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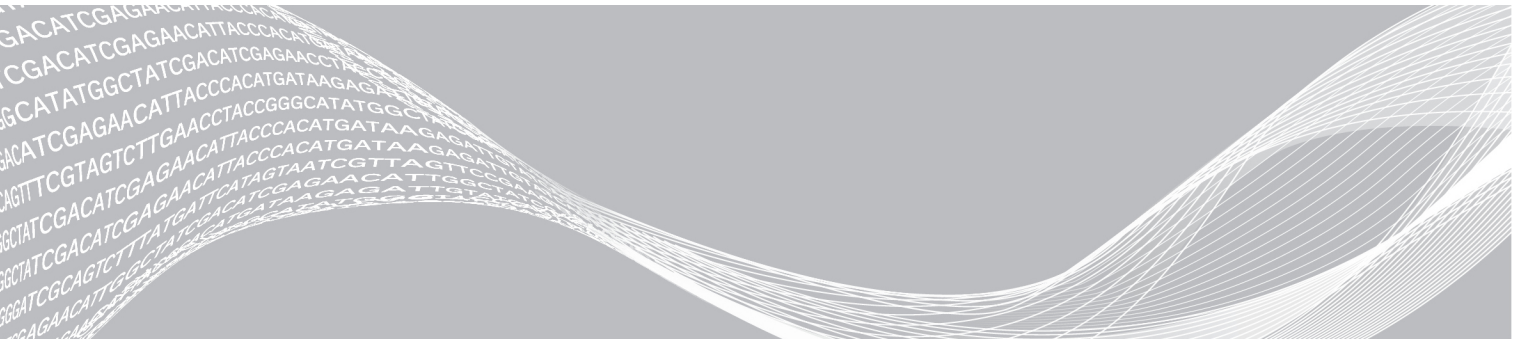
TruSeq Methyl Capture EPIC Library Prep Kit

Reference Guide



Document # 1000000001643 v01

May 2017

Revision History

Document	Date	Description of Change
Document # 1000000001643 v01	May 2017	Updated the name of this guide to *TruSeq Methyl Capture EPIC Library Prep Kit Reference Guide* to match the kit name. Updated the name of BaseSpace to BaseSpace Sequence Hub. Updated reagents information for the bisulfite conversion and amplified library cleanup steps to make sure that the correct beads are used. Added step to proceed immediately to bisulfite conversion after the second elution is complete. Removed SMB and SPB tube volumes, which vary by kit configuration and are not relevant to any procedure. Corrected reagents listed for Amplify Enriched Library in the workflow diagram.
Document # 1000000001643 v00	September 2016	Initial release.

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Chapter 1 Overview

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Introduction

This protocol explains how to prepare up to 48 targeted methylseq libraries using reagents provided in an Illumina® TruSeq® Methyl Capture EPIC Library Prep Kit. The purpose of this protocol is to add adapter sequences to the ends of DNA fragments to generate indexed libraries for subsequent clustering and sequencing.

The kit features:

- Unparalleled fixed panel spans the human methylome, including enhancer and promoter regions.
- Includes most reagents needed to prepare targeted methylseq libraries, including library prep reagents, bisulfite conversion reagents, index adapters, enrichment probes, and purification beads.
- Excellent data quality with only 500 ng input DNA.
- Fast and easy preparation of up to 48 enriched methylseq libraries in less than two days.
- High throughput-friendly procedure with bead-based enrichment and cleanup.
- Streamlined and fully integrated data analysis solution available in BaseSpace® Sequence Hub.

DNA Input Recommendations

For best results, use 500 ng input gDNA and follow all input recommendations. Quantify the input gDNA and assess quality before beginning library preparation.

Quantify Input DNA

Quantify input DNA per the following recommendations:

- Successful library prep depends on accurate quantification of input DNA. To verify, run samples in duplicates.
- Use fluorometric-based methods for quantification, such as Qubit or PicoGreen.
- DNA quantification methods that use intercalating fluorescent dyes measure double-stranded DNA only and are less subject to the presence of excess nucleic acids.
- Do not use spectrophotometric-based methods, such as NanoDrop, which measure the presence of nucleotides and can inaccurately measure gDNA.
- Quantification methods depend on accurate pipetting methods. Do not use pipettes at the extremes of volume specifications. Make sure that pipettes are calibrated.

Assess DNA Quality

Absorbance measurements at 260 nm are commonly used to assess DNA quality:

- The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. Values from 1.8 through 2.0 indicate relatively pure DNA.
- The presence of RNA or small nucleic acid fragments, such as nucleotides, can compromise both absorbance measurements.
- Make sure that samples are free of contaminants.

Positive Control

Use at least one of the following recommended positive control samples. These samples can be used for methylation status control.

- Normal samples:
  - HCC1187 normal (BL) (ATCC, catalog # CRL2323-D)
  - NA12878 (Coriell Institute, catalog # NA12878)
- Cancer samples:
  - HCC1187 breast cancer tumor (ATCC, catalog # CRL2322)
  - HeLa (Biochain, catalog # D1255811)
  - Jurkat (Biochain, catalog # D1255815)

Additional Resources

Visit the TruSeq Methyl Capture EPIC Library Prep Kit support page on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products.

The following documentation is available for download from the Illumina website.

Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
TruSeq Methyl Capture EPIC Library Prep Kit Checklist (document # 1000000001645)	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
TruSeq Methyl Capture EPIC Library Prep Consumables & Equipment (document # 1000000001646)	Provides an interactive checklist of user-provided consumables and equipment.

Chapter 2 Protocol

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Introduction

This chapter describes the TruSeq Methyl Capture EPIC protocol.

- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- Review Best Practices from the TruSeq Methyl Capture EPIC support page on the Illumina website.
- Before proceeding, confirm kit contents and make sure that you have the required equipment and consumables.
  - The polymerase required to amplify the enriched library is user-supplied and not included in the kit.
  - The kit contains sufficient reagents for the indicated number of samples *at four-plex only*.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between *each sample*.
- When adding adapters or primers, change tips between *each tube*.
- Remove unused index adapter tubes from the working area.

Capping the Tubes

- Always cap the tubes before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps

Tube Transfers

- When transferring volumes between tube strips, transfer the specified volume from each tube of a strip to the corresponding tube of the other strip.

