EpiSwitch® Explorer Array Kit

Handbook

Preparation of EpiSwitch® Libraries

OXFORD BIODYNAMICS

Product Name:	EpiSwitch® Explorer Array Kit
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1. Purpose

The purpose of this document is to describe in detail the technology adapted by Oxford BioDynamics to generate EpiSwitch® libraries extracted from human whole blood samples or other cellular inputs such that it can be used by any technician who has been trained in basic molecular biology techniques.

2. Overview of Product

2.1 Chromosome Conformation Signatures

Oxford BioDynamics's EpiSwitch® technology is based on epigenetics, mechanisms that alter gene expression without altering the underlying DNA sequence and whose deregulation plays a role in the development of cancer, autoimmune, and neurologic diseases. Although DNA is often illustrated as a simple linear strand, the reality is that it is packaged into a complex three-dimensional structure that brings DNA sequences that are distant from each other in linear genomic space into close physical proximity. These long-range interactions, occurring both within and between gene loci, reflect genetic epistasis and the regulatory network imposed on the genome and as result directly modulate gene expression in the context of the established phenotype.

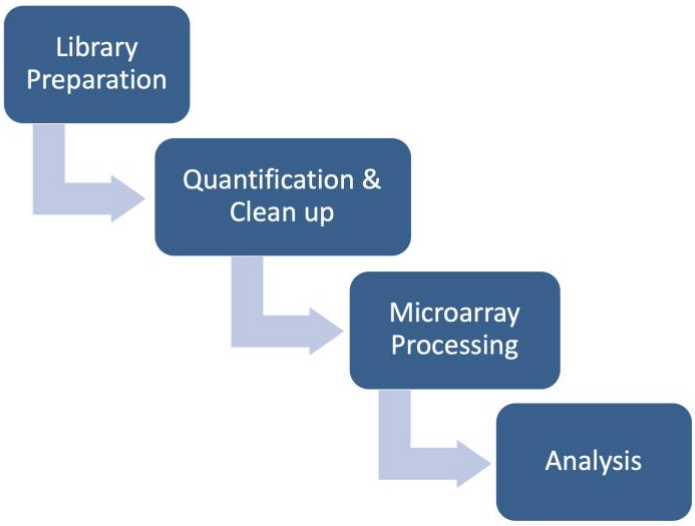
Collectively, functionally relevant combinations of discrete chromosomal conformations (CC) across the genome (chromatin "bar-codes") are called a chromosome conformation signature (CCS). CCSs can be monitored by means of molecular biology and therefore represent an ideal biological marker for assessing disease pathophysiology and response to therapeutic intervention.

2.2 EpiSwitch® Technology

EpiSwitch® proprietary industrial platform for detecting CCSs uses well-accepted principles of CC detection, proprietary molecular biology techniques and operates under ISO compliant standards. The platform generates readouts within hours and can handle diverse types of source material (peripheral blood, tissue biopsies and cellular isolates) in high throughput at high resolution and sensitivity, using a manual or robotic/automated platform. Detection is based on established molecular 3C biology techniques. In short, without disrupting the cells, the CCs are chemically stabilized, extracted as a chromatin template and the distant genomic sites found in proximity by stable juxtaposition are converted into adjacent sequence tags on an artificially generated chromatin template. Quantification of the segments can be done by comparative genomic hybridization (CGH) on an Agilent array platform, by next generation sequencing (NGS) and by nested or Real-Time PCR and provides the readout of the CCS present in a sample.

The regents and protocols included in this product are for the processing of the EpiSwitch Explorer Array Kit. This protocol details the library preparation protocol, for the microarray processing see the accompanying protocol.

Figure1. Overview of workflow



The EpiSwitch® technology packaged in this product includes the technical protocols and proprietary reagents supplied in the product kit to generate the EpiSwitch libraries from cellular biological inputs including, whole blood and blood fractions (buffy coat, PBMC), cultured cell lines and primary cells. Contact Oxford BioDynamics for information and protocols for processing other desired cellular inputs.

For cultured cells, the cells should be washed in PBS to remove the media before proceeding.

