Single cell analysis of haematopoietic stem cells

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i) Running title: Single cell analysis of HSCs

ii) Abstract

The study of haematopoiesis has been revolutionized in recent years by the application of single cell RNA sequencing technologies. The technique coupled with rapidly developing bioinformatic analysis has provided great insight into the cell type compositions of many populations previously defined by their cell surface phenotype. Moreover, transcriptomic information enables the identification of individual molecules and pathways which define novel cell populations and their transitions including cell lineage decisions. Combining single cell transcriptional profiling with molecular perturbations allows functional analysis of individual factors in gene regulatory networks and better understanding of the earliest stages of malignant transformation. In this chapter we describe a comprehensive protocol for scRNA-Seq analysis of the mouse bone marrow, using both plate-based (low throughput) and droplet-based (high throughput) methods. The protocol includes instructions for sample preparation, an antibody panel for flow cytometric purification of haematopoietic progenitors with index sorting for plate-based analysis or in bulk for droplet-based methods. The plate-based protocol described in this chapter is a combination of the Smart-Seq2 and mcSCRB-Seq protocols, optimized in our laboratory. It utilizes off-the-shelf reagents for cDNA preparation, is amenable to automation using a liquid handler, and takes 4 days from preparation of the cells for sorting to producing a sequencing-ready library. The droplet-based method (using for instance the 10x Genomics platform) relies on the manufacturer's user guide and commercial reagents, and takes 3 days from isolation of the cells to the production of a library ready for sequencing.

iii) Key Words

Haematopoiesis, scRNA-Seq, Transcriptome, gene regulatory networks, mcSCRB-Seq, Smart-Seq2, mcSmart-Seq2, Haematopoietic stem cell, Haematopoietic stem/progenitor cell, Bone marrow.

1) Introduction

Haematopoiesis is characterised by a hierarchical differentiation process, wherein stem cells and progenitors choose either to self-renew, or enter differentiation pathways towards more than 10 distinct cell lineages. Traditional descriptions of this differentiation hierarchy have depended on immunophenotyping, using combinations of several cell-surface markers to delineate cellular populations with defined differentiation and self-renewal potentials. Sorting strategies have been refined over decades with the identification of new combinations of cell surface markers, but the purified populations remain functionally heterogeneous. For instance, only a proportion of 'phenotypic haematopoietic stem cells' display long term multilineage reconstitution properties. The application of single cell RNA sequencing (scRNA-Seq) is transforming our ability to classify the haematopoietic system in detail. Such analyses permit interrogation of molecular profiles at an unprecedented resolution. The transcriptome-wide coverage allows unbiased correlation of self-renewal and differentiation properties with specific gene signatures, providing insight into mechanisms controlling cell state [1–4]. Computational analysis of thousands of transcriptomes can identify rare novel populations which would be lost in bulk analysis, while also allowing an overview at tissue or whole organ level [3, 5]. Transcriptomic analysis of haematopoiesis has demonstrated that most populations defined by cell-surface phenotype are indeed heterogeneous and revealed that lineage decisions are made earlier than previously anticipated [2, 6–9].

Starting from the premise that scRNA-Seq captures a snapshot of cells at various stages of a differentiation trajectory, computational methods can arrange single cell profiles into coherent landscapes progressing from a multipotent state through uni-lineage states to mature populations. This in turn enables identification of putative branchpoints, at which cells "choose" a specific differentiation potential [10–12]. Using this approach, entry points into distinct haematopoietic lineages have been identified, resulting in a comprehensive single cell landscape of the haematopoietic compartment within mouse bone marrow [8, 13, 14]. Navigating this landscape allows observation of transitions and inference of cellular origins, while unbiased capture of the entire transcriptome uncovers previously inaccessible and novel molecular pathways and gene combinations defining distinct cell transitions. Comparative analysis of specific cell populations facilitates identification of new cell surface markers for prospective cell isolation and correlative functional analysis [2]. The combination of single

cell transcriptomics with genetic perturbations allows an insight into the earliest stages of leukemogenesis, and molecular co-operation which may underly disease states [13, 15, 16]. Using high-throughput droplet-based methods, hundreds of thousands of single cells can be analysed in a time and cost efficient manner, generating sufficient resolution to draw conclusions about even very rare populations [17, 18]. High throughput techniques (e.g. InDrops [19], Drop-Seq [20], 10x Genomics [21] and SPLiT-seq [22]) allow profiling of over 10,000 cells per experiment. While these methods tend to detect fewer genes per cell, the higher throughput permits analysis of a vast diversity of cell-types at a much higher scale, allowing refinement of cellular populations. Conversely, lower-throughput plate-based techniques (e.g. Smart-Seq2 [23], Smart-seq3 [24], mcSCRB-Seq [25], Quartz-Seq2 [26], CEL-Seq2 [27], MARS-seq [28] and RamDA-seq [29]) are very effective to profile fewer cells per experiment (in the range of 100s) but with a higher sensitivity for the detection of genes per cell, offer the advantage of index sorting data (a feature which enables the recording of every measured parameter by the sorter) which can be integrated with transcriptomic data. Some plate based methods additionally offer the advantage of full-length transcript coverage.

Modifications of these techniques have permitted multimodal measurements, such as transcriptomics alongside genomic or epigenomic profiling in a single cell in so-called 'multi-omics' protocols [30]. One of the most clinically relevant applications thus far has been the combined analysis of single cell gene expression and mutation status. Giustaccini et al modified the Smart-Seq2 protocol to simultaneously genotype for the BCR-ABL fusion gene and capture transcriptomes of over 2,000 stem cells from chronic myeloid leukaemia patients [31]. This allowed characterisation of a subgroup of BCR-ABL mutant stem cells which persist through therapy, and also transcriptionally aberrant wild type HSCs present in CML patients whose presence predict the response to therapy. TARGET-Seq, a further refinement of this protocol from the Mead group, allows parallel targeted mutation analysis from cDNA and gDNA in addition to scRNA-Seq [32]. Applied to over 4,500 haematopoietic stem/progenitor cells (HSPCs) from patients with myeloproliferative disorders, this technique reveals clonal heterogeneity correlated with aberrant transcriptional profiles, and can resolve the order of acquisition of mutations.

A further study of Acute Myeloid Leukaemia (AML) has employed nanowell technology and nanopore long read sequencing to integrate targeted genotyping with transcriptional profiling [33]. While genotyping data remained

relatively sparse, integrating these with single cell transcriptomes from over 38,000 cells in identified six malignant AML phenotypes which shared features with healthy HSPCs. Intra-tumoural diversity can be dissected by this technique as demonstrated by the detection of different differentiation states associated with FLT3-ITD and FLT3-TKI mutations in the same patient.

Nam et al [34] modified the 10x Genomics scRNA-Seq protocol by spiking in primers for specific mutant transcripts associated with myeloproliferative disorders, which allowed the authors to demonstrate the progenitor-specific fitness effects of driver mutations and correlate these with patient phenotypes. The application of single cell transcriptomics and mutational profiling techniques to cancer biopsy samples illuminates the molecular programmes underlying carcinogenesis and clonal evolution. The ability to separate tumour cells from their non-mutant counterparts also extends insights into the tumour microenvironment and non-cell-autonomous effects of driver mutations.

Here we present comprehensive experimental protocols for plate-based scRNA-Seq (mcSmart-Seq2) and droplet-based scRNA-Seq (using the 10x Genomics platform). These techniques are complementary and can be combined in a single experiment if sample quantity permits. A droplet-based workflow has several advantages; its higher throughput provides an overview of organs at tissue level, the high cell number yield confers greater power to detect rare cell populations, and finally the use of unique molecular identifiers reduces amplification-related noise. However, the method provides only minimal splicing data. By default, the 10x Genomics platform does not allow genotyping of mutant cells in mixed populations, but several labs have tried to enable this by adding further processing steps [34, 35]. By contrast, plate-based techniques are more labour-intensive, expensive and the number of cells which can be profiled is more limited. Nevertheless, the method provides full length transcript sequencing, which requires a greater read depth but confers increased sensitivity and permits more even coverage with the ability to distinguish RNA isoforms. The plate-based approach is generally better suited when looking at a specific population of cells or when cell numbers are a limiting factor.