- If beads are aspirated into the pipette tips, dispense back to the tube on the magnetic stand and wait until the liquid is clear (~2 minutes).

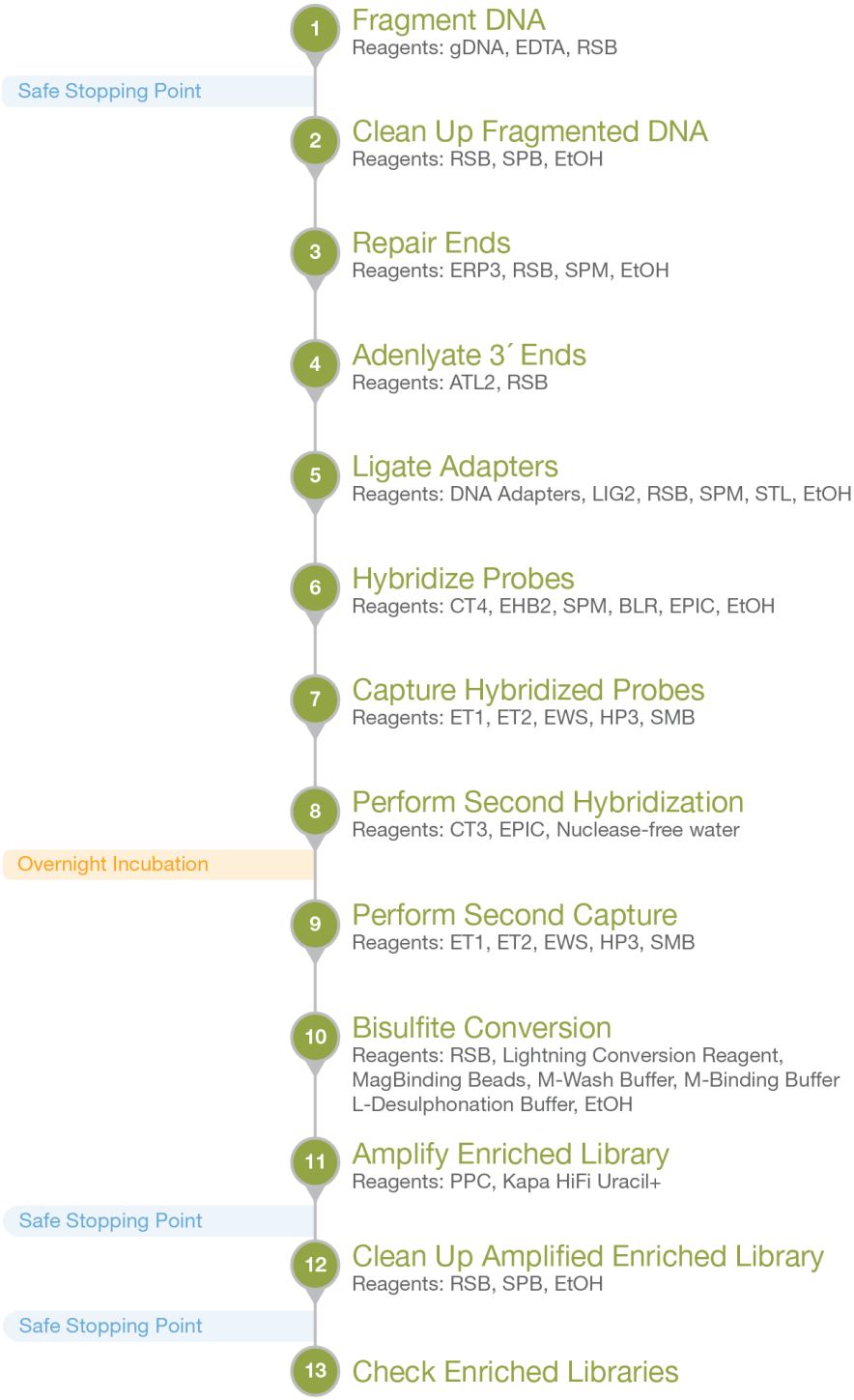
Centrifugation

- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the tube, and to prevent sample loss.

Library Prep Workflow

The following diagram illustrates the workflow using a TruSeq Methyl Capture EPIC kit. Safe stopping points are marked between steps.

Figure 1 TruSeq Methyl Capture EPIC Workflow



Fragment DNA

This step optimally fragments gDNA. Covaris shearing generates dsDNA fragments that contain a mixture of 3′ or 5′ overhangs or blunt ends.

Consumables

- gDNA samples (10 ng/µl, 500 ng total)
- 0.5 M EDTA
- RSB (Resuspension Buffer)
- Covaris tubes (1 per sample)
- Eight-tube strips
- 15 ml conical tube

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
RSB	-25°C to -15°C	Thaw at room temperature. Store at 2°C to 8°C after the initial thaw.
EDTA	-25°C to -15°C	Thaw at room temperature.

2 Turn on and set up the Covaris instrument per manufacturer guidelines.

Procedure

Normalize gDNA

1 Quantify gDNA using a fluorometric-based method within 24 hours of starting the protocol.
Accurate quantification of input DNA is critical. The amount of DNA input is directly proportional to the amount of DNA that is multiplexed with other samples later in the protocol.

NOTE

Requantify frozen samples before shearing. There can be loss of dsDNA over time while in storage.

2 Create shearing buffer premix in a 15 ml conical tube.

Reagent	Volume per Sample
RSB	5 ml
0.5 M EDTA	10 µl

The premix can be stored at 4°C for up to 6 months.

3 Normalize the total input of 500 ng gDNA to 10 ng/µl using the shearing buffer premix for a final volume of 50 µl. Pipette or vortex to mix.

4 Centrifuge briefly.

Fragment DNA

1 Transfer 50 µl DNA samples to separate Covaris tubes.

2 Centrifuge at 280 × g for 5 seconds.

3 Fragment the DNA. Regardless of the shearing instrument used, make sure that the results meet the following criteria:
- A peak between 155–170 bp.
- > 60% of all fragments are between 100–300 bp.
- In the range of 100–300 bp, the mean fragment size is between 180–200 bp.

The following Covaris S220 settings were used for the recommended positive control samples (see *Positive Control* on page 2).