3. Materials and Equipment

The protocol detailed in this work instruction uses proprietary EpiSwitch technology buffers supplied in the Explorer Array kit and third-party reagents that must be purchased separately.

3.1 EpiSwitch Reagents

The list of chemicals supplied in the Explorer Array kit is given below. The reagents are shipped at ambient temperature and should be transferred to the storage conditions stated below on receipt. Each Explorer Array kit is supplied in bottles with sufficient volumes of reagents for 50 individual reactions for ease of use.

All EpiSwitch® reagents are supplied as general laboratory reagents manufactured in accordance with OBD's ISO13485:2016 certification. Please consult the safety data sheets and warning signs for all EpiSwitch® reagents.

Full Name	Supplier	Part number	Molecular Weight or Concentration	Storage
EpiMix Buffer DE-A	OBD	DE-A-50	-	Ambient
EpiMix Buffer DE-B	OBD	DE-B-50	-	Ambient
10X EpiMix Buffer DE-C	OBD	DE-C-50	10x	2˚C to 8°C
EpiMix Buffer DE-D	OBD	DE-D-50	-	2˚C to 8°C
EpiMix Buffer DE-E	OBD	DE-E-50	-	2˚C to 8°C
25X EpiMix Buffer DE-F	OBD	DE-F-50	25x	Ambient
2X EpiMix Buffer DE-G	OBD	DE-G-50	2x	2˚C to 8°C

3.1.1 EpiMix Buffer DE-A

EpiMix Buffer DE-A contains formaldehyde for the initial fixation of the patient whole blood samples and as such is toxic. All operations with EpiMix Buffer DE-A should be performed in a suitable fume/chemical hood. The addition of EpiMix Buffer DE-B renders the mix inert and any supernatant can be disposed of through standard clinical waste.

3.1.2 EpiMix Buffer DE-C

EpiMix Buffer DE-C is supplied as a 10x concentrate. It is used in the protocol at this 10x concentration as well as at a 1x concentration. It is advised that a suitable volume of 1x EpiMix DE-C is diluted with Nuclease free water before commencing the protocol (see section 4). Any 1x EpiMix DE-C generated should be stored at 2˚C to 8°C and have the same expiry date as the 10x EpiMix Buffer DE-C it is derived.

3.1.3 EpiMix Buffer DE-F

EpiMix Buffer DE-F is supplied as a 25x concentrate. It is used in the protocol at this 25x concentration as well as at a 1x concentration. It is advised that a suitable volume of 1x EpiMix DE-F is diluted with Nuclease free water before commencing the protocol. Only small volumes of this reagent is required (see section 4.1.5). Any 1x EpiMix Buffer DE-F diluted should be stored at ambient temperature and have the same expiry as the 25x EpiMix Buffer DE-F.

A white precipitate may form in this buffer. This can be re-suspended by gentle heating. Do not vortex as this will produce large amounts of foam.

3.1.4 EpiMix Buffer DE-G

EpiMix Buffer DE-G is supplied as a 2x concentrate. It is used in the protocol at this 2x concentration as well as at a 1x concentration. It is advised that a suitable volume of 1x EpiMix DE-G is diluted with Nuclease free water before commencing the protocol (see section 4.1.5). Only small volumes of this reagent is required. Any 1x EpiMix Buffer DE-G diluted should be stored at 2˚C to 8°C and have the same expiry as the 2x EpiMix Buffer DE-G.

3.2 Third Party Reagents

The list of required third party reagents for this protocol is given below.

Full Name	Supplier	Part number	Molecular Weight or Concentration	Storage
cOmplete Protease Inhibitor cocktail tablets	Sigma-Aldrich	04693132001	-	2˚C to 8°C
Phosphate Buffered Saline (Cell Culture)	Sigma-Aldrich	P5493-1L (1L) P5493-4L (4L)	20x	Ambient
Nuclease-free water (NOT DEPC-treated)	Ambion (Thermo-Fisher) or equiv.	AM9937 (10 x 50 mL) AM9932 (1 x 1L) 4387936 (4x 1L)	-	Ambient
QIAamp DNA FFPE Tissue Kit	Qiagen	56404	-	See manufacturers guidance
Ethanol (200 proof)	Sigma	E7023	-	Ambient
RNase A	Sigma	10109142001	-	-20˚C
Tris-EDTA Solution Buffer pH8	Sigma	93283	-	Ambient
20X TE pH 7.5	Invitrogen	T11493	-	2˚C to 8°C
FastDigest® TaqI-v2 Kit	New England Biolabs (NEB)	R0149S/ R0149L	20,000 units/mL	-20˚C
T4 DNA Ligase Enzyme Kit	TaKaRa/Clontech	2011A	350,000 units/mL	-20˚C
Approved Alternate Enzymes				
FastDigest® TaqI	ThermoFisher	FD0674	-	-20°C
T4 DNA Ligase Enzyme	New England Biolabs (NEB)	M0202S M0202L	400,000 units/mL	-20°C