The plate-based scRNA-Seq protocol that we describe in this chapter is a combination of two previously published protocols from Picelli et al [23] and Bagnoli et al [25]. This protocol has also been successfully used for small pools of cells (up to 100) without any modification to the reagents; only reducing the number of PCR cycles for the amplification of the cDNA library will be required, to account for larger starting material. The plate-

based method described in this chapter captures polyadenylated transcripts and the signal detected encompasses the full length cDNAs. As with all polyA capture methods, the obtained sequencing reads are 3' biased, but it is possible to analyse all the exons of highly expressed genes, different spliced variants and even the spliced vs un-spliced transcripts (although the latter is a small proportion of the detected material). This method takes advantage of template-switching during the reverse transcriptase reaction and therefore the choice of reverse transcriptase (RT) is essential as not all RT enzymes are able to add untemplated nucleotides onto the end of the cDNA molecule.

Whilst this protocol is plate based and therefore ultimately more expensive per cell than the droplet methods, it allows the retention of additional metadata (such as index sorted data) which the droplet methods do not. Of note, droplet methods can now be combined with oligo-labelled antibodies therefore allowing surface phenotype to be measured in parallel with the transcriptome [36]. Two major advantages of this plate-based protocol are that all the cDNA amplification steps use off-the-shelf reagents and the entire protocol takes around 4 days, from preparation of the cells for sorting to producing a final library ready to be sequenced. Whilst we present a manual version of the protocol, automation could be implemented at several stages (3.3.5 PCR purification and 3.3.8 Library preparation). Automation at the clean-up stages will ensure that repetitive pipetting steps are performed uniformly, and miniaturisation of the library preparation reaction offers large savings in commercial reagents.

2) Materials

2.1 Bone Marrow Harvest

1. PBS (Calcium and Magnesium free) supplemented with 2% heat-inactivated FCS
2. Pestle and mortar
3. 15 ml centrifuge Tube
4. 50 µM filter
5. p1000 pipette
6. p200 pipette
7. p20 pipette
8. p2 pipette

9. p1000 filter tips
10. p200 filter tips
11. p20 filter tips
12. p2 filter tips
13. Pipette boy
14. Stripettes
15. Ammonium chloride
16. 5 ml round bottom polystyrene tube
17. 5 ml round bottom polypropylene tube
18. Hematopoietic progenitor enrichment kit (For example EasySep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit, Stemcell Technologies)
19. Magnet (this will depend on the progenitor enrichment kit used - for example EasySep™ Magnet, Stemcell Technologies)
20. Antibodies (see Antibodies section 2.2)
21. 1.5 ml Eppendorf Tubes
22. FACs sorter

2.2 Antibodies

1. For dilutions of the individual antibodies used see Table 2 and **Note 1**.
2. Mouse Hematopoietic Progenitor Isolation Cocktail PN (19856C) part of the EasySep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit (Stemcell Technologies)
3. EPCR PE, Clone RMEPCR1560
4. CD48 APC, Clone HM48-1
5. CD150 PE-Cy7, Clone TC15-12F12.2
6. c-kit APC-Cy7, Clone 2B8
7. Sca1 BV421, Clone D7
8. CD45 FITC, Clone 30-F11
9. BV510 Streptavidin
10. 7AAD

2.3 scRNA-Seq

1. qPCR machine
2. Spectro fluorometer or fluorescence microplate reader
3. Bioanalyser or Tapestation (for instance from Agilent)
4. Temperature controlled Microcentrifuge
5. Temperature controlled Bench Top Centrifuge (15 ml tube)
6. 96- well PCR plate (for example: non- skirted Starlab or skirted Framestar 96 4titude)
7. Adhesive film lid (see **Note 2**)
8. Optical caps for 96-well plate (qPCR)
9. 8-strip 0.2 ml PCR tubes with cap
10. 15 ml Centrifuge tubes Tubes
11. Index Plate Fixture (Illumina)
12. Microplate, 384-well, PS, Flat-bottom, Fluotrac, medium binding, black (Greiner Bio-One)
13. Magnetic stand (for example: Life Technologies DynaMag-96 side)
14. Magnetic stand (for example: Life Technologies DynaMag-2)
15. 8-channel multichannel pipette 1-10 µl (pre-amp)
16. 8-channel multichannel pipette 1-10 µl (post-amp)
17. 8-channel multichannel pipette 5-50 µl (post-amp)
18. 8-channel multichannel pipette 3-300 µl (post-amp)
19. 12-channel multichannel pipette 1-10 µl (post-amp)
20. Eppendorf Multipette® E3 (Eppendorf) – optional
21. p1000 pipette
22. p200 pipette
23. p20 pipette
24. P2 pipette
25. p1000 filter tips low retention
26. p200 filter tips low retention
27. p20 filter tips low retention
28. p2 filter tips low retention

29. Index Adapter Replacement Caps (Illumina)
30. 1x PBS (Calcium and Magnesium free) supplemented with 0.04% BSA solution (400 µg/ml)
31. 10 % Triton X-100
32. DEPC treated Distilled water
33. Distilled water – PCR grade
34. RNase Inhibitor (for example: SUPERase-In, Ambion)
35. dNTP mix (10 mM each)
36. ERCC RNA Spike-In Mix (ThermoFisher) (see **Note 3**)
37. Maxima H minus Reverse Transcriptase (200 U/µl) (ThermoFisher)
38. Terra PCR Direct Polymerase Mix (250 U) (Takara Bio)
39. AMPure XP beads (Beckman Coulter)
40. Elution buffer (EB) solution: 10 mM Tris-Cl, pH 8.5
41. 80% Ethanol (see **Note 4**)
42. Bioanalyser High Sensitivity DNA reagents or Tapestation High Sensitivity DNA Reagents (Agilent)
43. Quant-iT™ Picogreen double stranded DNA assay kit (ThermoFisher)
44. 1 x TE
45. Nextera XT DNA sample preparation kit 96 samples (Illumina)
46. Nextera XT Index Kit v2 (96 Indexes, 384 samples) (Illumina)
47. KAPA Library Quantification Kit (Roche)
48. KAPA dilution buffer: 10 mM Tris-HCl, pH 8.0 – 8.5 + 0.05% Tween® 20

2.4 Oligonucleotides

1. Oligos – for details of sequence for individual oligos see Table 1. Oligo-dT30VN and IS PCR oligo were ordered from Integrated DNA Technologies, Inc (IDT). LNA-modified TSO oligo was ordered from Qiagen. All oligos were ordered with HPLC purification. Avoid repeated freezing-thawing cycles for all oligos.
2. Resuspend oligonucleotides in 1x TE as described in Table 1.
3. Oligos can be stored for up to 6 months at -80°C.

