

Appendix 1: Single cell sequencing methods

(Adapted from protocol by Hayley Bennett / Maria Duque)

The full method for single cell sequencing is outlined below, but as a brief overview of the process; iHO were grown in two 24 well plates for 5 days, one plate was stimulated with rhIL-22 100ng / mL 18 hours prior to commencement of the assay. iHO were isolated from Matrigel with Cell Recovery Solution, pelleted via centrifugation at 750rpm and washed with DPBS (No Ca²⁺ or Mg²⁺). iHO were treated for 15 minutes with TrypLE to dissociate cells, washed with DPBS, transferred to Eppendorf tubes and stained with Calcein blue AM (0.5µg /mL, GeneCopoeia) and fixable viability dye eFluor 780 (1:2000; eBioscience). Cells were then washed with FACS buffer and samples run on the Becton Dickinson FACSAria11 using FACS Diva software. Three 100 cell and 3 zero cell well controls were sorted on each plate (see plate design below); the remaining 90 wells received a single cell during the sort. Once sorted, cells were lysed in 0.8% Triton-X, and mRNA was polyadenylated using OligoDT anchored 30 (Sigma-Aldrich), then reverse transcribed and amplified by SmartSeq-2 PCR. Resultant cDNA was cleaned up using Agencourt Ampure XP beads (Beckman Coulter). Quality control of selected wells was performed using Agilent High Sensitivity DNA kit. Nextera libraries were prepared using Nextera XT DNA Library Prep Kit (Illumina) and then underwent a further PCR amplification step. Plates were split into 3 pools, which were again cleaned up with Agencourt Ampure beads, and QC of pools was undertaken with Agilent High Sensitivity DNA kits. Pools were submitted to the WTSI sequencing pipeline. Pools were sequenced on the Illumina-C HiSeq V4, generating 125bp paired-end reads. Read outputs were quality assessed using numerous parameters in an automatic standard pipeline managed by the DNA Pipeline Informatics team at WTSI. Reads were aligned to the human (hg19) reference genome. Quality control (QC) metrics of >100,000 reads per cell, <20% mitochondrial (MT) content and >2000 genes detected per cell were used to select cells for further analysis. 89% of cells sequenced passed QC. Data were processed using the Seurat¹ and Single Cell Consensus Clustering (SC3)² packages, and principal components analyses run after scaling data for mitochondrial content and number of unique molecular identifiers (UMIs). Identification of cell types was attempted by clustering of cells by marker genes and enrichment analysis was done using the G:Profiler

(<https://biit.cs.ut.ee/gprofiler/gost>) resource to identify differences between unstimulated and rhIL-22 stimulated cells.

1. Plate preparation for sorting

- Eighteen hours prior to commencing sort, pre-stimulate half of plates to be sorted with rhIL-22 100 ng/mL (R&D).
- In addition, make up 10% stock of Triton X-100 (Sigma-Aldrich), diluting in Ambion nuclease-free water (Thermo-Fisher) and place on rotator to mix overnight. Dilute 800 µL of 10% stock in 9.2 mL Ambion nuclease-free water (Thermo-Fisher) in a 50 mL falcon tube to produce a final concentration of 0.8%.
- Put the tube under UV light in the UV Stratalinker 2400 (Stratagene) at 2000 joules/cm for 30 minutes.
- Prepare a master mix of the following in a 1.5 mL DNA LoBind Eppendorf tube in a clean microbiological safety cabinet (MSC):

Reagents	For 1 Reaction (µL)	For 100 reactions (µL)
0.8% Triton X-100	2	200
dNTP mixes (Thermo Scientific™)	1	100
OligoDT anchored 30. HPLC purified, 100 µM. 5'-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTVN-3' (Sigma-Aldrich)	0.1	10
SUPERase In™ RNase inhibitor (Thermo-Fisher)	0.1	10
Ambion nuclease-free water (Thermo-Fisher)	0.8	80

- Using the Eppendorf Multipipette Xstream dispenser with 0.2 mL Combitip advanced®, distribute 4 µL of the above mix per well of a nuclease-free non-skirted 96 well plate (Thermo Scientific™) and cover plate with a sterile film (AlumaSeal® CS Films for cold storage, Sigma-Aldrich).
- Spin briefly at 1000 rpm and keep the plate on wet ice until cell sort.
- Repeat for number of plates required for sort.