3.2.1 Water

All solutions prepared in the laboratory must be prepared using water that has not been treated with Diethyl pyrocarbonate (DEPC) and that has been shown to be free from non-specific endonuclease, exonuclease and RNase activities.

3.2.2 NEB TaqI Restriction enzyme Kit

NEB TaqI kits are designed for fast DNA digestion and convenience. In this WI Taq$^{\alpha}$I is used. The kit comes with its own 10X CutSmart® Buffer. If using the approved FastDigest TaqI alternative, the protocol can be followed without amendment, substituting the buffer and enzyme at the appropriate steps of this protocol.

3.2.3 TaKaRa Ligase enzyme kit

TaKaRa T4 DNA ligase enzyme at the concentration of 350 units per micro litre is used for ligation. The kit comes with its own 10X DNA ligase buffer. If using the approved NEB T4 DNA Ligase alternative, the protocol can be followed without amendment, substituting the buffer and enzyme at the appropriate steps of this protocol.

3.2.4 QIAamp® DNA FFPE Tissue Kit

The QIAamp® FFPE kit is designed to purify genomic DNA from formalin-fixed, paraffin embedded tissue sections, the first parts of the protocol have been removed to accommodate the purification of EpiSwitch template. The remaining steps of the protocol follow the manufacturers protocol.

As per the Manufacturer's instructions, do not store the ENTIRE kit at 4°C - remove all of the buffers from the box and store at room temperature, then place FFPE columns in a fridge.

3.2.5 RNase A

RNase should be dissolved to 100mg/mL in TE buffer pH 8, this can be done by adding 250ul of TE pH 8.0 to the bottle of RNase and mixing vigorously until dissolved. 100mg/mL RNase should then be aliquoted in volumes of 10µL or appropriate volume into strip tubes and stored at -20°C for up to 12 months.

3.2.6 PBS

The PBS detailed in this WI is purchased from a third party at 20x concentrate. Sufficient volumes should be diluted to a 1x concentration with the water detailed above before proceeding with the protocol (see section 4).

3.2.7 Inhibitor solutions

To prevent degradation of the sample during the first stages of the protocol cOmplete Protease Inhibitor cocktail is used. For this protocol a 25x solution of cOmplete in PBS and 10x EpiMix Buffer DE-C is required.

Stocks of 25x cOmplete PBS and 25x cOmplete 10x EpiMix Buffer DE-C should be made up and stored -15°C to-30°C. These stocks can be stored for up to 12 weeks.

- To make 25x cOmplete PBS add 1 inhibitor tablet to 2mL of 1x PBS.
- To make 25x cOmplete 10x EpiMix Buffer DE-C add 1 inhibitor tablet to 2mL of 10x EpiMix Buffer DE-C.

3.2.8 TE

The TE Buffer (ph7.5) utilised to elute the samples from the Qiagen FFPE columns is a 1x concentration diluted from the 20x TE buffer that is detailed above. Sufficient volumes should be diluted to a 1x concentration with the water detailed above before proceeding with the protocol (see section 4.2).

3.3 Plasticware

The disposable plasticware used in this Work Instruction are given below. All plasticware described are RNase, DNase, DNA and pyrogen free. Alternative plasticware may be used as required.