3) Methods

3.1 Sample preparation

1. Cool down the centrifuge before use (4 - 8°C).
2. Ensure PBS+2% FCS and Ammonium chloride are pre-cooled.

3.1.1 Harvest bone marrow

1. Sacrifice the mouse/mice according to local rules.
2. Dissect femur, tibia and pelvic bones ensuring to remove all tissue.
3. Harvest cells from femurs, tibia and pelvic bones of a mouse by crushing bones with a pestle and mortar in 3 ml cold PBS+2% FCS (see **Note 5**).
4. Place 50 µM filter onto a 15 ml centrifuge tube (see **Note 6**).
5. Ensure complete resuspension of the crushed bones by pipetting the PBS+2% FCS up and down using a p1000 pipette.
6. Using a p1000 pipette collect cell suspension and filter into a 15 ml centrifuge tube.
7. Add 3 ml of cold PBS+2% FCS to mortar and pipette up and down with a p1000 pipette. Filter solution into 15 ml centrifuge tube, combining all bone marrow.
8. Repeat step 7 (total of two washes).
9. Top up to final volume of 10 ml in cold PBS+2% FCS.
10. Centrifuge for 5 minutes at 300x g at 4°C.
11. Discard supernatant and resuspend in 3 ml of cold PBS+2% FCS.
12. Lyse the red blood cells by adding 5 ml of cold Ammonium chloride.
13. Incubate on ice for 5 mins, shake well or vortex, then incubate for a further 5 minutes (10 minutes total) (see **Note 7**).
14. Top up to 15 ml with cold PBS+2% FCS (to dilute the Ammonium Chloride) (add ~ 7ml).
15. Centrifuge for 5 minutes at 300x g at 4°C.
16. Discard supernatant and resuspend the pellet in 500 µl of cold PBS+2% FCS.
17. Transfer all the bone marrow sample to a 5 ml round bottom polystyrene tube and place on ice.

18. Keep the centrifuge tube as remaining cells in the centrifuge tube will be used for antibody single stain controls (remaining volume and volume/cells stuck to walls of tube).

3.1.2 Single Antibody Staining Controls

1. The volume in which to resuspend the controls will depend on the number of different antibodies used for FACS; add 100 µl cold PBS+2% FCS per control to the 15ml centrifuge tube which contained the red cell lysed bone marrow (retained in step 18 of 3.1.1 Harvest bone marrow) (see **Note 8**).
2. The controls are not lineage depleted.
3. Add 1 ml cold PBS+2% FCS for the 10 control tubes to the 15 ml centrifuge tube which contained all the red cell lysed bone marrow (retained in step 18 of 3.1.1 Harvest bone marrow).
4. Place a 50 µM filter into 15 ml centrifuge tube.
5. Filter samples into 15 ml centrifuge tube (see **Note 9**).
6. Label control tubes (unstained, all individual antibodies as in Table 2 and all stain control).
7. Aliquot 100 µl into each of the 10 tubes (unstained, single stains, all stain control).
8. Add antibodies as described (see Table 2), for all stain control add 4.3 µl of Antibody Master Mix (see **Note 10**).
9. Mix and incubate on ice, in the dark for 30 minutes.
10. After incubation, wash by adding 1 ml of cold PBS+2% FCS to each tube (except the viability stain or unstained sample as these contain no antibodies).
11. Centrifuge samples at 300x g for 5 minutes at 4°C.
12. Remove supernatants, being careful not to disturb the cell pellet. The cell pellet can be almost invisible so a small volume of supernatant can be left behind.
13. Resuspend the cell pellet by flicking the tubes.
14. Keep “Lineage” single stain and “All stain” control tubes to one side.
15. Resuspend remaining controls in 500 µl cold PBS+2% FCS.
16. Place on ice and in darkness.
17. To Lineage single stain and all stain control add 100 µl cold PBS+2% FCS.
18. Add 0.5 µl Streptavidin conjugated antibody (BV510 Streptavidin).
19. Mix and incubate on ice for 15 minutes.
20. Add 1 ml of cold PBS+2% FCS and centrifuge at 300x g for 5 minutes at 4°C.

21. Remove supernatants, being careful not to disturb the cell pellet. The cell pellet can be almost invisible so a small volume of supernatant can be left behind.
22. Resuspend the cell pellet by flicking the tubes.
23. Resuspend in 500 µl of cold PBS+2% FCS.
24. Add 0.5 µl 7AAD to viability single stain and All stain control tubes (see **Note 11**).
25. These control samples are now ready to be used to set voltages and gates on the flow cytometry sorter.

3.1.3 Lineage depletion

1. Lineage depletion protocol (see **Note 12**).
2. Use EasySep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit (Stemcell Technologies).
3. Retrieve samples from ice (step 18 of 3.1.1. Harvest bone marrow).
4. Make samples up to an equal volume if processing more than one mouse (normally 600 µl).
5. Add 1:100 of EasySep™ Mouse Hematopoietic Progenitor Isolation Cocktail. Mix and incubate on ice for 15 minutes.
6. Vortex EasySep™ Streptavidin RapidSpheres™.
7. Ensure the particles are in a uniform suspension with no visible aggregates.
8. Add 1:25 EasySep™ Streptavidin RapidSpheres™.
9. Mix and incubate on ice for 10 minutes.
10. Add cold PBS+2% FCS to bring volume up to 2.5 ml, pipette up and down, and place the tube (without lid) into EasySep™ magnet.
11. Incubate for 3 minutes at room temperature.
12. Pick up magnet, bracing the tube against the side of the magnet using index finger, and in one continuous motion pour into a new 5 ml round bottom tube (see **Note 13**). Do not shake or blot.
13. The supernatant contains the enriched bone marrow cell suspension (lineage depleted).
14. Centrifuge for 5 minutes at 300x g at 4°C.
15. Resuspend in 300 µl of cold PBS+2% FCS.

3.1.4 Specific antibody staining of samples

1. Add antibody master mix to sample (see Table 3), mix and incubate on ice, in the dark for 30 minutes.
2. After incubation, wash by adding 3 ml of cold PBS+2% FCS.
3. Centrifuge samples at 300x g for 5 minutes at 4°C.

4. Remove supernatant, being careful not to disturb the cell pellet.
5. Resuspend the cell pellet by flicking the tube.
6. Resuspend sample in 300 µl cold PBS+2% FCS.
7. Add 1.5 µl Streptavidin conjugated antibody (BV510 Streptavidin).
8. Mix and incubate on ice for 15 minutes.
9. Add 3 ml of cold PBS+2% FCS and centrifuge at 300x g for 5 minutes at 4°C.
10. Remove supernatant, being careful not to disturb the cell pellet.
11. Resuspend the cell pellet by flicking the tube.
12. Resuspend in 500 µl of cold PBS+2% FCS.
13. Place a 50 µM filter onto a new 5 ml round bottom tube.
14. Filter antibody stained enriched bone marrow sample into 5 ml round bottom tube.
15. Wash the original 5 ml round bottom tube with an additional 500 µl cold PBS+2% FCS by pipetting up and down.
16. Filter supernatant into tube containing first supernatant of antibody stained enriched bone marrow sample.
17. Add 1 µl 7AAD to sample (see **Note 11**).
18. The sample is now ready to be sorted.
19. Specifications for each FACs sorter will be specific to the individual machine, please speak to your local flow cytometry expert for details.
20. Ensure to use index sorting mode on the sorting machine to allow retrospective review of the surface phenotype of each individual single cell sorted into a 96-well plate [37].
21. Each plate should be sealed with an adhesive film lid and clearly labelled. If cell quantities permit, cells prepared as above can be FACS sorted in bulk for droplet-based scRNA-Seq 10x Genomics (section 3.2) in parallel to index sorting individual cells for plate-based scRNA-Seq (section 3.3).

3.2 Droplet-based scRNA-Seq using the 10x Genomics platform

1. Droplet-based scRNA-Seq (see **Note 15**).

2. Collect sorted cells for this experiment into 1.5 ml Eppendorf tube(s) containing 200 µl cold PBS+2% FCS.
3. FACS sort cells in bulk (see **Note 16**).
4. Harvest cells by centrifuging at 300x g for 7 minutes at 4°C.
5. Remove supernatant, taking care not to touch the cell pellet (normally not visible).
6. Flick the tube to resuspend the cells.
7. Resuspend the cells in 1x PBS + 0.04% BSA solution (400 µg/ml) (see **Note 17**).
8. Process the samples following the 10x Genomics user guide.