2. Cell preparation for sorting

- Remove media from iHO and dissolve Matrigel using Corning® Cell Recovery Solution (Sigma-Aldrich) for 1 hour.
- Pellet cells via centrifugation at 750 rpm, remove overlying media and wash x1 in PBS (No calcium or Magnesium – Gibco)
- Treat iHO for approximately 15 minutes with 5 mL TrypLE™ Express (Gibco) to dissociate cells, then add 5 mL base growth media to inactivate.
- Centrifuge cells at 750 rpm and wash x1 with PBS
- Split suspended cells into 1.5 mL Eppendorf tubes and label for the following conditions:
 1. Unstimulated - Unstained
 2. Unstimulated - Calcein blue
 3. Unstimulated - Fixable viability dye
 4. Unstimulated - Calcein blue + Fixable viability dye (experimental sample for sort)
 5. IL-22 stimulated - Unstained
 6. IL-22 stimulated - Calcein blue
 7. IL-22 stimulated - Fixable viability dye
 8. IL-22 stimulated - Calcein blue + Fixable viability dye (experimental sample for sort)
- Spin down cells in Eppendorf tubes and incubate in 100 µL of 0.5µg/mL Calcein blue AM (GeneCopoeia) or Fixable viability dye eFluor 780 (1:2000; eBioscience) in PBS under conditions outlined above, for 15 minutes at room temperature, protected from light.
- Add 1 mL FACS buffer (containing 2mM EDTA (Sigma) in DPBS and Trustain FcX block (5 µL/ 100µL buffer; BioLegend)), spin and wash again with 1 mL FACS buffer and re-suspend in 0.5 mL FACS buffer for sorting.

3. Cell sorting

- Decontaminate surfaces in FACS hood with RNaseZap (Thermo-Fisher).
- Complete sorting using Becton Dickinson FACSAria11 and FACS Diva software; plate plan as outlined in **Figure A1** (100 cell and 0 cell wells used as controls).

- Once sort is complete, seal each plate in the hood (MicroAmp™ Clear Adhesive Film, Thermo-Fisher), spin briefly at 1000 rpm in Star Lab plate centrifuge and transfer immediately to a bed of dry ice.

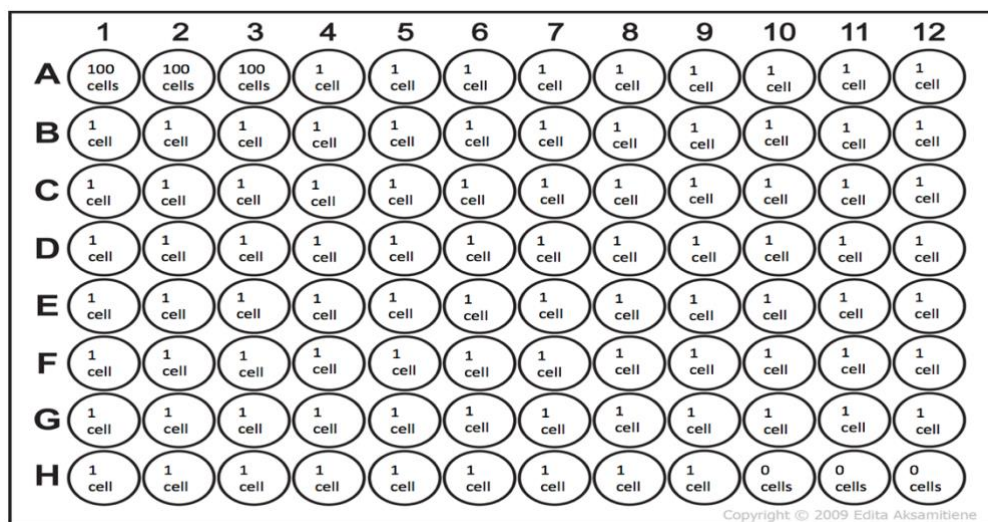


Figure A1: Plate plan for single cell sequencing

4. Reverse transcription and PCR

- Transport samples on dry ice to a clean PCR hood which has not been used for generating post-PCR NexteraXT libraries and has been cleaned thoroughly with RNaseZap and DNA Away (Fisher Scientific).
- Prepare reverse transcription (RT) mastermix for Smart-seq2 (adding reagents in order below) in 1.5 mL DNA LoBind Eppendorf tube, invert tube, spin briefly then store on ice until use:

