST WASH BUFFER 1 USING THE TWIST TARGETED METHYLATION SYSTEM

As with all applications using the Twist Fast Hybridization workflow, adjusting the temperature of the Fast Wash Buffer 1 will change the underlying Picard performance metrics in a predictable manner. Specificity, uniformity, coverage and lack-of-coverage (represented by Off-Target, Fold-80 Base Penalty, 30X Coverage, and Zero Coverage Targets Picard metrics, respectively) are shown in the figure below. Please refer to this figure and the Fast Wash Buffer 1 temperatures on the x-axis when optimizing panel performance around these metrics. Results shown below are using a 4-hour hybridization time with no Methylation Enhancer. Panel design, target size, GC content, and gDNA methylation levels will vary results slightly. Twist recommends starting with a Fast Wash Buffer 1 temperature of 65°C; however, the system is compatible with a temperature range of 63°C to 66°C, allowing the temperature to be tuned for your specific custom panel.



END OF APPENDIX

APPENDIX B: USE OF THE TWIST HUMAN METHYLOME PANEL

The Twist Human Methylome Panel is a target enrichment panel that spans 123 Mb of the human genome, with a target area covering over 3.98M CpG sites. Researchers can use the panel to perform robust methylation detection in a diverse range of research applications, such as cancer genomics, human development, and functional genomics.
The following steps found in the Twist Targeted Methylation Sequencing Protocol need to be modified when using the Twist Human Methylome Panel:

Before You Begin

- When using the Twist supplied NEBNext Enzymatic Methyl-seq Library Preparation Protocol, the length of the mechanically sheared gDNA should be between 200 to 250bp, giving a final library size of 350 to 400bp.
- When generating libraries using the Twist supplied NEBNext Enzymatic Methyl-seq Library Preparation Protocol, the number of amplification cycles used in Step 7.3 should be set to 9.

MODIFIED STEPS

PREPARE THE METHYLATION PRE-HYBRIDIZATION SOLUTION (PG. 9)

1.3 Add the following volumes of reagents to each amplified indexed library to create a methylation prehybridization solution. Mix by flicking the tube(s).

REAGENT	VOLUME
Twist Custom Methylation Panel	4 µl
Universal Blockers	8 µl
Blocker Solution	5 µl
Methylation Enhancer	2 µl

PERFORM THE HYBRIDIZATION REACTION (PG. 11)

2.6 Transfer the tube(s) to a preheated thermal cycler with the following conditions:

TEMPERATURE	TIME
95°C	HOLD
95°C	5 minutes
60°C	16 hours

BIND THE TARGETS (PG. 13)

3.12 Remove the tube(s) from the magnetic stand and add 200 µl preheated (63°C) Fast Wash Buffer 1. Mix by pipetting.

3.13 Incubate the tube(s) for 5 minutes at 63°C.

3.16 Remove the tube(s) from the magnetic stand and add 200 µl preheated (63°C) Fast Wash Buffer 1. Mix by pipetting.

3.17 Incubate the tube(s) for 5 minutes at 63°C.

PREPARE THE BEADS, THERMAL CYCLER, AND PCR MIX (PG. 15)

4.1 Perform a thermal cycler with the following conditions. Set the heated lid to 105°C.

STEP	TEMPERATURE	TIME	NUMBER CYCLES
1 Initialization	98°C	45 seconds	1
2 Denaturation	98°C	15 seconds	8-plex: 6
Annealing	60°C	30 seconds	Single plex: 8
Extension	72°C	30 seconds	
3 Final Extension	72°C	1 minute	1
4 Final Hold	4°C	HOLD	–

Final Analysis

While the best aligners and methylation callers will depend on the overall application defined prior to using the workflow, Twist recommends starting by using the BWA-meth aligner at a depth from 150X - 250X raw coverage. We also recommend using MethylDackel to generate CpG methylation states with a minimum depth of 10X and excluding likely variant sites >25%.

Product Information

CATALOG #	NAME	STORAGE
105517	Twist Human Methylome Panel, 2 Reaction	–25 to –15°C
105520	Twist Human Methylome Panel, 12 Reaction	–25 to –15°C
105521	Twist Human Methylome Panel, 96 Reaction	–25 to –15°C

END OF APPENDIX

LAST REVISED: May 11, 2022

REVISION	DATE	DESCRIPTION
4.0	May 16, 2022	· Updated with the Twist Human Methylome Protocol
3.0	Feb 10, 2022	· Minor language change to clarify Protocol Components
2.0	Oct 28, 2021	· Updated protocol for use with Equinox Library Amp Mix · Minor language changes