3.3 Plate based scRNA-Seq using molecular crowding Smart-Seq2 (mcSmart-Seq2)

Note that the protocol is adapted from Smart-Seq2 [23] and mcSCRB-Seq [25] (see **Note 18**). It is essential that all steps are performed at 4°C, unless otherwise stated. Cool down the centrifuge before use (4 - 8°C). All quantities in master mixes are in µl (see **Note 19**). Use a thermal cycler with a pre-heated lid set to 105°C for all incubations. Care should be taken to ensure that the same batch(s) of reagents are used for the entirety of a standalone experiment to reduce any potential batch effects.

3.3.1 Single Cell Sorting

1. This step should be performed in pre-amplification conditions.
2. Prepare lysis buffer, with reagents maintained on ice during preparation.

	x 1 well		x 100 wells
RNase Inhibitor (SUPERase-In) 20 U/µl	0.115		11.5
10% Triton X-100	0.046		4.6
DEPC-treated or RNase-free H_20	2.139		213.9
	2.3		**230**

3. Aliquot 2.3 µl of lysis buffer to each well of a 96-well PCR plate (see **Notes 20 and 21**).
4. Cover plates with adhesive film lid.
5. Centrifuge at 700x g for 1 minute in a pre-cooled centrifuge.
6. Keep plates on ice or in refrigerator until required (see **Note 22**).
7. Isolate single cells into 2.3 µl of lysis buffer by FACS (see **Notes 23, 24 and 25**).
8. Cover the plates with an adhesive film lid.

9. Ensure that each plate is labelled clearly.

10. Vortex well (see **Note 26**).

11. Centrifuge the plates in a pre-cooled centrifuge at 700x g for 2 minutes.

12. Store at -80°C for up to 6 months (see **Note 27**).

3.3.2 Cell lysis and hybridisation

1. This step should be performed in pre-amplification conditions.

2. Prepare the annealing mixture, with reagents maintained on ice during preparation (see **Note 28**):

	x 1 well		x 100 wells
ERCC (dilution 1:300,000)*	0.1		10
Oligo-dT (100 µM)	0.02		2
dNTP (10 mM)	1		100
DEPC-treated or RNase-free H_20	0.88		88
	2		**200**

* Dilution of ERCCs is batch specific; start with 1:300,000 (see **Note 2**).

3. Add 2 µl of annealing mix per well (Vf = 4.3 µl) (see Notes **29 and 30**).

4. Cover the plates with a new adhesive film lid.

5. Centrifuge at 700x g briefly (ensure that all the reagents are at the bottom of the well).

6. Incubate the samples at 72°C for 3 minutes and immediately place the samples on ice.

7. Centrifuge at 700x g briefly.

3.3.3 Reverse transcription

1. This step should be performed in pre-amplification conditions.

2. Prepare the reverse transcription mix, with reagents maintained on ice during preparation:

	x 1 well		x 100 wells
Maxima H Minus (200 U/ µl)	0.1		10
RNase inhibitor (20 U/ µl)	0.25		25
5x Maxima RT buffer	2		200
TSO (100 µM)	0.2		20
PEG 8000 (40% v/v)	1.875		187.5
DEPC-treated or RNase-free H_20	1.275		127.5
	5.7		**570**

3. Remove and discard the cover

4. Add 5.7 µl of reverse transcription mix per well (Vf = 10 µl) (see **Note 30**).

5. Cover the plates with an adhesive film lid.

6. Centrifuge at 700x g briefly (ensure that all the reagents are at the bottom of the well).

7. Run in the thermocycler with preheated lid:

Step	Temperature (°C)	Time	Cycle
RT and template switching	42	90 mins	1
Enzyme inactivation	70	15 mins	1
Storage	4	Hold	1

10. Centrifuge at 700x g for 1 minute.

Samples can be frozen at this stage, although it is preferable to continue to PCR stage.

3.3.4 PCR preamplification

1. This step should be performed in pre-amplification conditions.

2. If samples were frozen, defrost on ice and centrifuge at 700x g for 1 minute prior to PCR pre-amplification

3. Prepare the PCR mix, with reagents maintained on ice during preparation:

	x 1 well		x 100 wells
Terra PCR Direct Polymerase (1.25 U/µl)	1		100
2x Terra PCR Direct Buffer	25		2500
IS PCR primer (10 µM)	1		100
PCR grade dH_2O	13		1300
	40		**4000**

For preparation of IS PCR primer see **Note 31**.

4. Remove and discard the adhesive film lid.

5. Add 40 µl PCR mix per well (Vf = 50 µl) (see **Note 30**).

6. Cover the plates with a new adhesive film lid.

7. Centrifuge at 700x g briefly.

8. Perform the PCR in a thermocycler (with preheated lid) following the program:

Step	Temperature (°C)	Time	Cycle(s)
Denature	98	3 min	1
Denature	98	15 secs	19
Anneal	65	30 secs	

Extend	68	4 mins	
Extend	72	10 mins	1
Storage	4	hold	1

Number of PCR cycles (see **Note 32**).

9. Centrifuge at 700x g for 1 minute.

PCR products can be stored at -20°C for 6 months or -80°C for longer.

3.3.5 PCR purification

1. This step should be performed in post-amplification conditions.
2. Pipetting of PCR purification steps (see **Note 33**).
3. Equilibrate AMPure XP beads to room temperature for at least 15 minutes.
4. If samples were frozen, defrost on ice and centrifuge at 700x g for 1 minute prior to PCR purification.
5. Vortex room temperature equilibrated AMPure XP beads until all beads are resuspended (approximately 30 seconds).
6. Remove and discard the adhesive film lid.
7. Add 29 µl of AMPure XP beads to each sample (for 0.6x clean-up) (see **Note 34**).
8. Mix by pipetting up and down 10 times or until the solution appears homogeneous.
9. Incubate the mixture for 8 minutes at room temperature to allow the DNA to bind to the beads.
10. Place the 96-well plate on the magnetic stand for 5 minutes (check that the solution is clear and that all the beads are against the wall of the well where the tube is in contact with the magnet).
11. Carefully remove the supernatant without disturbing the beads.
12. Wash the beads with 200 µl of freshly prepared 80 % ethanol (vol/vol) (see **Note 4** and **Note 35**).
13. Incubate the samples for 30 seconds on the magnetic stand.
14. Remove the ethanol.
15. Repeat wash steps (12 to 14).
16. Leave the plate at room temperature for 5 minutes to remove any trace of ethanol and allow the beads to dry.
17. Avoid over drying of the beads as this makes resuspension difficult and leads to loss of material.
18. Add 26 µl of elution buffer (EB) and pipette up and down ten times to resuspend the beads (see **Note 36**).

19. Incubate the 96-well plate off the magnet at room temperature for 2 minutes.
20. Place the 96-well plate on the magnet and leave it for 2 minutes at room temperature (see **Note 37**).
21. Transfer 25 µl of the supernatant without disturbing the beads to a fresh 96-well plate (see **Notes 38 and 39**).
22. Cover the plates with a new adhesive film lid.
23. Samples can be placed at -20°C for longer term storage or 4°C overnight.

3.3.6 Quality check the cDNA library

1. This step should be performed in post-amplification conditions.
2. If samples were frozen, ensure that they are fully defrosted and then centrifuge at 700x g for 1 minute prior quality check.
3. Check the size distribution of the amplified cDNA library on an Agilent Bioanalyzer high-sensitivity DNA chip (see **Note 40**). The library should be free of products smaller than 500 base pairs (bp) (primer dimers etc) and whilst will have a distribution of fragment sizes, should show a peak around 1.5 – 2 kb (Figure 3).

