Protocol title

An optimized protocol to detect high-throughput DNA methylation from custom targeted sequences

Authors

Nathalie Iannuccelli[1*], Remy-Felix Serre[2,3], Céline Vandecasteele[3], Alonzo Lee[4], Yann Merlet[4], Cécile Donnadieu[3] and Julie Demars[1]

[1]GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326 Castanet-Tolosan, France
[2]Qualyse, Le Treuil, INRAE, 19000, Tulle, France
[3]INRAE, GeT-PlaGe, Genotoul, F-31326 Castanet-Tolosan, France (doi: 10.15454/1.5572370921303193E12)
[4]Twist Bioscience, South San Francisco, CA 94080, United States

[*]Correspondence: nathalie.iannuccelli@inrae.fr

ORCID Nathalie Iannuccelli : https://orcid.org/0000-0002-7822-3250
ORCID Céline Vandecasteele : https://orcid.org/0000-0002-5262-0709
ORCID Cécile Donnadieu : https://orcid.org/0000-0002-5164-3095
ORCID Julie Demars: https://orcid.org/0000-0003-2451-0059

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Abstract

The protocol describes a targeted methylation library preparation upstream short read sequencing with an Illumina instrument. The protocol includes the New England Biolabs Next Enzymatic Methyl-seq Library Preparation workflow combined with the Twist Bioscience Targeted Methylation Sequencing workflow. The protocol is divided into 8 steps: fragmentation, library preparation, enzymatic conversion, indexing, pooling, hybridization, capture and amplification. Main advantages are *(i)* the limitation of DNA degradation using enzymatic conversion, *(ii)* both the specificity and efficiency of the capture especially in CpG highly rich regions although this step is the critical step due to the temperature control, and *(iii)* the pooling of samples into 8-plex reducing handling time and experimental costs. However, the workflow takes three working days with several break points. This protocol can be performed in standard molecular biology laboratories and it's suitable for 96 samples. This approach can be adapted to any interesting regions using a custom panel for agronomic species and model organisms but also in human as a diagnostic tool.

Introduction

Studies of epigenetic modifications such as DNA methylation are essential for understanding the regulation of gene expression. Many approaches are developed to identify and quantify DNA methylation across the whole genome or targeted regions. The methylation is detected thanks to the conversion of unmethylated 5-cytosines to uracil either by chemical bisulfite or enzymatic reaction. The different available technologies, based on microarray or sequencing methods, are benchmarked on numerous recent scientific publications (Morrison et al. 2021; Tanić et al. 2022; Shu et al. 2020; Han et al. 2022; Olohan et al. 2018). However, the existing technologies do not allow the simultaneous targeting of large, numerous and specific CpG highly rich regions across the genome. Most of them are based on bisulfite conversion known to damage DNA molecules. In addition, the capture is usually performed before the conversion step that reduces the performance of the capture to the targeted regions.

To overcome these limitations, we optimized a protocol to target specific CpG highly rich regions using enzymatic conversion followed by a custom capture. We adapted two existing protocols : New England Biolabs (NEB) Next Enzymatic Methyl-seq Library Preparation Protocol and Twist Bioscience Targeted Methylation Sequencing Protocol. The workflow is divided into 8 steps: fragmentation, library preparation, enzymatic conversion, indexing, pooling, hybridization, capture and amplification. Here, we described a step-by-step process from genomic DNA fragmentation to sequencing. DNA was mechanically fragmented and ends were repaired. After ligation of methylated adapters, fragments were purified. Then, the 5mC were oxidized by TET2 enzyme and an additional APOBEC treatment deaminated the unmethylated cytosines to uracils. After purification, the

converted library was indexed by PCR amplification. A quality control was done, and eight libraries were pooled in equimolar quantity. Then, hybridization with a custom double-stranded DNA panel was performed to target specific regions. Fragments of interest were captured with streptavidin beads. After the PCR amplification, the pool of libraries was quantified by qPCR before sequencing.

This protocol could be adapted to any region, as it uses customs probes. However, we recommend to adapt *(i)* the number of PCR cycles according to the size of the panel and *(ii)* the hybridization washing temperature to adjust the panel specificity. This approach could be used for methylation studies of any organisms that have a reference genome and also in human as a diagnostic tool.

Reagents and equipment

Reagents

- Twist Targeted Methylation Sequencing Workflow, 96x12 Reactions (Twist Bioscience, 103496) including :
  - NEBNext® EM-seq™ Kit for Twist Targeted Methylation Sequencing, 96 Samples (Twist Bioscience, 101976)
  - Twist Methylation Enhancer, 12 Reactions (Twist Bioscience, 103557)
  - Twist Universal Blocker, 12 Reactions (Twist Bioscience, 100578)
  - Twist Binding and Purification Beads Kit, 12 Reactions (Twist Bioscience, 100983)
  - Twist Fast Hybridization and Wash Kit, 12 Reactions (Twist Bioscience, 101174)
- Twist Methyl Custom panel (Twist Bioscience, 103504) 0.22 fmol/probe/4µl
- Equinox Library Amplification Kit without Primers (WatchMaker Genomics, 7K0021-096, Twist Bioscience, 104108)
- EB buffer : 10mM Tris HCl pH 8.5 (Qiagen, 19086)
- Ethanol absolute anhydrous (Carlo Erba, 4146072)
- Water DEPC treated nuclease free (Fisher BioReagents, BP561-1)
- Formamide (Sigma, F9037)
- Qubit DS DNA Broad Range Assay kit (Invitrogen, ThermoFisher Scientific, Q32850, Qubit™ dsDNA BR Assay Kit)
- Qubit DS DNA High Sensitivity Assay kit (Invitrogen, ThermoFisher Scientific, Q32851, Qubit™ dsDNA HS Assay Kit)
- DNA 7500 Kit (Agilent Technologies, ThermoFisher Scientific, 5067-1506, Agilent DNA 7500 and DNA 12000 Kit Quick Start Guide G2938-90025)