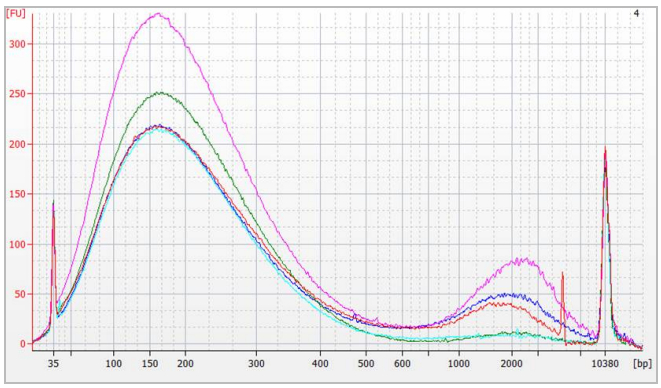
Setting	Value
Duty Factor (%)	10
Peak/Displayed Power (W)	175
Cycles/Burst	200
Duration (seconds)	280
Temperature (°C)	7
Water Level	12

4 Centrifuge at 280 × g for 5 seconds.

5 Transfer 50 µl sample volume from each Covaris tube to a new eight-tube strip.

6 [Recommended] Run 1 µl on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip. If Bioanalyzer peaks differ by > 50%, requantify input DNA using a fluorometric-based method before proceeding.
The following graph includes multiple independent samples to show variability and consistency.

Figure 2 Example Bioanalyzer Result After Covaris Shearing



SAFE STOPPING POINT

If you are stopping, cap the tubes and store at 2°C to 8°C overnight.

Clean Up Fragmented DNA

Resuspension Buffer, Sample Purification Beads, and EtOH are used to clean up the fragmented DNA.

Consumables

- RSB (Resuspension Buffer)

- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.
- Visually inspect SPB to make sure that it is not frozen. Do not use if frozen.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Keep at room temperature until the end of the day.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Keep at room temperature until the end of the day.

2 Prepare fresh 80% ethanol.

Procedure

1 Vortex SPB until well-dispersed.

2 Add 80 µl SPB to each well of the eight-tube strip containing sample. Pipette using a P200 tip or vortex to mix.

3 Incubate at room temperature for 3 minutes.

4 Place on a 96-well magnetic stand and wait until the liquid is clear (~5 minutes).
- Keep on the magnetic stand until step 7.
- Use a 96-well magnetic stand until *First Bind* on page 16.

5 Remove and discard all supernatant from the tube.

6 Wash two times as follows.

a Add 200 µl freshly prepared 80% EtOH to the tube.

b Incubate on the magnetic stand for 30 seconds.

c Remove and discard EtOH.

7 Centrifuge briefly, and then place on a magnetic stand.

8 Remove and discard residual EtOH from the tube.

9 Air-dry on the magnetic stand for 5 minutes.

10 Add 60 µl RSB to the tube.

11 Remove from the magnetic stand, and then pipette or vortex to mix.

12 Incubate at room temperature for 1 minute.
Beads are retained with the elution and carried through to the next step.

Repair Ends

This step uses ERP3 (End Repair Mix 3) to convert the overhangs resulting from fragmentation into blunt ends. The 3′ to 5′ exonuclease activity of this mix removes the 3′ overhangs and the 5′ to 3′ polymerase activity completes the 5′ overhangs.The 5′ ends of the DNA fragments are phosphorylated.

Consumables

- ERP3 (End Repair Mix 3)
- RSB (Resuspension Buffer)
- SPM (Sample Purification Mix)
- Freshly prepared 80% ethanol (EtOH)

About Reagents

- Aspirate and dispense SPM slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ERP3	-25°C to -15°C	Thaw at room temperature, and then place on ice. Return to storage after use.
SPM	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Keep at room temperature until the end of the day.

2 Save the following ERP program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 30°C for 30 minutes
- Hold at 4°C
- Each well contains 100 µl

Procedure

Convert Overhangs

1 Invert ERP3 to mix, and then gently tap the tube.

2 Add 40 µl ERP3 to each sample. Pipette or vortex to mix.

3 Place on the thermal cycler and run the ERP program. Remove when samples reach 4°C.

4 Briefly centrifuge at 280 × g.

Optimize Fragment Length

1 Add 120 µl SPM, which brings the tube to capacity.

2 Pipette carefully to mix, and make sure that the mixture is homogenous.
When the tube is at capacity, the volume is close to the top and spills easily.

3 Incubate at room temperature for 3 minutes.

4 Place on a magnetic stand and wait until the liquid is clear (at least 10 minutes). Keep on the magnetic stand until step 7.

5 Remove and discard supernatant.

6 Wash two times as follows.

   a Add 200 µl freshly prepared 80% EtOH to the tube.

   b Incubate on the magnetic stand for 30 seconds.

   c Remove and discard supernatant.

7 Centrifuge briefly.

8 Place on a magnetic stand. Remove and discard residual EtOH from the tube.

9 Air-dry on the magnetic stand for 5 minutes.

10 Remove from the magnetic stand.

11 Add 17.5 µl RSB to the tube. Pipette or vortex to mix.

12 Incubate at room temperature for 1 minute.
Beads are retained with the elution and carried through to the next step.

Adenylate 3′ Ends

One adenine (A) nucleotide is added to the 3′ ends of the blunt fragments to prevent them from ligating to each other during adapter ligation. A corresponding thymine (T) nucleotide on the 3′ end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

- ATL2 (A-Tailing Mix)

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ATL2	-25°C to -15°C	Thaw at room temperature, and then place on ice.

2 Save the following ATAIL70 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 37°C for 30 minutes
- 70°C for 5 minutes
- 4°C for 5 minutes
- Hold at 4°C
- Each well contains 30 µl

Procedure

1 Add 12.5 µl ATL2 for a total volume of 30 µl.

2 Pipette or vortex to mix.

3 Place on the thermal cycler and run the ATAIL70 program.

Ligate Adapters

This process ligates index adapters to the ends of the DNA fragments, which allow them to hybridize to a flow cell.