Reagents	For 1 Reaction (µL)	For 100 reactions (µL)
Pre-RT Ambion nuclease-free water (Thermo-Fisher)	0.29	29
TSO, HPLC purified, stock 100 µM (Exiqon) 5'AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3'	0.1	10
1M MgCl ₂ (nuclease-free, Ambion)	0.06	6
5M ultrapure Betaine solution (VWR International)	2	200
SMARTScribe™ 5x RT Buffer (Clontech)	2	200
20mM DTT (dithiothreitol; part of SMARTScribe™ kit, Clontech)	0.5	50
SUPERase In™ RNase inhibitor (Thermo-Fisher)	0.25	25
SMARTScribe™ Reverse Transcriptase (Clontech)	0.5	50

- Place first plate on thermocycler and run the denaturing step at 72 °C for 3 min. Immediately afterwards, spin down plate for 10 seconds at 1000 rpm and place on CoolRack® XT PCR 96 (BioCision®). (If working with more than one plate, place the ones not in use on dry ice).
- Dispense 5.5 µL of RT mastermix into each well using the Eppendorf Multipette Xstream dispenser with 0.5 mL Combitip advanced®, seal plate using MicroAmp™ Clear Adhesive Film using a plate roller to ensure tight seal.
- Spin down plate at 1000 rpm and run the following programme on the Tetrad 2 thermal cycler (Bio-Rad) to reverse transcribe:
 - Step 1: 42 °C 1.5hr
 - Step 2: 42 °C 2 min
 - Step 3: 50 °C 2 min
 - Step 4: Go to Step 2 x9
 - Step 5: 70 °C 15 min
 - Step 6: 4 °C forever
- Following RT, proceed to Smart-seq-2 PCR step. Spin down plate at 1000 rpm and place on CoolRack® XT PCR 96.
- Prepare PCR mastermix in 1.5 mL DNA LoBind Eppendorf tube, invert tube, spin briefly then store on ice until use:

Reagents	For 1 Reaction (µL)	For 100 reactions (µL)
KAPA Hotstart HiFi 2X ReadyMix (Kapa Biosystems)	12.5	1250
ISO SMART primer (100 µM), HPLC purified 5'-AAGCAGTGGTATCAACGCAGAGT-3 (Sigma Genosys)	0.25	25
Post-RT Ambion nuclease-free water (Thermo-Fisher)*	2.25	225

*NB: This must be a different bottle of nuclease-free water from that used in RT step

- Add 15 µL of PCR mastermix per well to each plate, using Rainer multichannel pipette and fresh individual 20 µL tip for each well. Seal plate with MicroAmp™ Clear Adhesive Film, spin down at 1000 rpm and run the following programme on the Tetrad 2 thermal cycler for PCR:

Step 1: 98 °C 3 min
Step 2: 98 °C 20 sec
Step 3: 67 °C 15 sec
Step 4: 72 °C 6 min
Step 5: Go to Step 2 x 22
Step 6: 72 °C 5 min
Step 7: 4 °C forever

- Plates can be frozen at -20 °C at this point prior to completing next steps.

5. Bead clean-up 1

- Warm Agencourt AMPure XP beads (Beckman Coulter) for 20 min until at room temperature. Ensure beads are well mixed in suspension.
- Make up 40 mL of 80% ethanol (add 32 mL 100% molecular biology grade ethanol (Fisher Scientific) to 8 mL Ambion nuclease-free water (Thermo-Fisher)) per plate.
 - Use beads at 1x ratio for clean-up; therefore, add 25 µL beads per well, using multi-channel pipette and reagent boat and allow to bind at room temperature for 6 minutes
 - Place plate on 96 well plate magnet and allow beads to settle for 5 minutes
 - Remove supernatant using multichannel pipette
 - Without removing the plate from the magnet, add 200 µL 80% ethanol, then remove. Do not attempt to resuspend beads.
 - Repeat ethanol wash and carefully remove any remaining ethanol from wells.
 - Allow the beads to dry for 8 minutes.
 - Remove plate from magnet and using a multi-channel pipette, resuspend beads in 10 µL Ambion nuclease-free water per well.
 - Place on magnet and leave for at least 5 minutes for beads to settle. Remove eluted amplified cDNA (being careful to not transfer beads) to a new plate (skirted

SuperPlate 96 well plate, Thermo-Fisher) and seal with AlumaSeal® CS Film for cold storage.

