**Targeted bisulfite sequencing for biomarker discovery in Pteropodids**

1. **Introduction**

Cytosine methylation has been identified as a key epigenetic change that occurs with aging.

This work aims to optimize existing pipelines to develop a workflow to create an epigenetic clock from bat tissue samples.

Here, we use Reduced-Representation Bisulfite Sequencing (RRBS) featuring hybridization enrichment using biotinylated RNA probes to capture a select part of the genome containing CpG sites purported to be correlated with age.

**2.1 Reagents**

| **Reagent** | **Supplier** | **Catalog #** | **Notes** |
| --- | --- | --- | --- |
| **Tissue Collection, DNA Extraction and Quantification** |  |  |  |
| Bead based DNA extraction |  |  | or equivalent |
| Qubit dsDNA HS Assay  Content:   * Qubit dsDNA HS Reagent (200x concentrate in DMSO) * Qubit dsDNA HS Buffer * Qubit dsDNA HS Standard #1 (0 ng/uL in TE buffer) * Qubit dsDNA HS Standard #2 (10 ng/uL in TE buffer) | ThermoFisher Scientific | Q32854 |  |
| Qubit dsDNA BR Assay  Content:   * Qubit dsDNA BR Reagent (200x concentrate in DMSO) * Qubit dsDNA BR Buffer * Qubit dsDNA BR Standard #1 (0 ng/uL in TE buffer)   Qubit dsDNA BR Standard #2 (100 ng/uL in TE buffer) | ThermoFisher Scientific | Q32853 |  |
| Unmethylated lambda phage genomic DNA (250 ug\*) | Promega | D1521 | \*Measure the concentration of the DNA using the Qubit BR dsDNA Assay. Genome sequence: GenBank #J02459 |
| **Library Preparation** |  |  |  |
| UltraPure 1 M Tris-HCl, pH 8.0 | ThermoFisher Scientific | 15568025 | or equivalent |
| Bioanalyzer Assay |  |  | or tape station |
| NEBNext Ultra II DNA Library Prep with Sample Purification Beads  Content:   * NEBNext Ligation Enhancer * NEBNext Ultra II End Prep Enzyme Mix * NEBNext Ultra II End Prep Reaction Buffer * NEBNext Ultra II Ligation Master Mix * NEBNext Ultra II Q5 Master Mix (2x) * NEBNext Sample Purification Beads (store at room temperature) | New England Biolabs | E7103S/L (24/96 samples) | Alternative formats: NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645S/L) and separate Purification Beads (see Note 9.3) |
| Ethanol, Absolute (200 Proof), Molecular Biology Grade | Fisher Scientific | 64-17-5 | or equivalent |
| **Bisulfite Conversion, Indexing, and Library Amplification** |  |  |  |
| EZ DNA methylation-Lightning kit  Content:   * Lightning Conversion Reagent * M-Binding Buffer * M-Wash Buffer * L-Desulphonation Buffer * M-Elution Buffer * Zymo-Spin IC Columns * Collection Tubes | Zymo Research | D5030 | Other formats are available |
| KAPA HiFi HotStart Uracil + ReadyMix Kit (we need indexing PCR) | Roche Sequencing | 7959052001 | KAPA cat #: KK2801 |
| **Hybridization Capture** |  |  |  |
| UltraPure DNase/RNase-Free Distilled Water | ThermoFisher Scientific | 10977015 | or equivalent |
| myBaits Custom 1-20k DNA-seq kit  Content:   * Hyb N (red cap) * Hyb S (teal cap) * Beads (streptavidin beads) * Binding Buffer * Wash Buffer * Hyb D (yellow cap) * Hyb R (purple cap) * Block C (green cap) * Block O (blue cap) * Block A (orange cap) * Baits (white cap) | Arbor Biosciences | 300116 (16 captures) | Other formats are available. Each reaction corresponds to a capture.  Order TruSeq-style double-index blockers. |
| Tween-20 (10% solution) | Sigma-Aldrich | 11332465001 | or equivalent |
| EDTA 0.5 M, pH 8 | Sigma-Aldrich | 03690-100ML | or equivalent |