Consumables

- DNA Adapter tubes
- LIG2 (Ligation Mix 2)
- RSB (Resuspension Buffer)
- SPM (Sample Purification Mix)
- STL (Stop Ligation Buffer)
- Freshly prepared 80% ethanol (EtOH)
- Eight-tube strips

About Reagents

- Do not remove LIG2 from storage until instructed to do so in the procedure.
- Return LIG2 to storage immediately after use.
- Aspirate and dispense SPM slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
DNA Adapters	-25°C to -15°C	Thaw at room temperature for 10 minutes. Return to storage after use.
STL	-25°C to -15°C	Thaw at room temperature.

2 Prepare fresh 80% ethanol.

3 Save the following LIG program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 30°C for 10 minutes
- Hold at 4°C
- Each well contains 38 µl

Procedure

Add Index Adapters

1 Briefly centrifuge the DNA adapter tubes.

2 Remove LIG2 from -25°C to -15°C storage.

3 Add the following reagents in the order listed to the 8-tube strip containing sample.
   - RSB (1.5 µl)
   - LIG2 (2.5 µl)
   - DNA adapters (4 µl)

   NOTE
   A different DNA adapter is added to each sample. Make sure that DNA adapters are compatible with other samples when multiplexing.

4 Pipette or vortex to mix, and then centrifuge briefly.

5 Place on the thermal cycler and run the LIG program. Remove when samples reach 4°C.

6 Centrifuge briefly.

7 Vortex STL to mix.

8 Add 5 µl STL to the tube. Pipette or vortex to mix.

Clean Up Ligated Fragments

1 Add 43 µl SPM to the tube. Pipette or vortex to mix and make sure that the mixture is homogenous.

2 Incubate at room temperature for 3 minutes.

3 Place on a magnetic stand and wait until the liquid is clear (~5 minutes). Keep on the magnetic stand until step .

4 Remove and discard supernatant.

5 Wash two times as follows.

   a Add 200 µl freshly prepared 80% EtOH to the tube.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard EtOH.

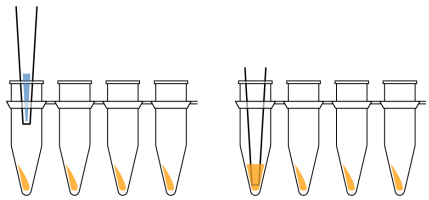
6 Centrifuge briefly, and then place on a magnetic stand.

7 Remove and discard residual EtOH from the tube.

8 Air-dry on the magnetic stand for 5 minutes.

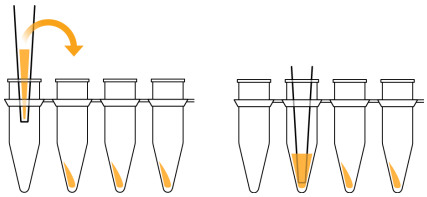
9 Add 40 µl RSB to the first of four samples for multiplexing, and then pipette to mix.

Figure 3 Add 40 µl RSB and Pipette



10 Transfer all contents from the first sample to the second sample, repeating until all four samples are resuspended together.

Figure 4 Transfer Contents and Resuspend



11 Make sure that all liquid and beads have been transferred from each of the corresponding four tubes to the final DNA library pool.
The final elution volume is 40 µl for each set of multiplexed samples. For example, four indexed samples are resuspended in 40 µl RSB.

NOTE

Beads are retained with the elution and carried through to the next step.

Hybridize Probes

This step mixes the DNA library with capture probes to targeted regions of interest. Following the recommended hybridization time ensures that targeted regions bind to the capture probes thoroughly.

Consumables

- CT4 (Capture Target Buffer 4)
- EHB2 (Enrichment Hybridization Buffer 2)
- SPM (Sample Purification Mix)
- BLR (Blocker)
- EPIC Oligos
- Freshly prepared 80% ethanol (EtOH)
- 1.7 ml microcentrifuge tubes and eight-tube strips

About Reagents

- Make sure that you use CT4. CT3 is not used until the second hybridization step.
- Before using CT4, vortex to resuspend the thawed solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
BLR	-25°C to -15°C	Thaw at room temperature.
CT4	-25°C to -15°C	Thaw at room temperature.
EPIC	-25°C to -15°C	Thaw at room temperature. Keep on ice until the end of the day.

Item	Storage	Instructions
EHB2	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Save the following MC HYB1 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 95°C for 10 minutes
- Hold at 58°C
- Each well contains 10 µl

Procedure

1 Add the following reagents in the order listed to the eight-tube strip containing 40 µl DNA library pool, which brings the tube to capacity.
- Blocker (10 µl)
- EPIC Oligos (10 µl)
- SPM (150 µl)

2 Pipette carefully to mix, and make sure that the mixture is homogenous.
When the tube is at capacity, the volume is close to the top and spills easily.

3 Incubate at room temperature for 10 minutes.

4 Place on a magnetic stand and wait until the liquid is clear (~10 minutes). Keep on the magnetic stand until step 7.

5 Remove and discard supernatant.

6 Wash two times as follows.

a Add 200 µl freshly prepared 80% EtOH to the tube.

b Incubate on the magnetic stand for 30 seconds.

c Remove and discard all supernatant from each well.

7 Centrifuge briefly.

8 Place on a magnetic stand. Remove and discard residual EtOH from the tube.

9 Air-dry on the magnetic stand for 5 minutes.

10 Add 7.7 µl CT4. Pipette or vortex to mix.

11 Incubate at room temperature for 2 minutes.

12 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

13 Add 2.5 µl EHB2 to a new eight-tube strip.

14 Transfer 7.5 µl of the concentrated DNA mixture to the eight-tube strip containing 2.5 µl EHB2.
Beads are *not* retained with the elution.

15 Pipette or vortex to mix.

16 Place on the preprogrammed thermal cycler and run the MC HYB1 program.
Each tube contains 10 µl.

17 Keep at the 58°C holding temperature for 35 minutes–2 hours. Do not remove from 58°C until ready for the next step.

Capture Hybridized Probes

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. Two heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

- ET1 (Elute Target Buffer 1)
- ET2 (Elute Target Buffer 2)
- EWS (Enrichment Wash Solution)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 1.7 ml microcentrifuge tubes and 8-tube strips

About Reagents

- ET1 and ET2 have similar labels. Make sure that you use the correct reagent.
- EWS is expected to be cloudy after reaching room temperature.
- Make sure that you use SMB for this procedure, not SPB.
- Discard elution premix after use.
- Inspect SMB to make sure that the beads are not frozen. Do not use if frozen.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ET1	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
EWS	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
ET2	2°C to 8°C	Let stand at room temperature. Return to storage after use.
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex SMB before use. Return to storage after use.

