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**Protocol and materials**

**<https://github.com/alanfoleynibrt/SingleCellmtDNA>**

## **1 Materials and Equipment**

### **2.1 CHO cell culture**

1. 125mL bioreactor flasks (Nalgene 10266432).
2. Appropriate CHO cell culture medium (e.g. Gibco CD FortiCHO 10887640).
3. CHO cell lines of interest (e.g. **Table 1**).

### **2.2 Immunolabelling and Staining**

1. DPBS
2. Nuclease-free water
3. Trypan Blue 0.4% (Gibco 15250061)
4. Luna II (or other appropriate cell counter)
5. DAPI (Invitrogen D1306)
6. Anti-Human IgG F(ab')<sub>2</sub>-Fluorescein antibody produced in goat (Sigma Aldrich SAB3701254-2MG). Or other appropriate fluorescent stain (e.g. CellTracker Green Invitrogen 11570166).

### **2.3 FACS**

1. 70% IPA.

2. FACS with appropriate lasers for DAPI and FITC. Here, a BD FACS Melody was used.
3. FACS polystyrene tubes (Falcon Corning 1018640)
4. U-bottom 96-well plates (Corning 3799)
5. Parafilm
6. TCL Buffer (QIAGEN 1070498)

## **2.4 AMPure purification**

1. AMPure XP Beads (10136224)
2. 70% ethanol
3. Elution buffer (QIAGEN 19086)
4. Sterile PCR tubes (autoclaved)
5. 0.2mL tube magnetic stand (New England Biolabs S1515S)
6. 10uL multichannel pipette (optional)

## **2.5 Long Range PCR**

Primers were designed using NCBI Primer-BLAST to specifically bind to mtDNA, and no known CHO nuclear DNA sequences to minimise Numt contamination.

1. SuperFi II Plat Taq (Invitrogen 12361010).
2. PCR thermocycler
3. 10uM forward and reverse primers (**Table 2**) (IDT)
4. 10mM dNTP Mix (Thermo Scientific R0192)

## **2.6 Agarose Gel**

1. Agarose powder.
2. TAE buffer.
3. SafeView (NBS Biologicals). Ethidium Bromide is an alternative.
4. GeneRuler 1kb Plus Ladder (Thermo Scientific SM1333).
5. Gel Viewer/transilluminator.
6. Disposable lab scalpel.
7. Eppendorf tubes.

## **2.7 Gel Purification**

1. QIAquick Gel Extraction Kit (QIAGEN 28706X4). Other gel extraction kits would be equally sufficient.

## **2.8 Qubit**

1. Qubit 4 Fluorometer (Invitrogen)
2. Qubit 1x dsDNA HS Kit (Invitrogen Q33230)

## **2.9 Sequencing**

1. iSeq100 (Illumina) PE150, 8 million reads
2. Illumina DNA Prep, (M) Tagmentation (24 Samples) (Illumina 20018704)
3. IDT for Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples) (Illumina 20027213)
4. iSeq 100 i1 Reagent v2 (300-cycle) (Illumina 20031371)
5. PhiX v3 (Illumina FC-110-3001)

## 2 Methods

All steps up to the completion of the LRPCR for the 4 samples (**Table 1**) were performed in sterile conditions (BSC).

### 2.1.1 PCR Component Storage

Since the LRPCR amplifies from less than 5,000 copies of mtDNA, PCR components must have optimal efficacy. This can be ensured by making small (20uL) aliquots of dNTPs (Thermo Scientific R0192) and primers (IDT) and storing at -80°C. New aliquots must be used for each lot of PCR performed and subsequently discarded – do not re-freeze aliquots.

1. After receiving primers (**Table 2**) and dNTPs, promptly make 20uL aliquots of 10uM primers and 10mM dNTPs in microcentrifuge tubes.
2. Store at -80°C.
3. Discard excess primers and dNTPs.
4. For each PCR performed, take a fresh aliquot from -80°C. Discard after use, do not store for re-use.

### 2.1.2 CHO cell culture

4. Suspension CHO cells are cultured in FORTICHO (Gibco CD FortiCHO 10887640) at 37°C, 5% CO<sub>2</sub>, 85% humidity, 125rpm with 25mm orbit in a shaking incubator in 125mL bioreactor flasks (Nalgene 10266432). Different cell lines may require different media/incubation conditions.
1. Every 3-4 days passage cells at  $0.2 \times 10^5$  cells/mL in 30mL media in 125mL culture shaking flasks.
2. A growth curve should be established to ensure samples are taken at the relevant cell phase (**Table 1**).

### 2.1.3 DAPI Stain

A working concentration of 0.1 $\mu$ g/mL is optimal for CHO cells.