- NEBNext® Enzymatic Methyl-seq (EM-seq™). A high-performance alternative to bisulfite sequencing for methylome analysis. 2019, New England Biolabs, Technical Note
- Highly Sensitive Methylation Detection Using Enzymatic Methyl-seq and Twist Target Enrichment. 2021, Twist Bioscience, Technical Note

Equipment

- ML230 Focused ultrasonicator (Covaris)
- Adaptive Focused Acoustics-Tube TPX Strip 8 (Covaris, 520292)
- Veriti Pro Thermal Cycler 96 well (Applied Biosystems)
- 0.2 ml PCR strip 8 tubes - domed cap (AB-Gene, AB-0266)
- Magnet DynaMag 0.2ml PCR (Invitrogen, Thermo Fisher Scientific, 492025)
- Speed Vacuum concentrator (ThermoFisher Scientific)
- Support Speed-Vac tube de 0.2ml (Eppendorf, 5425715005)
- 0.2 ml PCR tube - domed cap (AB-Gene, AB-0337)
- Microtube 1,5 ml pp safelock (Eppendorf, 0030120086)
- Magnet DynaMag-1.5-2ml (Invitrogen, Thermo Fisher Scientific, 12321D)
- Magnet DynaMag-96 (Invitrogen, Thermo Fisher Scientific, 12027)
- Rotator R2000 RotoFlex (Argos Technologies)
- ThermoMixer C (Eppendorf) Smart Block 1.5ml, 2 devices for 2 different temperatures
- Qubit 3 fluorometer (Invitrogen, Thermo Fisher Scientific, Q33216)
- 2100 Bioanalyzer (Agilent Technologies, G2939BA)
- NextSeq 2000 (Illumina)
- Ordinary lab equipments :
    - Pipettors (P10, P20, P200 and P1000)
    - Multi-channel pipettor (P10, P20, P200)
    - Vortex (StarLab, N2400-6010)
    - 25ml pipetting reservoir (Bio-Pure, Z679933)
    - Mini plate spinner centrifuge (Fisherbrand, 11766427)
    - Mini Microcentrifuge tube (Corning, 6770)

Procedure

An overview of the whole workflow is shown on Figure 1.

NEBNext Enzymatic Methyl-Seq Protocol Standard Insert Library (370-420bp[2])

☞ All reagents thaw on ice unless specific recommendations, then pulse-vortex 2sec to mix, and followed by pulse-spin

1. **Mechanical fragmentation** (Figure 1)

1.1. Genomic DNA shearing (Figure 1)

▢ Dilute genomic DNA (50ng* or 100ng**[2], fluorometric assay) to 50µl with EB buffer

🌱 *50ng per semence extraction and **100ng per blood extraction

▢ Transfer the diluted DNA in a tube appropriated to an ultrasonicator instrument

▢ Fragment the genomic DNA using Covaris ultrasonication parameters [2]

Repeat : 20 iterations

Process : dithering

Treatment :

Duration : 10sec

Peak Power : 210W

Duty Factor : 25%

Cycles Per Burst : 50

Average Power : 52.5W

● Possible stop point post-fragmentation but avoid the DNA freeze thaws

1.2. Optional QC fragmentation (Figure 1)

1.2.1. ***Quantification*** is performed on a Qubit 3.0 fluorometer with DS DNA High Sensitivity Quantitation Assay kit according manufacturer's recommendations (Agilent, ThermoFisher Scientific)

1.2.2. ***Validation*** is performed on a 2100 Bioanalyzer with DNA 7500 kit according manufacturer's recommendations (Agilent Technologies)

📝 Average size distribution should be approximately 240-290bp[2]

2. **Library preparation** (Figure 1)

2.1. End repair/dA-tailing (Figure 1)

▢ Transfer 50µl of the sheared DNA in a 0.2ml tube appropriated to a thermocycler instrument

▢ On ice, add the following components to the 0.2ml tube of sheared DNA :

▢ NEBNext Ultra II End prep Reaction Buffer (green) : 7µl

▢ NEBNext Ultra II End prep Enzyme Mix (green) : 3µl

Total volume : 60µl

▢ Set a 200µl pipette to 50µl and then gently pipette the entire volume up and down at least 10 times to mix thoroughly

▢ Perform a quick spin to collect all liquid from the sides of the 0.2ml tube

📝 The presence of small amount of bubbles will not interfere with performance

▢ Place the 0.2ml tube in a thermocycler and run the following program :

Temperature	Time
20°C	30min
65°C	30min
4°C	hold

2.2. Methylated adaptor ligation (Figure 1)

▢ On ice, add the following components to the 0.2ml tube of repaired DNA :

▢ NEBNext® EM-seq™ Adaptor (red) : 2.5µl

Total volume : 62.5µl

▢ Set a 200µl pipette to 50µl and then gently pipette the entire volume up and down at least 10 times to mix thoroughly

▢ Perform a quick spin to collect all liquid from the sides of the 0.2ml tube

▢ On ice, add the following components to the 0.2ml tube of repaired DNA :