2 Preheat a 1.7 ml microcentrifuge tube heat block to 50°C.

Procedure

First Bind

1 Centrifuge tubes at 280 × g for 1 minute.

> **NOTE**
> If you observe sample loss that is > 15%, do not proceed with the protocol. Poor sealing or insufficient heating of the lid can cause sample loss.

2 Vortex SMB to mix.

3 For each library pool, add 250 µl SMB to a new 1.7 ml microcentrifuge tube.

4 Transfer each pool (~10 µl) to the tubes of SMB.

5 To ensure complete transfer, rinse out tube as follows.

   a Transfer 200 µl from the 1.7 ml tube back to the empty eight-tube strip.
   b Pipette up and down one time.
   c Transfer the volume back to the 1.7 ml tube.

6 Vortex to mix.

7 Incubate at room temperature for 25 minutes.

8 Centrifuge briefly.

9 Place on a 1.7 ml magnetic stand and wait until the liquid is clear (~2 minutes).

> **NOTE**
> Use the 1.7 ml magnetic stand until *Clean Up Amplified Enriched Library* on page 23.

10 Remove and discard all supernatant from the tube.

11 Remove from the magnetic stand.

First Wash

1 Vortex EWS until fully mixed (~20 seconds).

2 Add 200 µl EWS to the tube containing sample and beads.

3 Pulse vortex to mix until SMB pellet is resuspended, which can be difficult.

4 Place on the 50°C heat block for 20 minutes.

5 Immediately place on a magnetic stand and wait until the liquid is clear (~2 minutes).

6 Remove and discard all supernatant from the tube.

7 Remove from the magnetic stand.

8 Repeat steps 2–7 for a total of two washes.

9 Centrifuge briefly.

10 Place on a magnetic stand and remove the remaining supernatant (~5 µl). Do not air dry the pellet.

First Elution

1 For each library pool, create elution premix in a 1.7 ml microcentrifuge tube.
   - ET1 (31.4 µl)
   - HP3 (1.7 µl)
2 Add 30 µl elution premix to the magnetic beads with bound sample. Pipette or vortex to mix.
3 Incubate at room temperature for 5 minutes, and then centrifuge briefly.
4 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
5 Add 5 µl ET2 to a new eight-tube strip.
6 Transfer 29 µl supernatant to the tube containing ET2. Pipette or vortex to mix.

Perform Second Hybridization

This step binds targeted regions of the enriched DNA with capture probes a second time. This second hybridization ensures high specificity of the captured regions.

Consumables

- CT3 (Capture Target Buffer 3)
- EPIC Oligos
- Nuclease-free water

About Reagents

- Make sure that you use CT3. CT4 is used for the first hybridization step.
- Before using CT3, vortex to resuspend the solution.
- Make sure that the reagent is free of crystals. If crystals or cloudiness are observed, vortex until the solution is clear. CT3 typically requires heavy vortexing to resuspend fully.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CT3	-25°C to -15°C	Thaw at room temperature. Before use, vortex until fully resuspended.

2 Save the following MC HYB2 program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 95°C for 10 minutes
   - 18 cycles of 1 minute each, starting at 93°C, then decreasing 2°C per cycle
   - Hold at 58°C
   - Each well contains 100 µl

Procedure

1 Add the following reagents in the order listed to the each tube of the eight-tube strip from the previous step.

- Nuclease-free water (6 µl)
- CT3 (50 µl)
- EPIC Oligos (10 µl)

2 Pipette or vortex to mix, and then centrifuge briefly.

3 Place on the preprogrammed thermal cycler and run the MC HYB2 program.
Each tube contains 100 µl.

SAFE STOPPING POINT

Keep at the 58°C holding temperature for at least 14.5 hours.

Perform Second Capture

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. Two heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for bisulfite conversion.

Consumables

- ET1 (Elute Target Buffer 1)
- ET2 (Elute Target Buffer 2)
- EWS (Enrichment Wash Solution)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 1.7 ml microcentrifuge tubes
- Eight-tube strips

About Reagents

- ET1 and ET2 have similar labels. Make sure that you are using the correct reagent.
- EWS is expected to be cloudy after reaching room temperature.
- Make sure that you use SMB for this procedure, not SPB.
- Discard elution premix after use.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ET1	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
EWS	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
ET2	2°C to 8°C	Let stand at room temperature. Return to storage after use.

Item	Storage	Instructions
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex SMB before use. Return to storage after use.

2 Preheat a 1.7 ml microcentrifuge tube heat block to 50°C.

Procedure

Second Bind

1 Centrifuge tubes at 280 × g for 1 minute.

NOTE

If you see a sample loss that is > 15%, do not proceed with the protocol. Poor sealing or insufficient heating of the lid can cause sample loss.

2 Vortex SMB to mix.

3 For each library pool, add 250 µl SMB to a new 1.7 ml microcentrifuge tube.

4 Transfer each pool (~100 µl) to the tubes containing SMB.

5 Vortex to mix.

6 Incubate at room temperature for 25 minutes.

7 Centrifuge briefly.

8 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

9 Remove and discard all supernatant from the tube.

10 Remove from the magnetic stand.

Second Wash

1 Vortex EWS until fully mixed (~20 seconds).

2 Add 200 µl EWS to the tube.

3 Pulse vortex to mix until SMB pellet is resuspended.

4 Place on the 50°C heat block for 30 minutes.
The second wash requires more incubation time than the first wash.

5 Immediately place on a magnetic stand and wait until the liquid is clear (~2 minutes).

6 Remove and discard all supernatant from the tube.

7 Remove from the magnetic stand.

8 Repeat steps 2–7 for a total of two washes.

9 Centrifuge briefly.

10 Place on a magnetic stand and remove the remaining supernatant (~5 µl). Do not air dry the pellet.

Second Elution

1 For each library pool, create elution premix in a 1.7 ml microcentrifuge tube.
   - ET1 (18.8 µl)
   - HP3 (1 µl)
2 Add 18 µl elution premix to the tube containing beads.
3 Pipette or vortex to mix.
4 Incubate at room temperature for 5 minutes, and then centrifuge briefly.
5 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
6 Add 2.9 µl ET2 to a new eight-tube strip.
7 Transfer 17.1 µl supernatant to the tube.
8 Pipette or vortex to mix. Proceed ***immediately*** to bisulfite conversion.