3.3.7 cDNA Quantification

1. This step should be performed in post-amplification conditions.
2. Use Quant-iT™ PicoGreen™ dsDNA Assay Kit (see **Note 41**).
3. Prepare “sample” plate – label a 96-well plate and add 28 µl of 1 x TE to each well.
4. To each well of the “sample” plate add 2 µl of the purified cDNA library (see **Note 42**).
5. Seal plate with adhesive film lid.
6. Prepare standard curve.
7. Dilute Lamdba DNA stock solution in 1 x TE to obtain 2000 pg/µl working solution (100 µl required per quantification plate).
8. Add 30 µl of the 2000 pg/µl working stock of Lamdba DNA solution to Std 1 and 2 of the standard curve (no 1 x TE will be added to Std 1) (see **Note 43**).
9. Prepare “standards” plate – label a 96-well plate and prepare serial dilutions of the Lamdba DNA working stock using 1 x TE as indicated in Table 4.
10. At each dilution step mix well by pipetting up and down 10 times.
11. Prepare Quant-iT™ PicoGreen™ working solution (see **Note 44**).

12. For one quantification plate add 31.7 µl of PicoGreen™ stock to 6304.3 µl of 1 x TE.
13. Pipette 30 µl of PicoGreen™ working solution into each well of the standard curve plate and the sample plate (see **Note 45**).
14. Seal all 96-well plates with adhesive film lids.
15. Vortex to ensure mixing and then centrifuge the plates at 700x g for 1 minute.
16. Transfer 25 µl of all samples and standards into a 384-well (PS, Flat-bottom, Fluotrac, medium binding, black) plate in duplicate as shown in Figure 4.
17. Cover the plates with an adhesive film lid.
18. Incubate the 384-well plate for 2-5 minutes at room temperature and protect from light.
19. Centrifuge the plate at 700x g for 1 minute.
20. Measure the fluorescence intensity in a 384-well fluorometer (excitation ~480 nm, emission ~520 nm).
21. Use 100-150 pg of DNA per sample for library preparation (see **Note 46**).

3.3.8 Library preparation

1. This step should be performed in post-amplification conditions.
2. Library preparation for multiple plates (see **Note 47**).
3. Equilibrate Tagment DNA Buffer and NT Buffer to room temperature.
4. Prepare the Tagmentation mix, with the Amplicon Tagment Mix maintained on ice during preparation:

	x 1 well		x 106 wells
Tagment DNA buffer	2.5		265
Amplicon Tagment Mix	1.25		132.5
	3.75		**397.5**

5. Pipette master mix up and down gently to mix all components.
6. Aliquot 49 µl of Tagmentation mix into each tube of an 8-strip PCR tube.
7. Take a clean 96-well plate.
8. Add 3.75 µl of Tagmentation mix per well into a 96-well plate using an 8-channel pipette.
9. Add 1.25 µl of sample (Vf= 5 µl) (see **Note 48**).
10. Seal the plate with an adhesive film lid and centrifuge at 700x g briefly.

11. Run the following program in a thermocycler:

Step	Temperature (°C)	Time	Cycle
Tagmentation	55	10 mins	1
Storage	10	Hold	1

12. Whilst tagmentation is taking place, aliquot NT buffer into an 8- strip PCR tube (16.5 µl per tube).

13. Defrost the Indexes (see **Note 49**), vortex and centrifuge briefly.

14. Place the index tubes into the Index Plate Fixture, taking care to place them in the correct order and location.

15. Remove and discard the adhesive film lid.

16. Using a multichannel pipette, add 1.25 µl of NT buffer per well as soon as sample reaches 10°C to neutralise the samples (Vf= 6.25 µl).

17. Seal the plate with an adhesive film lid and briefly centrifuge the plate at 700x g.

18. Place the plate onto the Index Plate Fixture

19. Remove and discard the adhesive film lid.

20. Add 1.25 µl of Index Primer 1 (N701-N707, N710-N712, N714, N715) to the corresponding well using a 12-channel pipette (Vf= 7.5 µl) (see **Note 50**) see Figure 5 for example layout of Indexes.

21. Add 1.25 µl of Index Primer 2 (S502, S503, S505-S508, S510, S511) to the corresponding well using an 8-channel pipette (Vf= 8.75 µl).

22. Aliquot 49.5 µl of Nextera PCR Master Mix (NPM) into an 8-strip PCR tube.

23. Add 3.75 µl of NPM to each well using an 8-channel pipette (Vf= 12.5 µl).

24. Seal the plate with an adhesive film lid and centrifuge at 700x g briefly.

25. Perform the PCR in a thermocycler (with preheated lid) following the program:

Step	Temperature (°C)	Time	Cycle(s)
Extend	72	3 mins	1
Denature	95	30 secs	1
Denature	95	10 secs	12
Anneal	55	30 secs	
Extend	72	60 secs	
Extend	72	5 mins	1

Storage	10	Hold	1

26. Centrifuge at 700x g for 1 minute and proceed with cleaning.

3.3.9 Library pooling and clean up

1. Equilibrate AMPure XP beads to room temperature for at least 15 minutes.
2. Samples will be cleaned up using a double-sided size selection (0.5x and 0.7x) (see **Note 51**).
3. This step should be performed in post-amplification conditions.
4. Vortex AMPure XP beads until all beads are resuspended (approximately 30 seconds).
5. At this step, the library of each cell is specifically labelled and can be pooled. For this, remove 2 µl from each sample. The final library should be in a 1.5 ml low bind tube (see **Note 52**).
6. Measure the final volume of the pooled sample (should be ~192 µl).
7. Add the required amount of AMPure XP beads to the sample (0.5x) (~96 µl).
8. Mix by pipetting up and down 5 - 10 times or until the solution appears homogeneous.
9. Incubate for 5 - 8 minutes at room temperature to allow the DNA to bind to the beads.
10. Place the 1.5 ml low bind tube on the magnetic stand for 2 minutes.
11. Do not discard supernatants.
12. Carefully transfer the supernatant to a fresh 1.5 ml low bind tube without disturbing the beads.
13. Add AMPure XP beads to the sample to obtain the desired ratio (0.7x) (~38.4 µl).
14. Mix by pipetting up and down 5 - 10 times or until the solution appears homogeneous.
15. Incubate for 5 - 8 minutes at room temperature to allow the DNA to bind to the beads.
16. Place the 1.5 ml low bind tube on the magnetic stand for 2 minutes.
17. Discard the supernatant without disturbing the beads.
18. Add 1 ml of freshly prepared 80 % ethanol (vol/vol) to the bead pellet.
19. Incubate for 30 seconds on the magnetic stand.
20. Remove the ethanol.
21. Repeat wash steps (18 to 20).
22. Leave the 1.5 ml low bind tube on the magnetic stand at room temperature for 5 - 7 minutes to remove any trace of ethanol but avoid over drying the beads as this makes resuspension difficult (see **Note 37**).

23. Resuspend the beads by adding 37.5 µl of EB (Qiagen) and mix 10 times or until the solution appears homogenous.
24. Incubate off the magnet for 2 minutes at room temperature.
25. Place the 1.5 ml low bind tube on the magnet and incubate for 2 minutes or until the solution appears clear.
26. Transfer 35 µl of supernatant to a fresh 1.5 ml low bind tube.
27. Samples can be stored at -20°C for up to 3 months.