**2.2 Solutions, master mixes, and buffers**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **ID** | **Buffer** | **Ingredients** | **Supplier** | **cat #** |
| 2.2.1 | DNA Extraction Stuff |  |  |  |
| 2.2.2 | DNA Extraction Stuff |  |  |  |
| 2.2.3 | EB Buffer | 10mM Tris-HCL pH 8  *Notes: Dilute with Nuclease-free water. Store at Room Temperature* | ThermoFisher Scientific | 15568025 |
| 2.2.4 | EndPrep Master Mix | N x 3 uL of NEBNext Ultra II End Prep Enzyme Mix  N x 7 uL of End Prep Reaction Buffer  *Notes: Prepare just before use. Mix thoroughly and store on ice until ready to use. N = number of samples* | New England Biolabs  New England Biolabs | Part of E7103S/L  Part of E7103S/L |
| 2.2.5 | Ligation Master Mix | N x 30 uL of NEBNext Ligation Master Mix  N x 1 uL of Ligation Enhancer  *Notes: Prepare just before use, stable up to 8 h at 4 C. Because of the viscosity of the solution, make sure it’s mixed thoroughly. Store in ice until ready to use. N = number of samples* | New England Biolabs  New England Biolabs | Part of E7103S/L  Part of E7103S/L |
| 2.2.6 | M-Wash Buffer | M-Wash Buffer (concentrate)  Ethanol (add as indicated on the bottle)  *Notes: Store at room temperature.* | Zymo Research  Fisher Scientific | Part of D5030  64-17-5 |
| 2.2.7 | PCR Master Mix | H x 25 uL of KAPA HiFi HotStart Uracil + ReadyMix  H x 1.5 uL of IDT xGen Primers (20 uM)  *Notes: Prepare just before use. Mix thoroughly and store in ice until ready to use. H = number of capture reactions. See 2.5.1 for primer sequences.* | Roche Sequencing  Integrated DNA Technologies | 7959052001 (KAPA cat #: KK2801)  1077675 |
| 2.2.8 | Blockers Mix | H x 0.5 uL of Block A | Arbor Biosciences | Part of 300116 |
| 2.2.9 | Hybridization Mix (HYB) |  |  |  |
| 2.2.10 | Wash Buffer X |  |  |  |
| 2.2.11 | SBE Buffer (Streptavidin-Beads Elution) |  |  |  |
| 2.2.12 | Sequencing Buffer | 10 mM Tris-HCl, pH 8  0.1% Tween-20  *Notes: Store at room temperature.* | ThermoFisher Scientific  Sigma-Aldrich | 15568025  11332465001 |

**2.3 Equipment and consumables**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **ID** | **Item** | **Name** | **Supplier** | **cat #** | **Notes** |
| 2.3.1 | Multichannel pipettes |  |  |  |  |
| 2.3.2 | Water bath incubator |  |  |  |  |
| 2.3.3 | Thermomixer |  |  |  |  |
| 2.3.4 | Fluorometer | Qubit Fluorometer | ThermoFisher Scientific |  |  |
| 2.3.5 | Tubes | Qubit Assay Tubes | ThermoFisher Scientific |  |  |
| 2.3.6 | Sonicator |  |  |  |  |
| 2.3.7 | Tubes | For sonicator |  |  |  |
| 2.3.8 | Thermocycler with heated lid |  |  |  |  |
| 2.3.9 | PCR tubes |  |  |  |  |
| 2.3.10 | Low-bind 1.7 mL tubes |  |  |  |  |
| 2.3.11 | Minicentrifuge tubes |  |  |  |  |
| 2.3.12 | Mini centrifuge |  |  |  |  |
| 2.3.13 | Benchtop centrifuge |  |  |  |  |
| 2.3.14 | Vacuum concentrator |  |  |  |  |
| 2.3.15 | Magnetic rack for PCR tubes |  |  |  |  |
| 2.3.16 | Magnetic rack for 1.5 mL tubes |  |  |  |  |
| 2.3.17 | Vortexer |  |  |  |  |

**2.4 Software Packages**

**2.5 Primers/oligonucleotides/adapters**

2.5.1 PCR Primers:

2.5.2 Adaptors:

2.5.3 Probes: Biotinylated RNA baits were synthesized by Arbor Biosciences (see Section 3: Experimental Design). The position of the targeted regions is available in Supplementary Table X.

**3. Experimental design**

Probe design (Wilkinson, phylogenetic similarity)

Number of probes (~2,000)

The amount of the recovered material after hybridization is usually very little and it will require more PCR cycles to obtain a sufficient concentration for NGS-sequencing, which will result in a higher rate of PCR duplicates that need to be filtered out before further analysis. This also means that we need to start with 500 – 1,000 ng per sample.

**4. DNA extraction and quantification**

**5. Library preparation**

**6. Bisulfite conversion and library amplification**

**7. Target enrichment through hybridization capture with RNA probes**

**8. Sequencing**

**9. Data Analysis**