Bisulfite Conversion

For preparation of unconverted libraries (not used for methylation calls), skip Bisulfite Conversion and proceed to *Amplify Enriched Library* on page 22.

This step deaminates unmethylated cytosine bases in the DNA to produce uracil bases. Methylated cytosines are protected from the conversion to uracil, allowing discrimination of methylated and unmethylated cytosines by sequencing. The bisulfite-treated library is then eluted from the beads in preparation for PCR amplification.

Consumables

- RSB (Resuspension Buffer)
- Lightning Conversion Reagent
- MagBinding Beads
- M-Wash Buffer
- M-Binding Buffer
- L-Desulphonation Buffer
- 100% ethanol (EtOH)
- 1.7 ml microcentrifuge tubes
- Eight-tube strips

About Reagents

- Store Lightning Conversion Reagent, MagBinding Beads, M-Wash buffer, M-Binding Buffer, and L-Desulphonation Buffer at room temperature.
- Store Lightning Conversion Reagent, which is light-sensitive, away from light.
- Check Lightning Conversion Reagent for precipitate before use. If precipitate is observed, vortex vigorously to resuspend. If necessary, incubate at 37°C.
- Make sure that MagBinding beads are used for this procedure, not SMB or SPB.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Keep at room temperature until the end of the day.

2 If you are using the kit for the first time, add the appropriate volume of 100% EtOH to each tube of M-Wash Buffer concentrate. Note the date on the M-Wash Buffer to indicate that EtOH was added.
- LT Kit—3.2 ml of 100% EtOH
- HT Kit—14.4 ml of 100% EtOH

3 Save the following BSF CON program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 54°C for 120 minutes
- Hold at 4°C
- Each well contains 150 µl

4 Preheat a 1.7 ml microcentrifuge tube heat block to 50°C.

Procedure

1 Add 130 µl Lightning Conversion Reagent to sample.

2 Pipette or vortex to mix, and then centrifuge briefly to remove liquid from sides of the tube.

3 Place on the preprogrammed thermal cycler and run the BSF CON program.

4 Add the following reagents to a new 1.7 ml microcentrifuge tube.
- M-Binding Buffer (600 µl)
- MagBinding Beads (10 µl)

5 Transfer entire sample (~150 µl) to the tube of M-Binding Buffer and MagBinding Beads, and then vortex to mix.

6 Incubate at room temperature for 5 minutes.

7 Centrifuge briefly to remove liquid from lid.

8 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

9 Remove and discard all supernatant from the tube.

10 Wash one time as follows.

   a Add 400 µl M-Wash Buffer.

   b Vortex to mix, and then centrifuge briefly.

   c Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

   d Remove and discard all supernatant from the tube.

11 Add 200 µl L-Desulphonation Buffer, and then vortex to mix.

12 Incubate at room temperature for 15 minutes.

13 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

14 Remove and discard all supernatant from the tube.

15 Wash two times as follows.

   a Add 400 µl M-Wash Buffer.
   b Vortex to mix, and then centrifuge briefly.
   c Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
   d Remove and discard supernatant (~400 µl) from the tube.

16 Centrifuge briefly, and then place on a magnetic stand.

17 Remove and discard supernatant (~10 µl).

18 With the tube cap open, place on the 50°C heat block until dry (~4 min).

19 Add 23 µl RSB. Pipette or vortex thoroughly to mix.

20 With the tube cap closed, place on the 50°C heat block for 4 minutes.

21 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

22 Transfer 20 µl DNA to a new eight-tube strip.

Amplify Enriched Library

This step uses one of the following PCR programs to amplify the enriched library:

- Eleven-cycle for four-plex.
- Twelve-cycle for two-plex and three-plex.
- Thirteen-cycle for single-plex.

NOTE

The kit contains sufficient reagents for the indicated number of samples at ***four-plex only***. You can perform this protocol at single-plex two-plex, and three-plex with additional PCR cycles, but some reagents run out when less than the maximum number of samples are used.

Consumables

- PPC (PCR Primer Cocktail)
- KAPA HiFi HotStart Uracil+ ReadyMix (2X)

About Reagents

- KAPA HiFi HotStart Uracil+ ReadyMix (2X) is user-supplied and not included with the kit.
- Of several tested and commercially available polymerases, only KAPA HiFi HotStart Uracil+ ReadyMix (2X) passed quality control specs. Do not use other polymerases.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
PPC	-25°C to -15°C	Thaw at room temperature, and then place on ice.
Kapa HiFi Uracil+	-25°C to -15°C	Thaw at room temperature, and then place on ice.

2 Save the following AMP MC program on the thermal cycler.

- Choose the preheat lid option and set to 100°C
- 95°C for 2 minutes
- 11 cycles (four-plex), 12 cycles (two-plex, three-plex), or 13 cycles (single-plex) of:
  - 98°C for 20 seconds
  - 60°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C
- Each well contains 50 µl

Procedure

1 Add the following reagents to 20 µl DNA from the previous step.
- PPC (5 µl)
- Kapa HiFi Uracil+ (25 µl)

2 Pipette or vortex to mix.

3 Place on the preprogrammed thermal cycler and run the AMP MC program.

SAFE STOPPING POINT

If you are stopping, cap the tube and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Clean Up Amplified Enriched Library

This step uses SPB (Sample Purification Beads) to purify the enriched library and remove unwanted products.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- 80% ethanol (EtOH)
- 1.7 ml microcentrifuge tubes or 8-tube strips

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.
- Inspect SPB to make sure that it is not frozen. Do not use if frozen.
- Make sure that SPB is used for this procedure, not SMB.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% ethanol. Alternatively, you can use ethanol from the previous day.