3.3.10 Quality check and quantification of the indexed library

1. This step should be performed in post-amplification conditions.
2. If samples were frozen, ensure that they are fully defrosted, vortex briefly and centrifuge at 700x g briefly prior to quantification.
3. Check the size distribution of the final library on an Agilent Bioanalyzer high-sensitivity DNA chip (see **Note 40**). Due to the double-sided cleaning a discrete peak should be visible with an average size between 450 – 600 bp (Figure 3). Determine the average size of the library by defining a range of 42 bp to 3561 bp.
4. Quantify pooled indexed library using KAPA Library Quantification Kit (Roche) according to the manufacturer's instructions.
5. Dilute 1 µl of the sample (product from 3.3.9) with KAPA dilution buffer (see **Note 52**); run the dilutions in triplicate.
6. Prepare enough Master Mix for two DNA dilutions per sample (run in triplicate), Standard Curve and No Template Controls (NTC) (water and KAPA dilution buffer) (run in duplicate).
7. Prepare the Master Mix 1x (volumes shown here are for two dilutions of one sample):

	x 1 well		x 18 wells
SYBR Fast Master Mix + Primer	12		216
Water	4		72
	16		**288**

8. Mix and briefly centrifuge the Master Mix.
9. To a 96-well plate, add 4 µl of DNA Standards, diluted samples and NTC to appropriate wells.

10. Briefly centrifuge the plate at 700x g.

11. Dispense 16 µl of the Master Mix into each well and pipette up and down several times. Fresh pipette tips must be used for each well to prevent well-to-well contamination.

12. Seal the plate with optical caps.

13. Briefly centrifuge the plate at 700x g.

14. Transfer to qPCR machine.

15. Run the following program on the qPCR machine (see **Note 54**):

<table>
<tr><th>Step</th><th>Temperature (°C)</th><th>Time</th><th>Cycle(s)</th></tr>
<tr><td>Initial denaturation</td><td>95</td><td>5 mins</td><td>1</td></tr>
<tr><td>Denaturation</td><td>95</td><td>30 secs</td><td rowspan="2">35</td></tr>
<tr><td>Annealing/Extension/Data acquisition</td><td>60</td><td>45 secs</td></tr>
<tr><td>Dissociation curve analysis</td><td colspan="3"></td></tr>
</table>

16. Follow instructions in the manufacturer's protocol for data analysis.

17. Use the average size determined in step 2 to calculate the final concentration.

3.3.11 Pooling of multiple samples for next generation sequencing

1. This step should be performed in post-amplification conditions.

2. If multiple 96-well plates (already pooled in section 3.3.9 Library pooling and clean up) are to be pooled and sequenced together, care must be taken to ensure that each pooled 96-well plate is evenly represented.

3. Distribute the number of moles of each library to be added to the sequencing pool according to the desired ratios, taking into consideration the number of cells in each pool. In that way, if 4 pooled libraries, each one containing 96 cells are to be pooled together, add equimolar quantities. 460 femtomoles in a volume of 20 µl corresponds to a final concentration of 23 nM. Typically, we would pool ~115 femtomoles of each library, which assuming a similar concentration of each single-cell library corresponds to ~1.2 femtomoles of each single-cell library.

4. Using these guidelines, the ratios can be adjusted to the number of cells, availability of material and desired ratio of sequencing.

3.3.12 Sequencing

1. We would routinely perform single end 50bp sequencing on scRNA-Seq libraries which reduces the expenses of sequencing without compromising the results.
2. The amount of sequencing required will depend on the number of single cells which were processed and of the desired depth of sequencing.
3. A standard lane of Illumina HiSeq 4000 would render approximately 400 Million reads. We find that around 0.5 million mappable reads per cell should be sufficient and we typically obtain between 50-60% mappability with this protocol.
4. We typically sequence 4 plates of 96 single cells (384 cells in total) in one lane of HiSeq 4000.

4. Notes

1. The dilutions for individual antibodies can be found in Table 2. Each dilution has been carefully titrated within our laboratory. It is important to remember that the precise optimal antibody concentration may vary depending on the supplier, the product lot and the experimental setting. We would recommend that antibody panels should be optimized by the end user.

2. We use optical adhesive film lids from Starlab, the lids do not need to be optical, but it is important to test the stickiness of the lids. The lids need to be reasonably easy to remove, otherwise removal of the lid can lead to the splashing of reagents and mixing of wells. But the lids also need to be able to withhold storage at -80°C and the extremes of the PCR conditions throughout the protocol. We recommend testing lids before proceeding.

3. Make dilutions of ERCC RNA spike-in mix in water with RNase inhibitor (1 U/µl). 1:300,000 dilution. Make a serial dilution. First prepare 1:10 dilution (this can be stored at -80°C). Then make a dilution 1:100 (1 µl + 99 µl of water + RNase inhibitor) and a further dilution 1:300 (4 µl + 1196 µl of water + RNase inhibitor) to obtain a final dilution 1:300,000. Make aliquots and store at -80°C – these aliquots can be used for multiple plates of the same experiment but should not be stored long term.

4. 80% Ethanol (vol/vol) needs to be made fresh every time, as ethanol absorbs moisture and therefore this will lead to changes in the final concentration if stored.

5. Bones can also be flushed using a 23G needle, but we have found that the number of cells harvested from the bone marrow is more reproducible when using a pestle and mortar. Also, the use of needles is an

additional biohazard to consider. If bones are crushed then the inclusion of a CD45 antibody is necessary within the stem cell antibody staining panel to ensure that all cells which are sorted are haematopoietic and not contaminating stromal cells.

6. Any 50 µM filter can be used. We prefer Partec filters as they fit snuggly into a 15 ml centrifuge tube and the nylon mesh inside the filter is at an angle to optimize recovery of cells.

7. Do not leave red cell lysis step longer than 15 min in total otherwise all granulocytes can be removed from the sample. Whilst red cell lysis is taking place, begin to label the tubes for the antibody single stains and prepare the antibody master mix – see Tables 2 and 3.

8. We do not include fluorescence minus one (FMO) controls in this protocol as we no longer routinely run them for these experiments. If establishing this protocol for the first time, FMO controls should be included in the control staining panel. FMOs are particularly important when setting up multicolor FACs panels, as they will facilitate the identification of a positive vs negative populations and will help to determine where the gates should be set.

9. When preparing single stains place the un-depleted bone marrow cells onto ice and begin with the lineage depletion step of the protocol (step 3.1.3) and then return to add control antibodies whilst the cells are incubating with the Mouse Hematopoietic Progenitor Isolation Cocktail.

10. For the All stain control and actual samples which will be sorted, it is better practice to prepare a master mix; this means that there is less chance that an antibody will be missed from the panel and the pipetting is generally more accurate as the volume to be pipetted is larger. Details of the antibody master mix are in Table 3.

11. The viability stain should only be added to the controls and samples just before proceeding to the flow cytometry facility as the signal can increase over time.

12. The lineage depletion performed in this protocol is very mild, this was undertaken to ensure that some of the more mature lineages which still expressed the markers used in the antibody staining panel would be included in the final analysis. If different populations are required we would recommend following the manufacturers' instructions for the lineage depletion as this will greatly save on sorting time.

13. At this stage the cells should be transferred into a tube suitable for the available flow cytometry sorter. In our case we use 5 ml round bottom polypropylene tube.

14. The master mix volume is 500 µl, this will allow for a single all stain control (100 µl), an individual sample (300 µl) and 100 µl excess. The additional/excess volume can be altered. The volume in which the sample is stained can also be adjusted to a larger volume, but we would not recommend staining the whole material obtained from one mouse in less than 300 µl.

15. We have always used the 10x Genomics platform to be able to investigate larger numbers of cells to be able to characterise the entire haematopoietic compartment of the mouse bone marrow. The antibody panel we use contains multiple additional antibodies which are used to be able to ensure that the different populations are present and at expected ratios within the bone marrow. We have always used the 3' reagents (referred to as single cell gene expression) from 10x Genomics but 5' reagents are also available (single cell immune profiling).