Description	Supplier	Catalogue Number	Grade
0.2 mL Single Thin Wall Tube + Cap	Starlab	L1402-4308	Natural
0.2mL 8-Strip PCR Tubes, Individually Attached Domed Shielded Caps	Starlab	S1602-2900	Natural
"Crystal Clear" 0.6 ml M/C Tube	Starlab	E1405-0610	Sterile
1.5 mL Single DNA LoBind Tube	Eppendorf	022431021	Natural
"Crystal Clear" 1.5 mL M/C Tube	Starlab	E1415-1510	Crystal Clear
2.0ml 'Crystal Clear' Microcentrifuge Tube	VWR	211-2165	Sterile
1.2mL Reaction Tubes in Rack	Starlab	E1760-8410 (in Racks) E1720-8000 (in 8-Strips)	Sterile
8-Strip Caps for 1.2mL Reaction Tubes	Starlab	E1702-8400	Sterile
Appropriate Micropipette tips	Any	N/A	Sterile

3.4 Equipment

The list of equipment used in this Work Instruction is given below. Alternative suitable equipment may be used. If using an alternative centrifuge ensure that it is using a suitable swing arm rotor. Use of fixed angle rotors can lead to the buildup of insoluble material which can interfere with efficient processing.

Description	Supplier
FugeOne Micro centrifuge	Starlab
Eppendorf MasterCycler Pro S or Eppendorf MasterCycler Nexus	Eppendorf
Eppendorf Centrifuge 5430R Or Eppendorf Centrifuge 5810R	Eppendorf
Scientific Industries G560-E Vortex	Jencons-PLS
Prism micro centrifuge	Labnet
Accu Block digital Dry Bath	Labnet
TEchne DRi-Block DB-2A	Jencons-PLS
3 Channel countdown timer	Fisher Scientific
Appropriate micropipette	Any

3.5 Incubation Programs

The following programs should be added to the MasterCycler Pro S/MasterCycler Nexus or appropriate alternative thermocycler.

Program One

Parameters	
Heated Lid:	105°C
Turn off Lid at low	Yes
Ramp Rate	100%
Program	
Step 1	65°C for 15 minutes
Step 2	4°C hold

Program Two

Parameters	
Heated Lid:	No
Turn off Lid at low	N/A
Ramp Rate	100%
Program	
Step 1	37°C for 15 minutes
Step 2	4°C hold

Program Three

Parameters	
Heated Lid:	105°C
Turn off Lid at low	Yes
Ramp Rate	100%
Program	
Step 1	65°C for 20 minutes
Step 2	4°C hold

Program Four

Parameters	
Heated Lid:	105°C
Turn off Lid at low	Yes
Ramp Rate	100%
Program	
Step 1	65°C for 5 minutes
Step 2	4°C hold

Program Five

Parameters	
Heated Lid:	No
Turn off Lid at low	N/A
Ramp Rate	100%
Program	
Step 1	37°C for 5 minutes
Step 2	4°C hold

Program Six

Parameters	
Heated Lid:	40°C
Turn off Lid at low	Yes
Ramp Rate	100%
Program	
Step 1	4°C pause
Step 2	16°C for 10 minutes
Step 3	80°C for 20 minutes
Step 4	4°C hold

4. Procedures

4.1 EpiSwitch Extraction

Before proceeding with the documented protocol:

- Prepare sufficient quantities of 1x PBS and 10x EpiMix Buffer DE-C, each containing 1x protease inhibitor from the prepared 25x inhibitor stock solutions (see 3.2.7).
- Pre-cool the centrifuges to 4°C

 To make sufficient 1x PBS with 1x protease inhibitor:

- Calculate the total volume required i.e., the number of reactions plus an additional 10% for wastage.

  ((no of reactions x 450µL) x1.1) = **Total Volume Required**

- Calculate the volume of 25x inhibitor in 1x PBS required.

  Total Volume Required / 25 = **Volume 25x inhibitor in 1x PBS**

- Calculate the volume of 1x PBS required and combine with the calculated volume of 25x inhibitor in 1x PBS stock.

  Total Volume Required - volume 25x inhibitor in 1x PBS = **volume 1x PBS**

 To make sufficient 10x EpiMix Buffer DE-C with protease inhibitor:

- Calculate the volume required i.e., the number of reactions plus an additional 10% for wastage.