Quality control:

Run an Agilent High-sensitivity DNA chip on the Agilent 2100 Bioanalyzer, following the manufacturer’s instructions, for one 0-cell well, one 100-cell well and 9 single cell wells to check for presence of cDNA in each well.

- Store all plates at -20 °C prior to library preparation.
- At this point 1 µL of PCR product can be used to prepare a 1:100 dilution for qRT-PCR.

6. Nextera XT Library preparation

- Using Nextera XT index kit v2 (Illumina), make up index plate as per manufacturer’s instructions. Depending on number of plates being processed, may need up to 4 different index kits (A, B, C and D). Seal with AlumaSeal® CS Film for cold storage; indexes can be stored at -20 °C prior to use.
- Prepare first mix using reagents contained in Nextera XT DNA Library Prep Kit in a 1.5 mL DNA LoBind Eppendorf tube (due to viscosity of reagents, prepare an excess of 125x reactions):

Reagents	For 1 Reaction (µL)	For 125 reactions (µL)
Tagment DNA buffer	2.5	312.5
Amplification tagment mix	1.25	156.25

- Add 3.75 µL of first mix per well of a new SuperPlate 96 well plate, using a multi-channel pipette, seal the plate with MicroAmp™ Clear Adhesive Film and spin briefly at 1000 rpm.
- Spin down the cDNA-containing plate at 1000 rpm and prepare a 1:50 dilution (in Ambion nuclease-free water) of the 100 cell controls. Add 1.25 µL of cDNA per well of the plate containing the first mix, pipetting onto the side of the well.

- Seal the first mix plate, briefly spin at 1000 rpm and incubate at 55 °C for 10 min in thermocycler. Let the block reach 10 °C, remove the plate and spin briefly again. During incubation step, thaw and spin down index plate at 1000 rpm.
- Add 1.25 µL NT buffer per well to the first mix plate, using a multichannel pipette and fresh tip for each well, seal and briefly spin at 1000 rpm.
- Add 3.75 µL NPM per well to the first mix plate, using a multichannel pipette and fresh tip for each well, seal and briefly spin at 1000 rpm.
- Add 2.5 µL index mix per well to the first mix plate, seal and briefly spin at 1000 rpm. Ensure index kit used for each plate is recorded.
- Run the following PCR programme on thermocycler:
 - Step 1: 72 °C 3 min
 - Step 2: 95 °C 30 sec
 - Step 3: 95 °C 10 sec
 - Step 4: 55 °C 30 sec
 - Step 5: 72 °C 1 min
 - Step 6: Go to Step 3 x 11
 - Step 7: 72 °C 5 min
 - Step 8: 10 °C forever

7. Bead clean-up 2

- Warm Agencourt AMPure XP beads (Beckman Coulter) for 20 min until at room temperature. Ensure beads are well mixed in suspension.
- In each of two 2 mL DNA LoBind Eppendorf tubes, pool half of the single cell and 0 cell wells (i.e. wells A4-D12 and E1-H12). Pool the 100 cell wells (A1-A3) in a 1.5 mL DNA LoBind Eppendorf tube.
- Use beads at 0.8x ratio for clean-up, therefore 12 µL of beads per sample. Add 36 µL beads to the tube containing A1-A3, 526 µL to tube containing A4-D12 and 540 µL to tube containing E1-H12. Pipette up and down 6 times, vortex tubes and spin down in benchtop centrifuge.