16. The number of cells required will depend on the population which has been sorted. If samples are precious, we would sort an exact number of cells and rely on the number specified by the sorter. If cells are plentiful, a higher number of cells can be sorted and counted after being resuspended in 1x PBS + 0.04% BSA solution (400 µg/ml). We find that bone marrow samples do not require filtering before being used in the 10x Genomics protocol, as after sorting they tend to be a homogenous single cell suspension. There are considerations to deciding on the cell number to use in the 10x Genomics protocol. As the number of cells increases, the number of droplets that will contain doublets will also increase. For many of our experiments characterising the Lineage negative c-kit+ compartment of the mouse bone marrow, we have always loaded ~16,000 cells which will produce an estimated doublet rate of ~ 8%. For more details, we recommend reading the 10x Genomics protocols/user manuals.

17. The volume in which to resuspend the cells for the 10x Genomics protocol will depend of the desired concentration of the cells. This will be determined by the total number of cells obtained from the sorting steps and by the desired number of cells to be used in the droplet protocol. Please refer to the 10x Genomics manual. Once the concentration has been chosen, we normally resuspend the cells in half of the required volume. We then count the cells in an improved Neubauer counting chamber and adjust the final volume to achieve the

desired concentration. For experiments using Chromium Next GEM Single Cell 3’ Reagent Kits v3.1, we would resuspend the cells at a concentration of ~370 cells/µl.

18. mcSmart-Seq2 experiments should be performed in RNase, RNA and DNA-free areas to minimise contamination. If possible, pre- and post-amplification work should be carried out in different laboratories. These experiments rely on the amplification of the RNA from single cells, so tiny amounts of contaminating material could drastically affect the output of the experiments. Be extremely careful in the handling of the samples if human material is processed to avoid contamination introduced by material from the operator. If separate laboratory space is not possible then the use of a UV PCR cabinet and separate equipment (particularly pipettes) is highly advisable to minimise the risk of contamination. If using UV PCR hood, all equipment should be sterilised by exposing to UV light before and after use.

19. All master mixes are enough for a 96-well plate, including an excess enough for 100 wells (~4% excess).

20. Aliquoting of the lysis buffer can be done using a multichannel pipette. We would normally aliquot the lysis buffer into 8-strip PCR tubes on a chilled metal block and then use a multi-channel pipette to aliquot the lysis buffer into all the wells of the 96-well plate. Care must be taken to ensure that each tip contains 2.3 µl, this should be verified visually for each pipetting step. The same pipette tips can be used for the entire 96-well plate as there are no cells present at this time.

21. The type of 96-well PCR plate will depend on the type of PCR machine which is available and the sorting options. We favour using non-skirted PCR plates, but this required our sorting facility to create a holder which could fit the 96-well plate in place whilst sorting [37]. Fully skirted PCR plates can generally be utilised in the FACS sorters as they have the same footprint as a 6-well culture plate.

22. Lysis plates should be kept cold until required, this can be either on ice or in the refrigerator. We do not recommend preparing the lysis plates the day before the experiment and storing in the refrigerator overnight as considerable evaporation occurs. We have found that it is very difficult to recuperate the volume back into the wells. It is much preferable to prepare the lysis plates fresh on the day of the experiment. The lysis buffer can be prepared in advance and stored at 4°C for 6 months or -20°C.

23. Use index sorting when possible, even if only one or two parameters are using in sorting as this will keep a record of the number of single cells that were sorted into wells. We would also recommend stringent settings

for the sorting procedure, it is preferable to have no cell in the well than two cells. It is very important to avoid re-filling the wells which are reported empty, as we have seen that event though no event may be recorded by the index sorter the scRNA-Seq may show data for this well [37].

24. Care should be taken when planning the layout of lysis plates. It is advisable to have self-contained experiments within each plate. Sorting different populations or conditions onto different plates can lead to batch effects, if two different conditions or populations are to be compared then they should be on the same plate. If multiple plates with similar content are to be used, it is also advisable to rotate the samples within the plate positions so that there are no plate effects when comparing across multiple plates (Figure 1).

25. We would also recommend that test plates are sorted for each sorting session. The test plates serve several purposes as they allow the investigator to: i) check that cells were successfully sorted on the day, ii) confirm that cells are of good quality, iii) test the quality of the batch of reagents to be used during the processing, and iv) optimise the number of PCR cycles to be used for the cDNA amplification (step 3.3.4). Each test plate normally comprises of single columns of single cells and one well of ten cells (Figure 2). A test should be sorted for each of the different cell types to be investigated within an experiment. The test plates are only processed up to step 3.3.6, once a successful cDNA trace can be seen the full 96-well plates can be processed. Several columns can be sorted on an individual 96-well plate (if using non-skirted plates) as these can be cut into separate columns and processed separately.

26. The vortexing of the sorted cells in the lysis buffer is controversial since the solution contains detergent which creates foam. We find that it does not seem to be an issue in such a small volume. The vortexing should help with the lysis of the cells, and specific protocols have shown it to be essential [38].

27. When freezing the lysis plates, we would recommend placing them onto a pre-cooled metal shelf within the -80°C freezer to accelerate the freezing process. Non-skirted 96-well plates tend to bend once stored at -80°C and if not properly sealed the adhesive lid can become unstuck. It is good practise that once frozen, the plates should be stored together in zip lock plastic bags to keep an experiment together, as individual plates can very easily be lost in the freezer. It is essential to avoid freeze-thawing of the plates as this will lead to degradation of the material.

28. We would normally prepare the master mixes for both the annealing and reverse transcription steps at the same time. This means that as soon as the cell lysis/annealing step has finished the reverse transcription mix can be added to the wells.

29. For the aliquoting of all reagents for reverse transcription and cDNA amplification in the protocol we suggest the use of a Multipette® (Eppendorf), as this saves the use of multiple pipette tips and repeated pipetting actions. The volume of retention within the combitips is also very small, which means that large excesses of reagents do not need to be used and the pipetting steps are more accurate.

30. Care should be taken when adding reagents to wells. Each well now contains a single cell and so touching inside of the wells could lead to cross-contamination. Reagents should be added to the wells dropwise to edge of well, touch very lightly if at all, capillary action should attract the drop to the side of the well without needing to physically need to touch the inside of the well. We do not vortex the plate after the addition of reagents.

31. The IS PCR primer should be prepared fresh. If multiple plates are going to be processed as a part of the same experiment then one dilution can be made and aliquoted, this should then be stored at -20ºC. Avoid freeze-thaw cycles.

32. The number of PCR cycles depends on the starting amount of RNA. Cells which are quiescent will contain less RNA than rapidly cycling cells, for example haematopoietic stem cells contain less RNA than more committed progenitor cells (i.e.: megakaryocyte–erythroid progenitors or granulocyte-monocyte progenitors). Typically, use 19 cycles for single haematopoietic cells. Try to maintain the PCR cycles at the minimum possible to minimise amplification bias and PCR duplicates. Not a lot of cDNA is required for library preparation. If multiple cell types are been compared within one experiment then the number of cycles of PCR should be fixed and remain constant for all cells from that experiment. This will allow bioinformatic comparison across the populations.

33. We would normally perform the PCR purification using a liquid handling robot. Whilst this is not essential, if many plates are being processed it ensures consistency between the plates and minimises pipetting. Care also must be taken to ensure that the beads do not over dry following the ethanol wash, as this can lead to a loss of material.

34. We have found that the ratio of AMPure XP beads:sample which works optimally for the PCR purification is 0.6x. We suggest to add 29 µl as there is normally a slight loss of volume from the wells, we find that on average

there is approximately 48 µl remaining in the wells. Care must be taken when pipetting the AMPure beads, as inaccuracy or droplets of beads on the side of tips will lead to a change in the ratio of beads to sample, and will alter the fragment sizes which are captured.

35. If manually washing an entire 96-well plate of AMPure XP beads, wash one column at a time to avoid over-drying of the beads which will reduce elution of the DNA.