1. Protect DAPI (Invitrogen D1306) solutions from light wherever possible.
2. In a BSC, dissolve 10mg DAPI powder completely in 2mL sterile deionised water to make a 5 mg/mL DAPI stock solution.
3. Aliquot and store at -20°C. Solutions are stable for at least six months.
4. Add 1 $\mu$ L of DAPI stock solution to 5mL DPBS for DAPI working solution for 1 $\mu$ g/mL stock 2 solution.
5. Add 1mL of 1 $\mu$ g/mL stock 2 solution to 9mL of DPBS to prepare a 0.1 $\mu$ g/mL DAPI working solution.

### 2.1.4 Staining Cells

Here, an AB-FITC conjugate is used which at 4°C can bind to IgG on the cell membrane in the process of being excreted by the cell. This allows the sorting of cells based on the productivity of an IgG-based antibody.

1. Prepare cell samples as per **Table 1**.
2. Count cells using trypan blue (Gibco 15250061) and a hemacytometer as per the manufacturer's instructions.
3. Centrifuge  $1 \times 10^6$  viable cells per sample at 200 x g for 5 minutes. Discard supernatant.
4. Wash the cells in 1mL DPBS, centrifuge at 200 x g for 5 minutes and discard the supernatant.
5. Repeat for a total of 2 washes.

6. Resuspend 2uL of anti-human IgG (Sigma Aldrich SAB3701254-2MG) (or DPBS) to samples. This concentration has been optimised for CHO cells, other cell lines may require optimisation.
7. Incubate at 4°C for 30 mins at 1000rpm, protected from light.
8. Wash cells twice with DPBS as per steps 6 & 7 for a total of 2 washes.
9. Resuspend in 1mL of cold DPBS or cold DAPI working solution.
10. Incubate on ice for 5 mins.
11. Transfer on ice to FACS lab for immediate analysis.

### **2.1.5 Setting Single cell Gating**

1. Setup FACS as per manufacturer's instructions.
2. Plan and prepare a U-bottom 96-well plate (Corning 3799). Pipette 5uL of 1x TCL buffer (QIAGEN 1070498) into the centre of each functional well using a multichannel pipette. Tap the plate firmly on a flat surface to encourage the central location of the TCL buffer.
3. Set size threshold on FACS to >12um (non-CHO cell types may differ).
4. Using Sample 4 (**Table 1**), set voltages to allow the representation of cells in a SSC-A against FSC-A logarithmic scale graph. Create a gate (G1) that excludes instrument noise and cell debris as per **Fig. 2A**.
5. Using Sample 4, bring forward the G1 gate and set a gate (G2) using FSC-H against FSC-A as per **Fig. 2B** to exclude doublets.
6. Using Samples 1 and 2, bring forward the G2 gate and set a range gate (G3) to only include live cells as per **Fig. 2C** and **2D**. DAPI positive means dead cells.
7. Using Samples 1 and 3, bring forward the G3 gate and set a gate (G4) for FITC-positive cells as per **Fig. 2E** and **2F**. G4 is the sorting gate for live, singlet cells. After

gates have been set, it is important to record data for a large number of events (e.g. 10,000 cells) and save FCS files.

### **2.1.6 Single cell Sort**

1. Keep the flow rate at a minimum to reduce the chance of doublets. If needed, make dilutions of the sorting sample to a minimum of  $0.5 \times 10^5$  cells/mL.
2. Load Sample 1 and set FACS to “single cell” and “96-well plate” modes.
3. Choose desired wells for sorting. Ensure the splash shield is present in the FACS.
7. Remove the lid and immediately insert the well plate into the FACS. Proceed with sorting. It may be useful to change the sort mode to “purity” for positive control.
4. After the sort is complete, remove the well plate and immediately cover it with the lid. Create an airtight seal around the edges with parafilm. Immediately place the plate in a  $-80^{\circ}\text{C}$  freezer.
5. Save FCS files for all samples.

STOPPING POINT: Samples can be stored for up to 6 months at  $-80^{\circ}\text{C}$ .

### **2.1.7 AMPure purification**

The QIAGEN DNA micro kit has previously been used to purify single cell samples (Ludwig et al., 2019) though not replicable by this team. The QIAGEN miniprep kit has been used for bulk analysis (Kelly et al., 2017). Both these methods utilise fixed-size mechanical filtration not designed for  $< 10\mu\text{L}$  single cell samples. AMPure beads (10136224) benefit from scalable purification – adapting to single cell samples, volumes can simply be reduced.

Sequences of mtDNA sometimes migrate and integrate into the nuclear genome – known as Numts. In a previous bulk analysis of mtDNA, the miniprep step purified the plasmid-like

mtDNA from contaminating linear nuclear DNA. Here, AMPure purification is used, leaving both mtDNA and nDNA in the sample. Blast searching primer sequences against the CHO cell line reference genome (taxid: 10029) and gel purifying 8.5kb bands provides adequate protection against Numts.