Procedure

1 Briefly centrifuge the eight-tube strip.

2 Vortex SPB until well-dispersed.

3 Add 50 µl SPB to the tube, and then pipette or vortex to mix.

4 Incubate at room temperature for 3 minutes.

5 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
Use either a 96-well or 1.7 ml magnetic stand, depending on the type of tube.

6 Remove and discard supernatant.

7 Wash two times as follows.

   a Add 200 µl freshly prepared 80% EtOH to the tube.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from the tube.

8 Centrifuge briefly.

9 Place on a magnetic stand and remove residual EtOH from the tube.

10 Air-dry on the magnetic stand until dry (~5 minutes).

11 Add 20 µl RSB, and then pipette or vortex to mix.

12 Incubate at room temperature for 1 minute.

13 Place on a magnetic stand and wait until the liquid is clear (2 minutes).

14 Transfer 19 µl supernatant to a new tube.

SAFE STOPPING POINT

If you are stopping, cap the tube and store at -25°C to -15°C for up to 6 months.

Check Enriched Libraries

Perform the following procedures to check the quality of the enriched library .

Quantify Libraries

Accurately quantify DNA libraries to ensure optimum cluster densities on the flow cell.

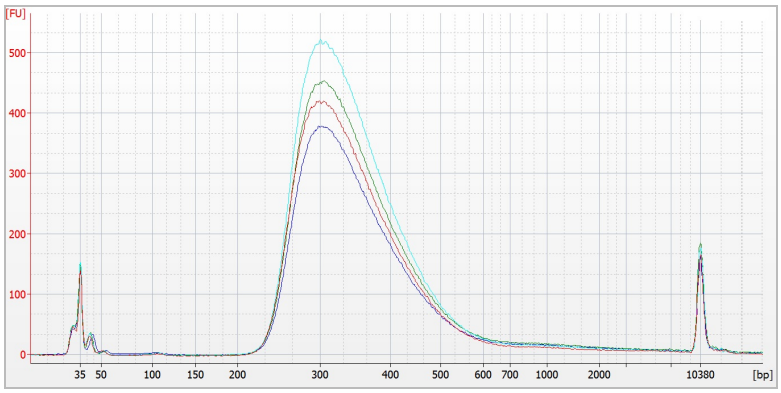
1 Quantify the postenriched library using the Qubit dsDNA HS Assay Kit.
   - Use 2 µl as the loading volume.
   - Use the dsDNA and high sensitivity settings.
   - Record STD1 (standard 1) and STD2 (standard 2) readings.
   - Measure the library concentration of the same tube twice and use the average of the two measurements.

Assess Quality

1 Run 1 µl of post enriched library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.

Expect a distribution of DNA fragments with a size range from ~250 bp through ~1 kb. Insert size distribution can vary slightly depending on the level of indexing. However, the sample peak must not be significantly shifted compared to the following example, which includes multiple independent samples to show variability and consistency.

Figure 5 Example Library Distribution



2 Use the results from the Bioanalyzer traces to determine the average size of the enriched library. Then use the following formula to convert from ng/µl to nM.

The average library size of fragments between 150–1500 bp is used.

$$\frac{\text{(concentration in ng/µl)}}{\text{(660 g/mol * average library size)}} \times 10^6 = \text{concentration in nM}$$

For example:

$$\frac{\text{(4 ng/µl)}}{\text{(660 g/mol * 300)}} \times 10^6 = 20 \text{ nM}$$

Supporting Information

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Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all the required consumables and equipment.

Acronyms

Acronym	Definition
ATL2	A-Tailing Mix
BLR	Blocker
CT3	Capture Target Buffer 3
CT4	Capture Target Buffer 4
EDTA	Ethylenediaminetetraacetic Acid
EPIC	Epigenetic Oligo Pool
ERP2	End Repair Mix 2
ET1	Elute Target Buffer 1
ET2	Elute Target Buffer 2
EWS	Enrichment Wash Solution
HP3	2 N NaOH
LIG2	Ligation Mix 2
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SPB	Sample Purification Beads
SPM	Sample Purification Mix
STL	Stop Ligation Buffer

Kit Contents

Make sure that you have all the reagents identified in this section before starting the protocol. The TruSeq Methyl Capture EPIC Library Prep Kit is available in the following configurations.

Kit Name	Number of Samples	Catalog #
TruSeq Methyl Capture EPIC LT Library Prep Kit (12 samples, 4 indexes)	12	FC-151-1002
TruSeq Methyl Capture EPIC HT Library Prep Kit (48 samples, 12 indexes)	48	FC-151-1003

TruSeq Methyl Capture EPIC LT Library Prep Kit Contents (12 samples, 4 indexes) (FC-151-1002)

This kit contains five boxes of reagents to support 12 samples in 3 × 4-plex reactions.

Box 1 – Bisulphite Conversion Reagents, Store at Room Temperature

Quantity	Reagent
1	Lightning Conversion Reagent
1	M-Binding Buffer
1	MagBinding Beads
1	M-Wash Buffer
1	L-Desulphonation Buffer

Box 2 – SPB Reagents, Store at 2°C to 8°C

Quantity	Reagent	Description
1	SMB	Streptavidin Magnetic Beads
2	SPB	Sample Purification Beads
1	EHB2	Enrichment Hybridization Buffer 2
1	ET2	Elute Target Buffer 2
1	SPM	Sample Purification Mix

Box 3 – Adapters, Store at -25°C to -15°C

Quantity	Reagent	Description
1	AD002	DNA Adapter Index 2
1	AD004	DNA Adapter Index 4
1	AD005	DNA Adapter Index 5
1	AD006	DNA Adapter Index 6

Box 4 – Core Reagents, Store at -25°C to -15°C

Quantity	Reagent	Description
1	ERP3	End Repair Mix 3
1	ATL2	A-Tailing Mix
1	LIG2	Ligation Mix 2
1	RSB	Resuspension Buffer
1	STL	Stop Ligation Buffer
1	CT3	Capture Target Buffer 3
1	CT4	Capture Target Buffer 4
1	EWS	Enrichment Wash Solution
1	BLR	Blocker

Quantity	Reagent	Description
1	HP3	2 N NaOH
1	ET1	Elute Target Buffer 1
1	PPC	PCR Primer Cocktail
1	EDTA	0.5 M EDTA

Box 5 – Probes, Store at -25°C to -15°C

Quantity	Reagent	Description
1	EPIC	Epigenetic Oligo Pool

TruSeq Methyl Capture EPIC HT Library Prep Kit Contents (48 samples, 12 indexes) (FC-151-1003)

This kit contains five boxes of reagents to support 48 samples in 12 × 4-plex reactions.