▢ NEBNext® Ligation Enhancer (red) : 1µl

▢ NEBNext® Ultra™ II Ligation Master Mix (red) : 30µl

Total volume : 93.5µl

📝 The Ligation Master Mix is **viscous**

👉 For multiple reactions, a master mix of above reaction components can be prepare before addition to the mix sample/adaptor

▢ Set a 200µl pipette to 80µl and then gently pipette the entire volume up and down at least 10 times to mix thoroughly

▢ Perform a quick spin to collect all liquid from the sides of the 0.2ml tube

📝 The presence of small amount of bubbles will not interfere with performance

▢ Incubate at 20°C for 15min in a thermocycler **with the heated lid turned off**

● Safe stopping point : Sample can be stored overnight at -20°C

2.3. Clean-up of adaptor ligated DNA (Figure 1)

▢ Equilibrate NEBNext® Sample Purification Beads to room temperature (30min)

▢ Vortex NEBNext® Sample Purification Beads until homogenization

▢ Add 110µl (1.18X) of homogenized beads to each sample

▢ Mix well by vortexing (until homogenization)

▢ Incubate 5min at room temperature

▢ Place 0.2ml tube against an appropriate magnetic stand for 5min (or when the solution is clear) to separate the beads from the supernatant

▢ Carefully remove and discard supernatant

📝 Do not discard beads pellet that contain DNA targets

▢ Add 200µl of 80% **freshly** prepared 80% ethanol to gently wash the beads pellet

📝 Do not disturb the bead pellet

▢ Incubate 1min

▢ Carefully remove and discard ethanol

📝 Do not discard beads that contain DNA targets

▢ Add 200µl of 80% **freshly** prepared 80% ethanol to gently wash the beads pellet

📝 Do not disturb the beads pellet

▢ Incubate 1min

▢ Carefully remove and discard ethanol

📝 Do not discard beads that contain DNA targets

📝 Be sure to remove all visible liquid using a 10µl pipette tip

▢ Air-dry the beads pellet for 2min (or until the beads pellet is dry)

👉 Do not over-dry the beads pellet to not reduce DNA recovery. The beads must still remain dark brown and glossy looking ; the beads must not turn lighter and start to crack

▢ Remove the 0.2ml tube from the magnetic stand

▢ Add 30µl of Elution Buffer (white) to the beads pellet

▢ Mix well by pipetting up and down 10 times (until homogenization)

▢ If necessary, quickly spin the sample to collect the liquid from the sides of the 0.2ml tube

▢ Incubate 2min at room temperature

▢ Place the 0.2ml tube against an appropriate magnetic stand for 3min (until the solution is clear) to separate the beads from the supernatant

▢ Transfer 28µl of the clear supernatant containing the adaptor ligated DNA to a new 0.2ml PCR tube

📝 Make sure not to disturb the beads pellet

● Safe stopping point : Sample can be stored overnight at -20°C

2.4. Optional QC ligation (Figure 1)

2.4.1. ***Quantification*** is performed on a Qubit 3.0 fluorometer with DS DNA High Sensitivity Quantitation Assay kit according manufacturer's recommendations (Agilent, ThermoFisher Scientific)

2.4.2. ***Validation*** is performed on a 2100 Bioanalyzer with DNA 7500 kit according manufacturer's recommendations (Agilent Technologies)

📝 Average size distribution should be approximately 370-420bp[2]

3. Enzymatic conversion (Figure 1)

3.1. Oxidation of 5mC and 5hmC (Figure 1)

▢ Add the entire volume of TET2 Reaction Buffer (yellow) to TET2 Reaction Buffer Supplement powder (yellow)

▢ Mix well and mention the date

☞ Reconstituted TET2 Reaction Buffer should be stored at -20°C and discarded after 4 months

▢ On ice, add the following components to the 28µl adaptor ligated DNA :

▢ **Reconstituted** TET2 Reaction Buffer (yellow)	10µl
▢ Oxidation Supplement (yellow)	1µl
▢ DTT (yellow)	1µl
▢ Oxidation Enhancer (yellow)	1µl
▢ TET2 (yellow)	4µl

Volume total : 45µl

☞ For multiple reactions, a master mix of above reaction components can be prepared before addition to the sample

▢ Mix thoroughly by vortexing

▢ Centrifuge briefly

▢ Dilute the 500nM Fe (II) solution (yellow) by adding 1µl to 1249µl of nuclease-free water

⚠ **Use the diluted Fe (II) solution immediately, discard after use**

▢ Add 5µl of **diluted** Fe (II) solution to the 0.2ml tube to the initiated oxidation reaction

Volume total : 50µl

▢ Mix well by vortexing

▢ Centrifuge briefly

▢ Incubate at 37°C for 1hr in a thermocycler

▢ Transfer the sample on ice

▢ Add 1µl of Stop Reagent (yellow)

▢ Mix well by vortexing

▢ Centrifuge briefly

▢ Incubate at 37°C for 30min then at 4°C in a thermocycler

● Possible stopping point : Sample can be stored overnight at either 4°C in the thermocycler or at -20°C in the freezer

3.2. Clean-up of oxidated DNA (Figure 1)

▢ Equilibrate NEBNext® Sample Purification Beads to room temperature (30min)