1. It is recommended to perform all steps in a BSC. The subsequent LRPCR is extremely sensitive and can potentially amplify small contaminations.
2. Thaw the 96-well plate at room temperature. Multiple samples can be taken through AMPure purification in batches (to a maximum of 12 samples). Multichannel pipettes are useful for high sample numbers.
3. Transfer the 5uL lysed sample into a labelled micro-centrifuge tube.
4. Fully resuspend AMPure beads by vortexing the bottle for 1 min.
5. Add 9uL of AMPure beads per sample (if lysed cell sample is greater, add 1.8x volume of AMPure beads) and pipette mix 10 times. Leave at room temperature for 5 mins.
6. Place tubes on a magnetic stand (New England Biolabs S1515S) for 2 minutes
7. Keeping the tubes on the magnetic stand, remove and discard cleared solution leaving the beads.
8. Wash with 40uL 70% ethanol. Discard supernatant, leaving the beads.
9. Repeat ethanol wash. On the second wash, ensure all ethanol is removed by using a P10 pipette. Avoid removing any beads.
10. Remove tubes from the magnetic stand. Add 18uL of elution buffer (QIAGEN 19086) to the bead aggregate and pipette mix 10 times or until fully resuspended.
11. Incubate for 5 minutes at room temperature.
12. Place tubes on the magnetic stand for 2 minutes.



13. Split eluate into two 8.5uL aliquots – leaving the bead aggregate. Label microcentrifuge tubes to identify which samples came from the same single cell. Proceed to PCR.

STOPPING POINT: Samples can be stored at -20°C

### **2.1.8 SuperFi II Plat Taq LRPCR**

The bottleneck of single cell sequencing is the DNA amplification. Amplification techniques that would work for bulk sequencing proved to be incompatible with single cell: mechanical purifications took too much of the sample, bacterial lysis buffers did not release enough mtDNA, and components lost effectiveness for the sensitive PCR. Once enough DNA is amplified, established protocols for bulk sequencing can be followed. The SuperFi II PCR kit (Invitrogen 12361010) has 300x fidelity compared to Plat Taq. SuperFi II was better able to amplify from small samples compared to Plat Taq. In addition, higher fidelity means greater confidence in lower-level heteroplasmies.

In addition to the below LRPCR protocol, single cell samples post AMPure purification are diluted to 1/10, 1/100, 1/1,000 and 1/10,000; to demonstrate the limits of the high fidelity LRPCR kit.

1. Ideally, all steps are performed in a BSC. Maintain samples at all steps on ice.
2. Thaw fresh aliquots of primers and dNTPs at room temperature then store on ice.

Equally, thaw SuperFi II 5x Buffer and store on ice. Do not remove DNA Polymerase from -20C, only remove briefly when needed. Briefly vortex and centrifuge components before use – except for the DNA Polymerase.

3. mtDNA LRPCR is performed in 2 separate fragments (termed X and Y). The eluate from a single cell has been split into 2 from AMPure Purification; 1 half is amplified using X primers, the other half by Y primers.

4. Make a mastermix with 10% overage, for each X primer and Y primer, as per the example in **Table 3**. Add SuperFi II DNA Polymerase last by briefly removing it from the -20°C freezer – to minimise time spent at room temperature. Gently vortex and centrifuge at low xG. Keep on ice.
5. Add 16.5uL mastermix to 8.5uL AMPure purified DNA. Gently vortex and centrifuge at low xG. Keep on ice.
6. Place samples in a PCR machine and set to PCR cycle as per **Table 4**. Set reaction volume to 25uL with lid temperature at 105°C.
7. Run cycle overnight.
8. On completion, remove samples and store them at 4°C.

STOPPING POINT: Samples can be stored for 2 weeks at 4°C.

### 2.1.9 Agarose Gel

Limit of detection: an 8.5kb band is still observable when taking a 1/1000 dilution of a single cell. You would expect around 100-10,000 mitochondria (Dhiman et al., 2019).

Theoretically, this PCR is on the edge of viability for single-mitochondrial sequencing.

1. Add 1g agarose to 100mL TAE buffer in a conical flask.
2. Microwave for 2.5 mins, or until fully dissolved.
3. Wait for the flask to cool to about 50°C.
4. Add 10uL SafeView (NBS Biologicals) and pour into the gel tray with a well comb.
5. Wait for the gel to cool and harden at room temperature.
6. Place gel in a gel box with TAE buffer just covering the gel.
7. Add loading dye to all samples as per the manufacturer's instructions.