  ((no of reactions x 130µL) x1.1) = **Total Volume Required**

- Calculate the volume of 25x inhibitor in 10x EpiMix Buffer DE-C required.

  Total Volume / 25 = **Volume 25x inhibitor in 10x EpiMix Buffer DE-C**

- Calculate the volume of 10x EpiMix Buffer DE-C required and combine with the calculated volume of 25x inhibitor in 10x EpiMix Buffer DE-C stock.

  Total Volume Required – volume 25x inhibitor in 10x EpiMix Buffer DE-C stock

  = **volume 10x EpiMix Buffer DE-C**

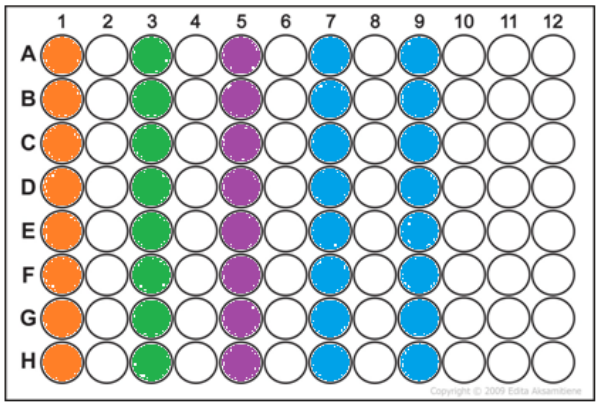
During the protocol avoid creating bubbles/froth – otherwise volumes may exceed the capacity of the tubes, presenting a cross contamination problem between samples.

- Transfer the enzyme buffers from the TaKaRa ligase or alternative ligase kit from storage at -20°C and place in a fridge at 2˚C-8°C to defrost.
- Transfer the enzyme buffers from the NEB restriction or alternative restriction kit from storage at -20°C and place at ambient temperature to defrost.

The initial steps of the protocol require certain buffers to be aliquoted into 1.2mL strip tubes to allow for the use of an 8-channel multi-pipette for dispensing and mixing.

Figure 2 documents the positions of 1.2mL strip tubes in a rack and the buffers to be aliquoted into each column. The volumes of buffers are shown in steps 1 – 4 below.

Figure2. 1.2mL tube rack Layout



Column 1: EpiMix Buffer DE-A
Column 3: EpiMix Buffer DE-B
Column 5: 10x EpiMix Buffer DE-C with inhibitor
Column 7 & 9: 1x PBS with inhibitor and Samples

The instructions below are based on the use of an input of 50µL of whole blood. If processing whole blood, perform multiple reactions to provide the required library yields. Do not increase the total whole blood input above 50µL. For other cellular inputs the volumes in steps 4 and 5 can be changed to provide the desired cell counts/concentrations. The final volume after step 5 should remain 500µL.

4.1.1 Chromosome Conformation fixation

Prepare the appropriate number of 1.2mL strip tubes for the number of reactions being processed according to figure 2. Each column of strip tubes should be capped when not in use. The aliquoted volumes below are based on performing 16 reactions, alter the volumes accordingly to allow for more reactions to be processed. Add additional strips of 1.2mL strip tubes to column 11 or other free columns for the additional reactions (figure2).

Steps 6 – 8 are best performed with an 8 channel 20-200µL pipette. Ensure a 3-channel countdown timer (or similar) is available close to hand with 15 minutes and 5 minutes programmed into separate countdown channels.

1. Aliquot 150µL of EpiMix Buffer DE-A into each well of Column 1.
2. Aliquot 350µL of EpiMix Buffer DE-B into each well of Column 3.
3. Aliquot 300µL of 10x EpiMix Buffer DE-C with inhibitor into each well of Column 5.

Note: if using input other than whole blood, I.E. cell lines, PBMC, primary cells, the volumes in steps 4 and 5 can be modified to provide the desired cell counts/concentrations. Ensure that the final volume going into step 6 remains 500µL for correct performance of the assay.