▢ Vortex NEBNext® Sample Purification Beads until homogenization

▢ Add 90µl (1.8X) of homogenized beads to each sample

▢ Mill well by vortexing (until homogenization)
▢ Incubate 5min at room temperature
▢ Place the 0.2ml tube against an appropriate magnetic stand for 5min (until the solution is clear) to separate the beads from the supernatant
▢ Carefully remove and discard supernatant
📝 Do not discard beads pellet that contain DNA targets
▢ Add 200µl of 80% **freshly** prepared ethanol to gently wash the beads pellet
📝 Do not disturb the beads pellet
▢ Incubate 1min
▢ Carefully remove and discard ethanol
📝 Do not discard beads that contain DNA targets
▢ Add 200µl of 80% **freshly** prepared ethanol to gently wash the beads pellet
📝 Do not disturb the beads pellet
▢ Incubate 1min
▢ Carefully remove and discard ethanol
📝 Do not discard beads that contain DNA targets
📝 Be sure to remove all visible liquid using a 10µl pipette tip
▢ Air-dry the beads pellet for 2min (until the beads pellet is dry)
👉 Do not over-dry the beads pellet to not reduce DNA recovery. The beads must still dark brown and glossy looking ; the beads must not turn lighter brown and start to crack
▢ Remove the 0.2ml tube from the magnetic stand
▢ Add 18µl of Elution Buffer (white) to the beads pellet
▢ If necessary, quickly spin the sample to collect the liquid from the sides of the 0.2ml tube
▢ Mix well by pipetting up and down 10 times (until homogenization)
▢ Incubate 2min at room temperature
▢ Place the 0.2ml tube against an appropriate magnetic stand for 3min (until the solution is clear) to separate the beads from the supernatant
▢ Transfer 16µl of the clear supernatant containing the converted DNA to a new 0.2ml PCR tube
📝 Make sure not to disturb the beads pellet
● Safe stopping point : Sample can be stored overnight at -20°C

3.3. Optional QC oxidation (Figure 1)

3.4.1. ***Quantification*** is performed on a Qubit 3.0 fluorometer with DS DNA High Sensitivity Quantitation Assay kit according manufacturer's recommendations (Agilent, ThermoFisher Scientific)

3.4.2. ***Validation*** is performed on a 2100 Bioanalyzer with DNA 7500 kit according manufacturer's recommendations (Agilent Technologies)

📝 Average size distribution should be approximately 370-420bp[2]

3.4. Denaturation (⚠ under chemical fume cupboard) (Figure 1)

☐ Pre-heat a thermocycler to 85°C

☐ Add 4µl formamide to the 16µl of oxidized DNA

☐ Mix well by vortexing

☐ Centrifuge briefly

☐ Incubate at 85°C for 10min in the ***pre-heated*** thermocycler

☐ **Immediately** place on ice

3.5. Deamination of C to U (⚠ under chemical fume cupboard) (Figure 1)

☐ On ice, add **immediately** the following components to the 20µl denatured DNA :

☐ Nuclease water (orange)	68µl
☐ APOBEC Reaction Buffer (orange)	10µl
☐ BSA (orange)	1µl
☐ APOBEC (orange)	1µl

Volume total : 100µl

👉 For multiple reactions, a master mix of above reaction components can be prepared before addition to the denatured DNA

☐ Mix well by vortexing

☐ Centrifuge briefly

☐ Incubate at 37°C for 3hr then at 4°C in a thermocycler

● Possible stopping point : Sample can be stored overnight at either 4°C in the thermocycler or at -20°C in the freezer

3.6. Clean-up of deaminated DNA (Figure 1) under chemical fume cupboard

☐ Equilibrate NEBNext® Sample Purification Beads to room temperature (30min)

☐ Vortex NEBNext® Sample Purification Beads until homogenization

☐ Add 100µl (1X) of homogenized beads to each sample

☐ Mill well by vortexing (until homogenization)

☐ Incubate 5min at room temperature

☐ Place the 0.2ml tube against an appropriate magnetic stand for 5min (until the solution is clear) to separate the beads from the supernatant

☐ Carefully remove and discard supernatant

📝 Do not discard beads pellet that contain DNA targets

☐ Add 200µl of 80% **freshly** prepared ethanol to gently wash the beads pellet

📝 Do not disturb the beads pellet

▢ Incubate 1min

▢ Carefully remove and discard ethanol

📝 Do not discard beads that contain DNA targets

▢ Add 200µl of 80% **freshly** prepared ethanol to gently wash the beads pellet

📝 Do not disturb the beads pellet

▢ Incubate 1min

▢ Carefully remove and discard ethanol

📝 Do not discard beads that contain DNA targets

👉 No more pipetting under fume cupboard at this step, so the beads don't become too dry

📝 Be sure to remove all visible liquid using a 10µl pipette tip

▢ Air-dry the beads pellet for 1.5min (until the beads pellet is dry)

👉 Do not over-dry the beads pellet to not reduce DNA recovery. The beads must still dark brown and glossy looking ; the beads must not turn lighter brown and start to crack

👉 **The beads behave differently during APOBEC clean-up, do not over-dry the beads as they become very difficult to resuspend**

▢ Remove the 0.2ml tube from the magnetic stand

▢ Add 22µl of Elution Buffer (white) to the beads pellet

▢ Mix well by pipetting up and down 10 times (until homogenization)

▢ If necessary, quickly spin the sample to collect the liquid from the sides of the 0.2ml tube

▢ Incubate 2min at room temperature

▢ Place the 0.2ml tube against an appropriate magnetic stand for 3min (until the solution is clear) to separate the beads from the supernatant

▢ Transfer 20µl of the clear supernatant containing the deaminated DNA to a new PCR 0.2ml tube

📝 Make sure not to disturb the beads pellet

● Safe stopping point : Sample can be stored overnight at -20°C

4. <u>Indexing</u> (Figure 1)

4.1. Indexation by PCR (Figure 1)

▢ On ice, add the following components to the 20µl deaminated DNA :