8. Carefully load the entire sample into gel wells with an appropriate DNA ladder (Thermo Scientific SM1333).
9. Run at 100V until bands are 70% down the gel.
10. Turn off the power and carefully place the gel in a gel viewer.
11. Ensure you observe bands at 8.5kb (**Fig. 2G**).
12. Take photos of the gel.

#### **2.1.10 Gel excision**

1. Under a gel visualiser, locate desired 8.5kb bands of single cell reactions, as illustrated by red rectangles in **Fig. 2G**. Minimise UV light exposure to minimise degradation of DNA.
2. Using a new disposable scalpel, excise the 8.5kb band of your X single cell sample and place it in a 1.5mL Eppendorf tube. Make sure to exclude any bands other than the desired 8.5kb band.
3. Clean the blade thoroughly with 70% IPA.
4. Excise the Y single cell sample and place it in a separate 1.5mL Eppendorf tube.

#### **2.1.11 Gel purification**

1. Here, the QIAquick Gel Extraction Kit (QIAGEN 28706X4) is used; other gel extraction kits should be equally sufficient.
2. Follow QIAquick Gel Extraction protocol for “QIAquick Gel Extraction using a Microcentrifuge”.
3. Use 10uL of elution buffer to encourage a higher final concentration

STOPPING POINT: Samples can be stored at -20°C for 2 weeks.

### **2.1.12 Equimolar combination**

1. Here, the Qubit 1x dsDNA HS kit (Invitrogen Q33230) is used to quantify dsDNA.
2. Allow kit components to equilibrate to room temperature for 30 mins.
3. Add 10uL of Standard 1 to a Qubit tube, and 10uL Standard 2 to a separate Qubit tube. Add 190uL of 1x buffer to each.
4. Add 1uL of each X and Y fragments to separate Qubit tubes and add 199uL of 1x buffer to each.
5. Vortex for 2-3 seconds and leave at room temperature for 2 mins.
6. Measure the concentration of standards 1 and 2 using Qubit (Invitrogen).
7. Measure the concentration of samples using Qubit.
8. Calculate the volume required to aliquot 1ng of the X fragment and Y fragment from the same cell and combine them in a new Lo-bind tube. There is now a total of 2ng of mtDNA from a single cell.
9. We expect low concentrations from our single cell samples. If >1ng of each fragment is achieved, a greater DNA mass can be used. The Illumina DNA prep suggests a total input of 100-500ng – but is still functional with less.

### **2.1.13 Library Prep**

1. Follow the Illumina DNA Prep protocol, using IDT for Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples) (Illumina 20027213). Each single cell should have a unique pair of indexes.
2. Check the library Quality of the cleaned-up library by running 1uL on a Tapestation D5000 microwell.
3. Combine and dilute the library to a 2nM starting concentration as per the manufacturer's instructions.

After library clean up, STOPPING POINT, can store at -20°C for 30 days

#### **2.1.14 Sequencing**

Libraries generated using Illumina DNA Prep are compatible with a wide range of Illumina sequencers including HiSeq, iSeq100, MiniSeq, NextSeq and NovaSeq technologies. For a full range refer to Illumina DNA Prep documentation. PE150, max 1.2G output.

1. Prepare the iSeq cartridge and flow cell as per the manufacturer's instructions (Illumina 20031371).
2. Add a 2% PhiX (Illumina FC-110-3001) spike-in.
3. Ensure the sample sheet loaded onto iSeq corresponds to the sample sheet from Library Prep.
4. Load cartridge and perform run as per manufacturer's instructions.
5. After running, download the data. Back-up data on an external hard drive.

#### **2.1.15 Data preprocessing**

GitHub repository: <https://github.com/alanfoleynibrt/SingleCellmtDNA>

The bioinformatics pipeline is available in the above GitHub repository. Initial processing of data is performed in Linux and figures are made in R. All raw FASTQ data analysed is made available in this reproducible pipeline.

Briefly, trim\_galore (0.4.3) trimmed adapter sequences in FASTQ files. bowtie-2 (2.3.4.1) mapped reads to the KX576660.1 CHO mtDNA reference genome. Picard (1.199) tools marked duplicates (MarkDuplicates), added read groups (AddOrReplaceReadGroups) and built a BAM index (BuildBamIndex). Gatk3.8-0 realigned indels (IndelRealigner) and recalibrated bases (BaseRecalibrator). 2 separate mutation calling software were used: lofreq\_star-2.1.2 and varscan.v2.3.9. When a mutation was called by both, it was brought forward for analysis. If a mutation allele frequency was between 4 and 96%, it was considered "heteroplasmic". SnpEff predicted mutation impact. In tandem, analysis was repeated using a shifted mtDNA reference genome to complete coverage over the D-loop region. Unshifted mutation calls were concatenated with shifted to provide full coverage. ggplot2 in R was used to generate figures.