- Incubate at room temperature for 6-10 minutes, periodically mixing tubes by hand. During this time, prepare 5ml 80% ethanol as described above.
- Briefly spin down tubes, place onto magnetic tube rack and allow beads to settle for 5 minutes.
- Discard supernatant, then without removing tubes from the magnet, add 1 mL 80% ethanol to both of the 2 mL Eppendorf tubes and 200 μ L to the 1.5 mL tube. Do not attempt to resuspend beads.
- Remove and discard the ethanol and repeat ethanol wash.
- Spin down tubes and replace on the magnetic rack. Carefully remove any remaining ethanol.
- Allow beads to dry for approximately 8 minutes, or until beads appear dull.
- Remove tubes from magnetic rack and resuspend beads in Ambion nuclease-free water. Use 10 μ L per 100 cell well (therefore 30 μ L for tube containing wells A1-A3), and 5 μ L per single cell well (therefore 225 μ L for tube containing A4-D12 and 240 μ L for tube containing E1-H12).
- Replace tubes onto magnetic rack and leave for at least 5 minutes, then transfer eluted amplified cDNA (being careful not to transfer beads) to fresh DNA LoBind 1.5 mL Eppendorf tubes.

Quality control:

- Run an Agilent High-sensitivity DNA chip on the Agilent 2100 Bioanalyzer, following the manufacturer's instructions for each pool to check for presence of cDNA.
- Store pools at -20 °C prior to submission to pipeline.

References:

1. Butler A, Hoffman P, Smibert P, Papalexi E, Satija R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol.* 2018;36(5):411-420.
2. Kiselev VY, Kirschner K, Schaub MT, et al. SC3: consensus clustering of single-cell RNA-seq data. *Nat Methods.* 2017;14(5):483-486.

Appendix 2: Generation of S100A9^{-/-} hiPSC line

S100A9 knockout was generated by WTSI CGaP facility, by a single T base insertion in the third exon containing the EF-hand motif at cDNA position 228 using CRISPR/Cas9 in the Kolf2 human iPSC line. This was achieved by nucleofection of 10⁶ cells with Cas9-crRNA-tracrRNA ribonucleoprotein (RNP) complexes. Synthetic RNA oligonucleotides (target site: 5'- AGACAAGCAGCTGAGCTTCG -3', WGE CRISPR ID: 915082321, 225 pmol crRNA/tracrRNA) were annealed by heating to 95°C for 2 min in duplex buffer (IDT) and cooling slowly, followed by addition of 122 pmol recombinant eSpCas9_1.1 protein (in 10 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT). Complexes were incubated at r.t. for 20 minutes before electroporation. After recovery, cells were plated at single cell density and colony were picked into 96 well plates. 96 clones were screened for heterozygous and homozygous mutations by high throughput sequencing of amplicons spanning the target site using an Illumina MiSeq instrument. Final cell lines were further validated by Illumina MiSeq. The homozygous targeted clone was used in downstream differentiation assays. Additional supporting data:

S100A9 WT sequence: GCAGCTGAGCTTCGAGGAGTTCA

S100A9 MUT sequence: GCAGCTGAGCTTTCGAGGAGTTCA

Insertion of T at position 228

PCR amplification primers:

F: TTTGGTATGTGCTCAGTGTCTG

R: GAAGAGGTGGAAGAAGCACAC

WT Protein

MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVIEHIMEDLDTNA
DKQLSFEEFIMLMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP

MUT Protein

MTCKMSQLERNIETIINTFHQYSVKLGHPTLNQGEFKELVRKDLQNFLKKNKNEKVIEHIMEDLDTNA
DKQLSF~~RGVHHADGEANLGLPREDARG.RGPWPPP.ARPRGGHPL~~

Appendix 3 - Bulk RNA-Seq data analysis methods

RNA-Seq analysis of hiPSC-derived macrophages infected with different *Salmonella enterica* serovars