4. Aliquot 450µL of 1x PBS with inhibitor into each well of Columns 7 & 9.
5. Add 50µL of sample to each well of Columns 7 & 9, using the appropriate number of replicates Mix by pipetting, 4-5 aspirate/dispense cycles. The samples in the 1.2mL strip-tubes should be homogeneous in colour.
6. Add 62.5µL of EpiMix Buffer DE-A from Column 1 to each well of the column of reactions (Column 7 & 9). Start the 15-minute timer after the addition of EpiMix Buffer DE-A to the first sample column.
7. When the 15-minute timer reaches zero add 167.5µL of EpiMix Buffer DE-B from column 3 to each well of the reaction columns (Column 7 & 9). Start the 5 minutes timer after the addition of EpiMix Buffer DE-B to the first reaction column.
8. When the 5 minutes timer reaches zero add 130µL of 10x EpiMix Buffer DE-C and inhibitor from column 5 to each well of the reaction columns (Column 7 & 9).

4.1.2 Cell lysis and debris removal

Steps 9-15 are performed in 2.0mL tubes. Ensure each tube has been labelled with the corresponding sample/reaction name. Reverse Pipetting should be used in steps 10, 14 and 16 to ensure the interface between the sample and buffers is not disrupted. The excess in the tip should for the reverse pipetting should be discarded along with the pipette tip.

9. Transfer the entire contents of each sample well from Columns 7 & 9 to individual 2.0mL tubes.
10. **Gently Underlay** 450µL of EpiMix Buffer DE-D to each 2.0mL tube by directing the pipette tip to the bottom of the tube and slowly dispensing the EpiMix Buffer DE-D. This will force the lysate to the top of the tube, leaving the clear dense EpiMix Buffer DE-D as a distinct lower phase. Do not Mix. Repeat process for all tubes.
11. Gently load the samples into a swing-arm rotor and centrifuge the samples for 15 minutes at 3,270 RCF at 4^{o}C.
12. Remove and discard the supernatant taking care not to disturb the pellet, leaving a sufficient volume to cover the pellet.
13. Re-suspend the pellet in 400µL 1x EpiMix Buffer DE-C. Mix by pipetting, 4-5 aspirate/dispense cycles.
14. **Underlay** 400µL of EpiMix Buffer DE-D to each 2.0mL tube as in step 10.
15. Centrifuge the samples for 15 minutes at 3,270 RCF at 4^{o}C.

Step 16 can be performed concurrently with Step 15.

16. Aliquot 90µL of EpiMix Buffer DE-E into an appropriate number of labelled 0.2mLstrip tubes, one for each 2.0mL tube.
17. Remove and discard the Supernatant from centrifuged samples (step 15). Taking care not to disturb the pellet, as before.
18. Re-suspend the pellet in 90µL 1x EpiMix Buffer DE-C. With the same pipette tip transfer the sample to the corresponding labelled 0.2mL strip tube, carefully **overlaying** the EpiMix Buffer DE-E inside the 0.2mL tube. This can be aided by letting a droplet of lysed cells run down the inside of the strip tube until it reaches the EpiMix Buffer DE-E. Then, in the same position, gently dispense the lysate so that it follows the same path.
19. Return to the rotor and centrifuge the samples for 15 minutes at 3,270 RCF at 4°C.

4.1.3 Chromosome Conformation capture

- **Step 20 and 21 can be performed concurrently with Step 19.**
- **After the completion of step 21 the 10x Cutsmart Buffer should be returned to storage at -20°C.**
- **1x EpiMix Buffer DE-F, 2x EpiMix Buffer DE-G and 1x EpiMix Buffer DE-G can be aliquoted into 0.2mL strip tubes to allow for the use of an 8 channel 1-10 µL or 2-20µL pipette in subsequent protocol steps. If aliquoted the reagents should be stored as documented in section 3.1 of this document.**

20. Prepare the appropriate volume of restriction master mix by adding 45µL Nuclease free water, 5µL NEB 10x Cutsmart buffer and 1µL 25x EpiMix Buffer DE-F for each reaction. It is advised to make an additional 10% master mix volume than required. Please note the enzyme is not added to the master mix at this stage.