▢ EM-Seq Index Primer tube (10µM) 5µl

▢ NEBNext® Q5U® Master Mix (blue) 25µl

Total volume : 50µl

▢ Mix well by vortexing

▢ Centrifuge briefly

▢ Place the 0.2ml tube in a thermocycler and run the following program :

Cycles step	Temperature	Time	Cycles[2]
Initial denaturation	98°C	30sec	1
Denaturation	98°C	10sec	
Annealing	62°C	30sec	9* or 11**
Extension	65°C	60sec	
Final extension	65°C	5min	1
Hold	4°C		

🏷 **11 cycles for 50ng input and *9 cycles for 100ng input

● Possible stopping point : Sample can be stored overnight at either 4°C in the thermocycler or at -20°C in the freezer

4.2. Clean-up indexed library (Figure 1)

▢ Vortex NEBNext® Sample Purification Beads until mixed well

▢ Add 45µl (0.9X[2]) of homogenized beads to each library

▢ Mill well by vortexing (until homogenization)

▢ Incubate 5min at room temperature

▢ Place the 0.2ml tube against an appropriate magnetic stand for 5min (until the solution is clear) to separate the beads from the supernatant

▢ Carefully remove and discard supernatant

📝 Do not discard beads that contain DNA targets

▢ Add 200µl of 80% freshly prepared ethanol to gently wash the beads pellet

📝 Do not disturb the beads pellet

▢ Incubate 1min

▢ Carefully remove and discard ethanol

📝 Do not discard beads that contain DNA targets

▢ Add 200µl of 80% freshly prepared ethanol to gently wash the beads pellet

📝 Do not disturb the beads pellet

▢ Incubate 1min

▢ Carefully remove and discard ethanol

📝 Do not discard beads that contain DNA targets

📝 Be sure to remove all visible liquid using a P10 pipette tip

▢ Air-dry the beads pellet for 2min (until the beads pellet is dry)

👉 Do not over-dry the beads pellet to not reduce DNA recovery. The beads must still dark brown and glossy looking ; the beads must not turn lighter brown and start to crack

▢ Remove the 0.2ml tube from the magnetic stand

▢ Add 22µl of Elution Buffer (white) to the beads pellet

▢ Mix well by pipetting up and down 10 times (until homogenization)

▢ If necessary, quickly spin the sample to collect the liquid from the sides of the 0.2ml tube

▢ Incubate 2min at room temperature

▢ Place the 0.2ml tube against an appropriate magnetic stand for 3min (until the solution is clear) to separate the beads from the supernatant

▢ Transfer 20µl of the clear supernatant containing the indexed DNA library to a new 0.2ml PCR tube

📝 Make sure not to disturb the beads pellet

● Safe stopping point : Sample can be stored overnight at -20°C

4.3. QC Libraries (Figure 1)

4.3.1. ***Quantification*** is performed on a Qubit 3.0 fluorometer with DS DNA Broad Range Quantitation Assay kit according manufacturer's recommendations (Agilent, ThermoFisher Scientific)

Average concentration per library should be approximately 30-50ng/µl

4.3.2. ***Validation*** is performed on a 2100 Bioanalyzer with DNA 7500 kit according manufacturer's recommendations (Agilent Technologies) (Figure 2)

📝 Average size distribution should be approximately 370-420bp[2]

📝 132 adaptor nucleotides shift between insert and library size

<u>Twist Bioscience Targeted Methylation Sequencing Protocol (DOC-001222 REV 4.0[2])</u>

👉 All reagents thaw on ice unless otherwise specified, then pulse-vortex 2sec to mix and pulse-spin

5. <u>Pooling</u> of 8 samples (Figure 1)

📌 Total DNA amount per pool depends on the number of libraries in the pool

📌 Total DNA amount per pool of 8 samples should be 1500ng

📌 The amount of each library per pool will be the same

▢ Transfer the calculated volumes from each indexed library **in a PCR 0.2ml tube appropriate for the hybridization reaction later**

▢ Add the following reagents to the pool of 8 indexed libraries :

▢ Twist Bioscience **Custom** Methylation Panel (0,01fmol/probe [2])	4µl
▢ Universal Blocker	8µl
▢ Blocker Solution (human Cot-1)	5µl
▢ Methylation Enhancer [2 custom]	2µl

▢ Mix by flicking the 0.2ml tube

▢ Perform a quick spin

👉 Ensure there are minimal bubbles present

▢ Dry the pre-hybridization solution using a vacuum concentrator into 0.2ml appropriate support at low heat (30°C)

● Safe stopping point : Dry pre-hybridization solution can be stored overnight at -20°C

6. <u>Hybridization</u> (Figure 1)

▢ Pre-heat a Thermal Cycler at 95°C

▢ Pre-heat a Thermal Cycler at 65°C

▢ Thaw Fast Hybridization Mix at room temperature

▢ Thaw Hybridization Enhancer at room temperature

▢ Heat 22µl of Fast Hybridization Mix at 65°C for 10min (for each pool)

📝 Do not allow the Fast Hybridization Mix to cool to room temperature

▢Resuspend the dried pre-hybridization material with 20µl pre-heated Fast Hybridization Mix

👉 Fast Hybridization Mix is **viscous**, pipette slowly to ensure accuracy

📝 The presence of small particles in the custom methylation panel will not interfere with performance

▢ Perform a quick spin

👉 Ensure there are no bubbles present

▢ Place the 0.2ml tube of pre-hybridization solution in the pre-heated 65°C Thermal Cycler

▢ Add 30µl of Hybridization Enhancer to the top of the pre-hybridization solution