Box 1 – Bisulphite Conversion Reagents, Store at Room Temperature

Quantity	Reagent
1	Lightning Conversion Reagent
1	M-Binding Buffer
1	MagBinding Beads
1	M-Wash Buffer
1	L-Desulphonation Buffer

Box 2 – SPB Reagents, Store at 2°C to 8°C

Quantity	Reagent	Description
3	SMB	Streptavidin Magnetic Beads
1	SPB	Sample Purification Beads
1	EHB2	Enrichment Hybridization Buffer 2
1	ET2	Elute Target Buffer 2
1	SPM	Sample Purification Mix

Box 3 – Adapters, Store at -25°C to -15°C

Quantity	Reagent	Description
1	AD002	DNA Adapter Index 2
1	AD004	DNA Adapter Index 4
1	AD005	DNA Adapter Index 5
1	AD006	DNA Adapter Index 6
1	AD007	DNA Adapter Index 7
1	AD012	DNA Adapter Index 12

Quantity	Reagent	Description
1	AD013	DNA Adapter Index 13
1	AD014	DNA Adapter Index 14
1	AD015	DNA Adapter Index 15
1	AD016	DNA Adapter Index 16
1	AD018	DNA Adapter Index 18
1	AD019	DNA Adapter Index 19

Box 4 – Core Reagents, Store at -25°C to -15°C

Quantity	Reagent	Description
1	ERP3	End Repair Mix 3
1	ATL2	A-Tailing Mix
1	LIG2	Ligation Mix 2
1	RSB	Resuspension Buffer
1	STL	Stop Ligation Buffer
2	CT3	Capture Target Buffer 3
1	EWS	Enrichment Wash Solution
1	BLR	Blocker
1	HP3	2 N NaOH
3	ET1	Elute Target Buffer 1
1	CT4	Capture Target Buffer 4
1	PPC	PCR Primer Cocktail
1	EDTA	0.5 M EDTA

Box 5 – Probes, Store at -25°C to -15°C

Quantity	Reagent	Description
4	EPIC	Epigenetic Oligo Pool

Consumables and Equipment

Make sure that you have the required user-supplied consumables and equipment before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
15 ml conical tubes	General lab supplier

Consumable	Supplier
10 µl barrier pipette tips	General lab supplier
20 µl barrier pipette tips	General lab supplier
200 µl barrier pipette tips	General lab supplier
1000 µl barrier pipette tips	General lab supplier
KAPA HiFi HotStart Uracil+ ReadyMix (2X)	KAPA HiFi HotStart Uracil+ ReadyMix (2X)—Kapa, catalog # KK2801
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
High Sensitivity DNA Kit	Agilent Technologies, part # 5067-4626
Ice bucket	General lab supplier
microTUBE AFA Fiber Pre-Slit Snap-Cap	Covaris, part # 520045
microTUBE Strips (8), package of 12	Covaris, part # 520053
Nuclease-free water	General lab supplier
Positive control sample (only 1 needed). Recommendations: • HeLa • Jurkat • HCC1187 normal (BL) • HCC1187 breast cancer tumor • NA12878	General lab supplier • Biochain, catalog # D1255811 • Biochain, catalog # D1255815 • ATCC, catalog # CRL2323-D • ATCC, catalog # CRL2322 • Coriell Institute, catalog # NA12878
Qubit dsDNA HS Assay Kit	Thermo Fisher, catalog # Q32851
RNaseZap (to decontaminate surfaces)	General lab supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier

Equipment

Equipment	Supplier/Description
Benchtop microcentrifuge for 8-tube strips	General lab supplier
2100 Bioanalyzer Desktop System	Agilent Technologies, part # G2940CA
Covaris S220, or equivalent	Covaris
Heat block for 1.7 ml microcentrifuge tubes	General lab supplier
Magnetic stand-96 well	Thermo Fisher, DynaMag-96 Side Magnet, catalog # 12331D
Magnetic stand for 1.7 ml tubes	General lab supplier Thermo Fisher, DynaMag-2 Magnet, catalog # 12321D
Qubit 2.0 Fluorometer	Thermo Fisher, catalog # Q32866
Thermal cycler (with heated lid) See *Thermal Cyclers*.	General lab supplier
Vortexer	General lab supplier

Thermal Cyclers

The following table lists the recommended specifications for the thermal cycler. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the protocol.

Make sure that your thermal cycler is compatible with the 8-tube strip and 8-tube strip cap.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, constant at 100°C	Plate
MJ Research PTC-225 DNA Engine Tetrad	Calculated	Heated, constant at 100°C	Plate
Bio-Rad S1000	N/A	Heated, constant at 100°C	Plate

Index Adapter Sequences

The TruSeq Methyl Capture EPIC Library Prep Kit contains the TruSeq LT Set A indexes.

- The index numbering is not contiguous. Indexes 3, 8, 9, 10, 11, and 17 are skipped.
- The sequence contains seven bases. The seventh base, which is shown in parenthesis, is not included in the Index Read. Record only the first six bases in the sample sheet. For indexes 13 and above, the seventh base might not be A, which is seen in cycle 7 of the Index Read.
- For more information on the number of cycles in an Index Read, see the system guide for your sequencing instrument.

Table 1 Index Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AD002	CGATGT(A)	AD013	AGTCAA(C)
AD004	TGACCA(A)	AD014	AGTTCC(G)
AD005	ACAGTG(A)	AD015	ATGTCA(G)
AD006	GCCAAT(A)	AD016	CCGTCC(C)
AD007	CAGATC(A)	AD018	GTCCGC(A)
AD012	CTTGTA(A)	AD019	GTGAAA(C)

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.635.9898	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
France	+33 805102193	+33 170770446
Germany	+49 8001014940	+49 8938035677
Hong Kong	800960230	
Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
Norway	+47 800 16836	+47 21939693
Singapore	+1.800.579.2745	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.