	1x Reaction	16x Reactions	16x Reaction *1.1
Nuclease Free Water	45	720	792
10x NEB Cutsmart Buffer	5	80	88
25x EpiMix Buffer DE-F	1	16	17.6
Total Volume	51	816	897.6

21. Mix and then aliquot the restriction master mix from step 20 into the appropriate number of 0.2mL strip tubes to allow for the use of an 8 channel 20-200µL pipette. Each well 0.2mL strip tube should contain approximately 110µL, allowing for two 51 µL aspirations to be taken.
22. Remove and discard supernatant from the centrifuged samples (step 19), taking care not to disturb the pellet.

Note: if required briefly centrifuge the tubes after each incubation to draw down any droplets in the lids.

23. Using an 8-channel pipette re-suspend each pellet in 51µL of restriction master mix. Place the tubes in a thermocycler and run Program One (65ºC for 15 minutes, 4ºC hold).
24. Remove the samples from the thermocycler and add 5µl of 2X EpiMix Buffer DE-G to each tube, using a multichannel pipette. Mix by pipetting with a larger volume. Place the tubes in a thermocycler and run Program Two (37ºC for 15 minutes, 4ºC hold).

4.1.4 Restriction

25. Add 2 µl of NEB TaqºI enzyme to each tube. Mix using a large volume multichannel pipette or vortex to mix. And briefly spin tubes down. Place into the thermocycler and run Program Three (65°C for 20 minutes, 4°C on hold).

Note: For subsequent steps, always place samples on a chill block (~4°c) when setting up reactions, transporting to or from thermocyclers or adding reagents. failure to regulate conditions between reaction steps may contribute to spurious and irreproducible results.

4.1.5 1X EpiMix Buffer DE-F Treatment

26. Add 3.25µl of 1X EpiMix Buffer DE-F to tubes, then mix using a larger volume (e.g. 30µl) or vortex to mix, pop spin the tubes to draw down any liquid from the lids. Return to the thermocyclers and run Program Four (5 min at 65°C, 4°C on hold).
27. Add 2µl of 1X EpiMix Buffer DE-G, then mix using a larger volume (e.g. 30µl) or vortex to mix, pop spin the tubes to draw down any liquid from the lids. Return to the thermocyclers and run Program Five (5 min at 37°C, 4°C on hold).

4.1.6 Ligation

Each TaKaRa ligation reaction consists of 122.75µL water, 20µL 10x Ligase Buffer and 2µL of TaKaRa Ligase.

28. To create a ligase master mix, add the appropriate multiples of each component with 10% surplus or a suitable number of additional reactions to account for loss to pipetting and plasticware retention.

	1x Reaction	16x Reactions	16x Reaction *1.1
Nuclease Free Water	122.75	1964	2160.4
10x Ligase Buffer	20	320	352
T4 DNA Ligase enzyme	2	32	35.2
Total Volume	144.75	2316	2547.6

29. Aliquot 150µL of the ligase master mix into 0.2mL strip tubes, one well for each extraction reaction.
30. Start Program Six on a thermocycler (pause and hold at 4°C, 16°C for 10 minutes, 80°C for 20 minutes, hold at 4°C).
31. Add 144.75µL of ligase master mix to the sample tubes (step 27) using a multichannel pipette. Pipette 4-6X to mix. Check the thermocycler has paused at the first 4°C step before adding samples to the block, place them into the thermocycler, then run the remainder of programme.
32. Centrifuge the samples for 15 minutes at 3,270 RCF at 4^{o}C.
33. Remove and discard the supernatant - leaving around 1mm on top of the pellet. Pellets can be stored in the fridge until the following day or if there is sufficient time, purified using the Qiagen FFPE columns.

4.2 Qiagen Column purification

The purification of the EpiSwitch libraries is performed to the manufacturer's instructions, starting at step 10 of the Isolation of Genomic DNA from FFPE Tissue Sections protocol. These instructions have been transcribed below.

Before starting the Qiagen purification, ensure that the buffers are fully prepared and free of precipitate

4.2.1 Preparing Buffer ATL

- Before starting the procedure, check whether precipitate has formed in Buffer ATL.
- If necessary, dissolve by heating to 70°C with gentle agitation.

4.2.2 Preparing Buffer AL

- Before starting the procedure, check whether precipitate has formed in Buffer AL.
- If necessary, dissolve by heating to 70°C with gentle agitation.