📝 Hybridization enhancer is mineral oil to prevent evaporation

▢ Perform a quick spin

📝 Hybridization enhancer settles on top of the hybridization reaction does not affect the final captured product

▢ Place the 0.2ml tube of hybridization solution in the **preheated** 95°C Thermal Cycler and run the following program :

Temperature	Time
95°C	5min
60°C[2 custom]	16hr[2 custom]

👉 Make sure the 0.2ml tube is sealed tightly to prevent evaporation during the incubation

7. <u>Capture</u> (Figure 1)

7.1. Streptavidin beads preparation (Figure 1)

▢ Equilibrate Streptavidin Binding beads to room temperature (at least 30min before use)

▢ Equilibrate Fast Binding Buffer to room temperature

▢ Vortex Streptavidin Binding Beads until mixed well

▢ **For each capture reaction**, add 100µl Streptavidin Binding Beads to a clean 1.5ml tube (1 tube per hybridization)
▢ Add 200µl Fast Binding Buffer
▢ Mix by pipetting until mixed well
▢ Place on a compatible magnetic stand
▢ Incube 1min at room temperature
▢ Carefully remove and discard supernatant
📝 Do not disturb the bead pellet
▢ Remove from the magnetic stand
▢ Add 200µl Fast Binding Buffer
▢ Mix by pipetting until mixed well
▢ Place on a compatible magnetic stand
▢ Incube 1min at room temperature
▢ Carefully remove and discard supernatant
📝 Do not disturb the bead pellet
▢ Remove from the magnetic stand
▢ Add 200µl Fast Binding Buffer
▢ Mix by pipetting until mixed well
▢ Place on a compatible magnetic stand
▢ Incube 1min at room temperature
▢ Carefully remove and discard supernatant
📝 Do not disturb the bead pellet
▢ Remove from the magnetic stand
▢ Add 200µl Fast Binding Buffer
▢ Resuspend washed beads by vortexing until homogenized

7.2. Streptavidin binding beads (Figure 1)
▢ After hybridization is complete, **quickly and directly** transfer the full volume (including hybridization enhancer) of an hybridization into a 1.5ml tube of **washed** streptavidin beads
⚠ **Rapid transfer directly from the Thermal Cycler is a critical step for minimizing off-target binding**
▢ Mix by pipetting and flicking
▢ Mix on a rotator mixer for 30min at room temperature at a sufficient speed to keep the solution mixed (22 rpm)

7.3. Wash non specific captured targets (Figure 1)
▢ If a precipitate is observed, heat at 48°C Fast Binding Buffer, Fast Wash Buffer 1 and Wash Buffer 2 until all precipitate is dissolved

☐ Pre-heat 500µl Fast Wash Buffer 1 to 63°C[2 custom] in a thermomixer (for each pool, 1 pool per 1.5ml tube)
☐ Pre-heat 700µl Wash Buffer 2 to 48°C in a thermomixer (for each pool, 1 pool per 1.5ml tube)
☐ Remove the 1.5ml tube containing the hybridization reaction with streptavidin binding beads from the rotator mixer
☐ Perform a quick spin to ensure that the whole solution is at the bottom of the 1.5ml tube
☐ Place on a compatible magnetic stand
☐ Incubate 1min at room temperature
☐ Carefully remove and discard supernatant including hybridization enhancer
📝 Do not discard beads pellet that contain DNA targets
📝 Trace amount of hybridization enhancer may be visible after supernatant removal, but it will not affect the final capture product
☐ Remove the 1.5ml tube from the magnetic stand
☐ Add 200µl **preheated 63°C**[2 custom] **Fast Wash Buffer 1**
☐ Mix by pipetting
☐ Perform a quick spin
☐ Incubate 5min at 63°C in the pre-heated thermomixer
☐ Place the 1.5ml tube on a compatible magnetic stand
☐ Incube 1min at room temperature
☐ Carefully remove and discard clear supernatant
📝 Do not discard beads pellet that contain DNA targets
☐ Remove the 1.5ml tube from the magnetic stand
☐ Add 200µl **preheated 63°C**[2 custom] **Fast Wash Buffer 1**
☐ Mix by pipetting
☐ Perform a quick spin
☐ Incubate 5min at 63°C in the pre-heated thermomixer
☐ Perform a quick spin to ensure all solution is at the bottom of the 1.5ml tube
☐ Transfer the entire volume to a new 1.5ml tube
👉 **This step reduces background resulting from non-specific binding to the surface of the 1.5ml tube**
☐ Place the 1.5ml tube on a compatible magnetic stand
☐ Incubate 1min at room temperature
☐ Carefully remove and discard clear supernatant
📝 Do not discard beads pellet that contain DNA targets
☐ Remove the 1.5ml tube from the magnetic stand
☐ Add 200µl **preheated 48°C Wash Buffer 2**
☐ Mix by pipetting