For all 12 samples, the mean sequencing depth was approximately 19.30 million reads. FASTQC was run on all samples to assess data quality. The 75bp paired-end sequencing reads were aligned to the human (hg19) reference genome, downloaded and indexed from the UCSC Genome Browser, using STAR RNA-Seq aligner version 2.5.3a.¹ Only the reads that mapped uniquely as pairs were retained for downstream analysis. The resulting BAM files containing the aligned reads were provided to *featureCounts* version 1.6.2² to obtain *gene-level read counts* using the reference annotation file (GTF format downloaded from the UCSC browser). Lowly expressed genes, defined as having less than 5 counts per million (CPM) reads in at least three samples, were removed. A total of 11,473 genes were retained for downstream analyses. Raw count data was then subjected to Trimmed Mean of M values (TMM) normalisation,³ followed by voom transformation⁴ that estimates the mean-variance relationship in the data. To identify differentially expressed genes between each stimulation condition and PBS control samples, a linear model was fitted to each gene using the *lmFit* function in the Limma R package.⁵ The technical differences between the three replicate groups (batch effects) were modelled as covariates in the analysis. Contrasts were defined for three pairwise comparisons (*S. Typhi* vs. PBS control; *S. Typhimurium* vs. PBS control; *S. Paratyphi A* vs. PBS control), and empirical Bayes moderated t-statistics, *log2-fold-change*, and *P*-values were computed for each comparison using the *eBayes* function in Limma. *P*-values were adjusted using the Benjamini and Hochberg FDR correction procedure.⁶ To obtain a list of biologically meaningful genes, in each comparison, differential analysis was further assessed for genes that achieved an absolute *log2-fold-change* greater than 0.58 and FDR < 0.05 using the TREAT method in Limma.⁷ Principal component analysis (PCA) was performed on *log2*-transformed FPKM Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values using the *prcomp* function in R software. Volcano plots for each pairwise comparison was produced using the *ggplot2* R package.

RNA-Seq analysis of hiPSC-derived intestinal organoids infected with different *Salmonella enterica* serovars

iHO samples were multiplexed across three sequencing lanes, generating 75bp paired-end reads, with each lane achieving a depth of approximately 5.84 – 8.06 million reads. Since the read depth was consistent across the lanes, lane-level FASTQ files for each sample were merged into a single file. FASTQC was run on all the 36 samples to assess data quality. The sequencing reads were aligned to the human (hg19) reference genome, downloaded and indexed from the UCSC Genome Browser, using STAR RNA-Seq aligner version 2.5.3a. Only the reads that mapped uniquely as pairs were retained for downstream analysis. The resulting BAM files containing the aligned reads were provided to *featureCounts version 1.6.2* to obtain *gene-level read counts* using the reference annotation file (GTF format downloaded from the UCSC browser). To separate the known transcriptional heterogeneity that exists between hiPSC cell lines⁸ from the stimulation-specific transcriptional differences, differential analysis in each of the three cell lines was performed separately. First, lowly expressed genes, defined as having less than 5 counts per million (CPM) reads in at least three samples, were removed. Approximately 12,000 genes were retained for downstream analyses in each cell line. Then, the raw count data was subjected to Trimmed Mean of M values (TMM) normalisation, followed by voom transformation. To identify differentially expressed genes between each stimulation condition and PBS control samples, a linear model was fitted to each gene using the *lmFit* function in the Limma R package. Contrasts were defined for three pairwise comparisons (*S. Typhi* vs. PBS control; *S. Typhimurium* vs. PBS control; *S. Paratyphi* vs. PBS control), and empirical Bayes moderated t-statistics, *log2-fold-change*, and *P*-values were computed for each comparison using the *eBayes* function in Limma. *P*-values were adjusted using the Benjamini and Hochberg FDR correction procedure. Principal component analysis (PCA) was performed on log₂-transformed FPKM Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values using the *prcomp* function in R software. Volcano plots for each pairwise comparison were produced using the *ggplot2* R package.

References:

1. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21.
2. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-930.
3. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol*. 2010;11(3):R25.
4. Law CW, Chen Y, Shi W, Smyth GK. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol*. 2014;15(2):R29.
5. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47.
6. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. . *J R Stat Soc Ser B* 1995;57(1):289-300.
7. McCarthy DJ, Smyth GK. Testing significance relative to a fold-change threshold is a TREAT. *Bioinformatics*. 2009;25(6):765-771.
8. Carcamo-Orive I, Hoffman GE, Cundiff P, et al. Analysis of Transcriptional Variability in a Large Human iPSC Library Reveals Genetic and Non-genetic Determinants of Heterogeneity. *Cell Stem Cell*. 2017;20(4):518-532 e519.