4.2.3 Preparing Buffer AW1

- Add 25 mL ethanol (200 proof) to the bottle containing 19 mL Buffer AW1 concentrate.
- Tick the check box on the bottle label to indicate that ethanol has been added.
- Reconstituted Buffer AW1 can be stored at room temperature (15–25°C) for up to 2 weeks.

Note: Before starting the procedure, mix reconstituted Buffer AW1 by shaking.

4.2.4 Preparing Buffer AW2

- Add 30 mL ethanol (200 proof) to the bottle containing 13 mL Buffer AW2 concentrate.
- Reconstituted Buffer AW2 can be stored at room temperature (15–25°C) for up to 2 weeks.

Note: Before starting the procedure, mix reconstituted Buffer AW2 by shaking

4.2.5 Purification Process

34. Resuspend 1-8 pellets from the same patient in 180µL ATL buffer, transfer to a 1.5mL DNA LoBind tube, add 20µL Qiagen proteinase K, then vortex to mix. Master mixes of ATL buffer and proteinase K can be made to avoid multiple pipetting steps.
35. Incubate at 56°C for 1 hour in a dry heat block
36. After 1 hour transfer the samples to a different heat block and incubate at 90°C for 1 hour.

    Note: If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 90°C.
37. Briefly centrifuge the 1.5 mL tube to remove drops from the inside of the lid.
38. Allow samples to cool to room temperature before adding RNase. Add 2µL RNase A, mixing with a larger volume pipette or vortex, and incubate at room temperature for 2 minutes.

39. Just before use, make a 10% surplus master mix of AL/ethanol buffer by adding, for each sample, 200µL AL buffer to 200µL 200 proof ethanol.

Note: It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. AL Buffer and Ethanol are both difficult liquids to pipette, especially for AL pipette slowly and allow the buffer to catch up with the air displacement before removing the pipette tip from the buffer bottle.

40. Add 400µL of this mixture to the RNase-treated sample then immediately mix by vortexing. When processing multiple tubes/samples ensure that the addition of the mixture and vortexing is completed for each tube before progressing to the next. Incomplete mixing can lead to reduced sample yields and differential results.

Note: A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the QIAamp procedure. If you run out of collection tubes, use 2mL centrifuge tubes with the lids removed - do not re-use collection tubes.

41. Briefly centrifuge the 1.5 mL tube to remove drops from the inside of the lid.
42. Carefully transfer the entire lysate to the QIAamp MinElute column (in a 2 mL collection tube) without wetting the rim, close the lid, and centrifuge at 6000 x *RCF* for 1 minute at room temperature.
43. Place the QIAamp MinElute column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

Note: If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.

44. Carefully open the QIAamp MinElute column and add 500µL Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x *RCF* for 1 minute at room temperature.
45. Place the QIAamp MinElute column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.
46. Carefully open the QIAamp MinElute column and add 500µL Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x RCF for 1 minute at room temperature.
47. Place the QIAamp MinElute column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

Note: Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.

48. Centrifuge at 20,000 x *g* for 3 min to dry the membrane completely.

Note: This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.

49. Place the QIAamp MinElute column in a clean 1.5 mL microcentrifuge tube and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply the following amounts of 1X TE (pH 7.5) (see section 3.2.8) Buffer to the center of the membrane.

    1 - 2 pellets purified using a single column: elute using 30µl 1X TE Buffer (pH 7.5)

    3 – 8 pellets purified using a single column: elute using 50µl 1X TE Buffer (pH 7.5)

50. Incubate at room temperature for 5 minutes (start the timer after the 1X TE Buffer has been added to all the samples).
51. Centrifuge the columns/collection tubes at 20,000 RCF for 1 minute at room temperature.
52. Without changing the collection tube, add the same volume of 1X TE Buffer again to the membrane and incubate at room temperature for a further 5 minutes.
53. Centrifuge at 20,000 RCF for 1 minute at room temperature.

Note: Where sample material may produce low concentration elution's, the second elution procedure (step 52 can be performed with the initial elution from step 51. This will slightly reduce the overall DNA Yield but increase the final concentration.

4.2.6 Storage of Extract

Extracts can be stored at 4°C for up to 5 days, after this time extracts should be stored at -80°C for long term storage.

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