▢ Perform a quick spin
▢ Incubate 5min at 48°C in the pre-thermomixer
▢ Place the 1.5ml tube on a compatible magnetic stand
▢ Incubate 1min at room temperature
▢ Carefully remove and discard clear supernatant
📝 Do not discard beads pellet that contain DNA targets
▢ Add 200µl **preheated 48°C Wash Buffer 2**
▢ Mix by pipetting
▢ Perform a quick spin
▢ Incubate 5min at 48°C in the pre-heated thermomixer
▢ Place the 1.5ml tube on a compatible magnetic stand
▢ Incubate 1min at room temperature
▢ Carefully remove and discard clear supernatant
📝 Do not discard beads pellet that contain DNA targets
Add 200µl preheated 48°C Wash Buffer 2
▢ Mix by pipetting
▢ Perform a quick spin
▢ Incubate 5min at 48°C in pre-heated thermomixer
▢ Place the 1.5ml tube on a compatible magnetic stand
▢ Incubate 1min at room temperature
▢ Carefully remove and discard clear supernatant
📝 Do not discard beads pellet that contain DNA targets
▢ Remove all traces of supernatant using a 10µl pipette tip
📝 before pipetting, the beads pellet may be briefly spun to collect supernatant at the bottom of the 1.5ml tube and returned to the magnetic stand
👉 **Proceed immediately to the next step, do not allow the beads to dry**
▢ Remove the 1.5ml tube from the magnetic stand
▢ Add 45µl molecular biology grade water
▢ Mix by pipetting until homogenization
▢ Place this solution of streptavidin binding beads **slurry** on ice
● Safe stopping point : slurry can be stored overnight at -20°C, possible stop point

8. Amplification (Figure 1)

8.1. PCR amplification (Figure 1)

▢ Mix by pipetting streptavidin binding beads slurry
▢ Transfer 22.5µl streptavidin binding beads slurry to a new 0.2ml tube appropriate to Thermal Cycler
👉 Store the remaining 22.5µl streptavidin binding beads slurry at -20°C for future use

☐ On ice, add the following reagents to the 0.2ml tube containing streptavidin binding beads slurry :

☐ P5P7 Primers Mix (10µM) : 2.5µl

☐ Equinox Library Amp Mix[2] (2X) : 25µl

Total volume : 50µl

☐ Mix gently by pipetting

☐ Perform a quick spin to collect all liquid from the sides of the 0.2ml tube

☐ Place the 0.2ml tube in a thermocycler and run the following program :

Cycles step	Temperature	Time	Cycles [2 custom]
Initial denaturation	98°C	45sec	1
Denaturation	98°C	15sec	
Annealing	60°C	30sec	10
Extension	72°C	30sec	
Final extension	72°C	1min	1
Hold	4°C		

☞ **Proceed immediately to the next step**

8.2. Clean-up of amplified library[2] (Figure 1)

☐ Equilibrate purification beads to room temperature (30min)

☐ Vortex purification beads until mixed well

☐ Add 90µl (1.8X) of homogenized beads to each library

☐ Mill well by vortexing (until homogenization)

📝 It's not necessary to recover supernatant or remove the streptavidin binding beads from the amplified product

☐ Incubate 5min at room temperature

☐ Place 0.2ml tube against an appropriate magnetic stand for 5min (or when the solution is clear) to separate the beads from the supernatant

☐ Carefully remove and discard supernatant

📝 Do not discard beads pellet that contain DNA targets

☐ Add 200µl of 80% **freshly** prepared 80% ethanol to gently wash the beads pellet

📝 Do not disturb the bead pellet

☐ Incubate 1min

☐ Carefully remove and discard ethanol

📝 Do not discard beads that contain DNA targets

☐ Add 200µl of 80% **freshly** prepared 80% ethanol to gently wash the beads pellet

📝 Do not disturb the beads pellet

☐ Incubate 1min

☐ Carefully remove and discard ethanol

📝 Do not discard beads that contain DNA targets

▢ Remove all traces of ethanol using a 10µl pipette tip

▢ Before pipetting, the beads pellet may be briefly spun to collect ethanol at the bottom of the 0.2ml tube and returned to the magnetic stand

▢ Air-dry the beads pellet for 2min (or until the beads pellet is dry)

📝 Do not over-dry the beads pellet

▢ Remove the 0.2ml tube from the magnetic stand

▢ Add 32µl of water to the beads pellet

▢ Mix well by pipetting up and down 10 times (until homogenization)

▢ If necessary, quickly spin the sample to collect the liquid from the sides of the 0.2ml tube

▢ Incubate 2min at room temperature

▢ Place the 0.2ml tube against an appropriate magnetic stand for 3min (until the solution is clear) to separate the beads from the supernatant

▢ Transfer 30µl of the clear supernatant containing the targets DNA to a new PCR 0.2ml tube

📝 Make sure not to disturb the beads pellet

● Safe stopping point : Sample can be stored overnight at -20°C

8.3. QC Library (Figure 1)

8.3.1. ***Quantification*** is performed on a Qubit 3.0 fluorometer with DS DNA High Sensitivity Quantitation Assay kit according manufacturer's recommendations (Agilent, ThermoFisher Scientific)

Average concentration of libraries's pool should be approximately 5-30ng/µl

8.3.2. ***Validation*** is performed on a 2100 Bioanalyzer with DNA 7500 kit according manufacturer's recommendations (Agilent Technologies) (Figure 3)

📝 Average size distribution should be approximately 370-420bp [2]

Protocol[2] is the functional protocol, after adapted of protocol[1]. Differences between protocol[1] and protocol[2] are listed below :

- Steps 1 to 4 title : Size library protocol : 470-520bp[1] => 370-420bp[2]
- Step 1.1. : Input DNA : 200ng[1] => 50ng or 100ng[2]
- Step 1.1. : Covaris ultrasonication parameters [1] with M220 Covaris :
  Peak Incident Power (W) = 75
  Duty Factor (%) = 10
  Cycles per Burst (cpb) = 200
  Time (sec) = 75sec
- Step 1.1. : Size fragmentation : 350-400bp[1]=> 240-290bp[2]
- Step 4.1. : Number of indexing cycles : 6 cycles [1] => 11 cycles or 9 cycles [2]
- Step 4.2. : Purification beads for clean up indexed library : 0.6X [1] => 0.9X [2]

- Step 4.3.2. : Size converted library : 470-520bp[1] => 370-420bp[2]
- Title steps 5 to 8 : Protocol version : DOC-001066 REV. 1.0[1] =>DOC-001222 REV 4.0[2]
- Step 5. : 0,22fmol/probe/4µl [1] => 0,01fmol/probe/4µl [2]
- Step 5. : Hybridization without Methylation Enhancer [1] => Methylation Enhancer [2]
- Step 6. : Hybridization temperature : 65°C[1] => 60°C[2]
- Step 6. : Hybridization time : 4hrs[1] => 16hrs[2]
- Step 3. : Wash Buffer 1 Temperature : 70°C [1] => 63°C [2]
- Step 8.1. : Enzyme of amplification : KAPA HiFi HotStart ReadyMix [1] (Roche Diagnostic) => Equinox Library Amp Mix[2] (Twist Bioscience)
- Step 8.1. : Number of amplification cycles : 9 cycles [1] => 10 cycles [2]
- Step 8.2. : Two Clean-up amplified library[1] (1.8X et 0.8X) => One Clean-up amplified library[2] (1.8X)
- Step 8.3.2. : Size captured library : 470-520bp [1] => 370-420bp [2]

Troubleshooting

Please refer troubleshooting to Twist Bioscience technical support.

Recommendations to be taken have been directly written into the procedure, but the most important recommendations for the use of custom panel are listed below :

- Step 5 : Custom : Methyl enhancer reduces off-target, the volume added depends on methylation and GC content of the custom panel, optimization may be needed
- Step 6 : Custom : Hybridization temperature depends on the size of the custom panel, optimization may be needed
- Step 6 Custom : Hybridization time depends on the size of the custom panel (from 30min to 4hrs or even overnight), optimization may be needed
- Step 7.3 Custom : Fast Wash Buffer 1 temperature depends on the custom panel, optimization may be needed
- Step 8.1 : Custom : Number of cycles depends of the custom panel size, optimization may be needed
- Step 8.3.2 : Depending on the presence of primer dimers after PCR amplification, it could be necessary to repeat the clean-up of the amplified library.
- Two quality controls are required (steps 4.3 and 8.3), and the other three (steps 1.2, 2.4 and 3.3) quality controls remain optional.

Time taken

The complete protocol takes 3 working days but there are several steps at which the procedure can be paused (stopping points), allowing some flexibility in the workflow. The protocol is carried out into 8 different steps before Illumina sequencing. For details of the

different steps and timing see the workflow (Figure 1). Depending on the number of Quality Controls performed (2 or more) the workflow time is different.

Anticipated results

- This protocol allows to obtain a valid DNA methyl-seq Illumina targeted library to send for sequencing
- This protocol works on mammalian blood and semen samples
- This protocol can be performed on 96 samples in plate format with 8-plex samples for hybridization/capture
- This protocol works with a large custom panel covering highly CpG rich regions
- This protocol will answer the scientific question by capturing interesting regions of the genome
- This protocol can be applied to all species for which a reference genome is available

References

Han, Yanan, Galina Yurevna Zheleznyakova, Yanara Marincevic-Zuniga, Majid Pahlevan Kakhki, Amanda Raine, Maria Needhamsen, et Maja Jagodic. 2022. « Comparison of EM-Seq and PBAT Methylome Library Methods for Low-Input DNA ». *Epigenetics* 17 (10): 1195-1204. https://doi.org/10.1080/15592294.2021.1997406.

Morrison, Jacob, Julie M. Koeman, Benjamin K. Johnson, Kelly K. Foy, Ian Beddows, Wanding Zhou, David W. Chesla, et al. 2021. « Evaluation of whole-genome DNA methylation sequencing library preparation protocols ». *Epigenetics & Chromatin* 14 (1): 28. https://doi.org/10.1186/s13072-021-00401-y.

Olohan, Lisa, Laura-Jayne Gardiner, Anita Lucaci, Burkhard Steuernagel, Brande Wulff, John Kenny, Neil Hall, et Anthony Hall. 2018. « A modified sequence capture approach allowing standard and methylation analyses of the same enriched genomic DNA sample ». *BMC Genomics* 19 (1): 250. https://doi.org/10.1186/s12864-018-4640-y.

Shu, Chang, Xinyu Zhang, Bradley E. Aouizerat, et Ke Xu. 2020. « Comparison of methylation capture sequencing and Infinium MethylationEPIC array in peripheral blood mononuclear cells ». *Epigenetics & Chromatin* 13 (1): 51. https://doi.org/10.1186/s13072-020-00372-6.

Tanić, Miljana, Ismail Moghul, Simon Rodney, Pawan Dhami, Heli Vaikkinen, John Ambrose, James Barrett, Andrew Feber, et Stephan Beck. 2022. « Comparison and Imputation-Aided Integration of Five Commercial Platforms for Targeted DNA Methylome Analysis ». *Nature Biotechnology* 40 (10): 1478-87. https://doi.org/10.1038/s41587-022-01336-9.

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Associated publication

This step-by-step protocol is related to the oral communication "Identification of parental allele-specific methylation in pigs through targeted sequence DNA methylation enrichment methods" presented at the AGBT congress (April 4-6, 2022, San Diego).

The procedure described in this Protocol Exchange chapter focuses on detailing the biological experiments, and is related to the scientific article entitled « A methyl-seq tool to capture genomic imprinted loci » that will be pre-published very soon in BioRxiv (Hubert et al.)