36. When resuspending AMPure beads ensure that all the sample is at the bottom of the well and no drops remain on the walls of the tube.

37. Care must be taken when drying AMPure beads. This protocol recommends leaving the beads for 2 minutes, but time may vary with the temperature of the laboratory, the amount of beads and plasticware used. Upon the removal of ethanol, the beads will appear shiny, as they dry they will lose this shiny appearance and a crack will begin to appear. If multiple cracks begin to appear this is a sign that the beads have begun to over dry and then elution efficiency may be reduced.

38. When transferring the eluted PCR material to a new 96-well plate maintain the well location, or keep clear records of which wells have moved to new plate locations. This will be essential to be able to track the individual cells in the sequencing files and be able to combine the scRNA-Seq to the metadata of each individual cell (flow cytometry index data etc).

39. When removing eluted material a small volume of EB will be left behind; this is to ensure that no beads are transferred with the cleaned cDNA library.

40. The size of the amplified cDNA library can also be checked on an Agilent TapeStation High Sensitivity DNA tape. We find that the trace of the amplified cDNA material is more accurate on the Bioanalyzer as the material can be larger than 5000 bp. It is not essential to accurately assign the size of the material, but we recommend using the Bioanalyzer unless it is performed by an experienced user of this technique.

41. For cDNA quantification we recommend following the manufacturers' protocol and specifications of the analysers used to read the assay.

42. If multiple plates are being processed it is not feasible that all wells can be quantified. As a result we would recommend to quantify as many samples as possible across a cross-section of the 96-well plate (typically 40

which is the equivalent to 5 columns). This will also depend on the design of your experiment and the layout of the individual 96-well plates. To characterise the haematopoietic stem and progenitor compartments of the mouse bone marrow we tend to have multiple different cell types on one 96-well plate. Attention should be paid to ensuring that similar numbers of cells of all cell types present in the plate are included in the quantification process. Multiple quantification plates can be run, but this is dependent on the researcher and the time and cost that you wish to dedicate to this. It is however very important to quantify the samples accurately since it will have a strong impact in the efficiency and quality of the final library.

43. If multiple PicoGreen™ quantification plates are being set up at once, the standard curve preparation should be multiplied as necessary, these should all be made up in the same "standards" plate to ensure comparison across the plates. The standards should then be transferred to adjacent wells to allow the separate addition of PicoGreen™ working solution.

44. When preparing Quant-iT™ PicoGreen™ working solution use a plastic container rather than glass as reagents may absorb to glass surfaces and ensure to protect the working solution from light by covering with foil or placing it in the dark.

45. If multiple quantification plates are being analysed the standards should have been transferred to adjacent wells. Add the PicoGreen™ working solution to one standard curve at a time.

46. For library preparation of single cells, use within the range of 100 - 150 pg DNA per sample for tagmentation. The amount of DNA has been optimised for the amount of Tagmentase recommended in this protocol. The use of too much starting material will produce under-tagmented products that will eventually result in suboptimal material for sequencing. Prepare dilutions of DNA samples in EB buffer, the dilutions should be made fresh as low concentrations of DNA are not stable for extended periods of time and run the risk of molecules sticking to the plastic of the plates.

47. If preparing libraries for multiple plates, process one plate at a time. It is very difficult to control the time that the Tagmentase is incubated with the DNA if multiple plates are processed at once.

48. 100 to 150 pg of cDNA should be used as input for tagmentation. Dilution of cDNA plates in EB buffer is normally required so that concentration of the majority of samples is within range. Prepare dilutions of DNA

samples in EB buffer, the dilutions should be made fresh as low concentrations of DNA are not stable for extended periods of time and run the risk of molecules sticking to the plastic of the plates.

49. There are four index options available from Illumina at the time of writing. The use of the four individual kits allows the combining of four 96-well plates. This can be useful, if a smaller amount of sequencing is required as multiple plates can be pooled into one single lane of sequencing. Care has to be taken to ensure that indexes are not repeated and that the order of the indexes is maintained, as it will be the indexes which will allow the researcher to link back to any metadata that exists for the individual cells. When adding indexes to individual wells it is essential to change tips between each row and each column. We normally discard all the lids from the index tubes and replace them with new lids so as not to cause any cross contamination between the different index tubes.

50. The indexes given as an example in the method and Figure 5 are from the Nextera XT Index Kit v2 Set A (Illumina). Nextera XT Index Kit v2 Set B, C and D are also available.

51. We have found that a single side clean up using the AMPure beads can leave some larger un-tagmented fragments within the sample that lead to a reduced performance of the sample in sequencing. We find that the indicated double-sided size clean up works best. The AMPure beads bind DNA depending on the ratio of AMPure beads used [39] which is determined by the volume of the sample to be cleaned (e.g. volume of AMPure beads/volume of DNA sample). Care must be taken when performing a double-sided size selection, as the supernatant from the first addition of AMPure beads must not be discarded and is transferred to a new tube. In this step, the larger fragments (bound to the beads) will be discarded. During the second clean, the beads are kept and the smallest fragments are discarded in the supernatant. The second addition of AMPure beads is accumulative, and therefore in our protocol to clean a 100 µl reaction with ratios 0.5 and 0.7, 50 µl of beads will be added in the first instance followed by the addition of 20 µl of beads (50 µl + 20 µl/100 µl = 0.7 x).

52. To reduce the number of samples which need to be cleaned, prepare a pool of the individual indexed libraries. Assume at this stage that the concentration of each individual library is equal. If preferred, samples can be cleaned up individually or in small pools prior to pooling all libraries, quantifying each library individually (or small pool) and pooling as desired. As only 1/5 of the each single-cell library will be pooled, it is always possible to repeat the process in the future using different ratios of each library. We would normally use an 8-channel multichannel pipette and transfer 2 µl from each well into an 8-tube strip, using a new pipette tip for each well

to avoid cross-contamination. Once all 12 columns have been pooled into the 8-tube strip, use a p200 pipette to pool the 8-tubes into one 1.5 ml low bind tube.

53. When quantifying pooled indexed libraries, dilutions and Standard Curves used will be qPCR machine dependent. We typically prepare dilutions 1:200,000 and 1:2,000,000 in KAPA dilution buffer, but it may vary according to the expected concentration of the libraries. Due to the dynamic range of our qPCR machine we only use Standards 3 to 6 for the Standard Curve. It is essential that the used dilutions are contained within the range of the standard curve. It is important to run control wells on the quantification plate using the KAPA dilution buffer and water used to prepare the dilutions and master mixes, to ensure that there is no contamination within the reagents.

54. When quantifying the library, please see additional notes within the manufacturers' protocol for longer fragment lengths and details of specific protocols for individual qPCR machines.

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Figure Legends

Figure 1: Planning of lysis plates. If comparisons need to be drawn from different variables then the layout of the sorting/lysis plates must be taken into careful consideration. Sorting one variable onto a single plate can lead to batch effects. Care must be taken to ensure that all the comparable variables are distributed across all plates, and that the correct number of total cells are sorted per variable.

Figure 2: Test plate. A test plate is essential to ensure that cells have been successfully sorted, to allow the batches of reagents to be tested and to optimize the number of PCR cycles for the cDNA amplification. Several columns can be sorted on an individual 96-well plate (if using non-skirted plates) as these can be cut into separate columns.

Figure 3: Examples of Bioanalyzer electropherograms of cleaned libraries. A) A representative electropherogram from the Bioanalyzer of a cleaned cDNA library, showing typical size distribution (bp). The example shows a single-cell. B) A representative electropherogram from the Bioanalyzer of a clean indexed library, showing a peak of the double-side selected library at around 500 bp. The example shows a pool of 96-cells.

Figure 4: Layout of PicoGreen Quantification plate. A) Layout of the "sample dilution" plates. B) Location of each diluted sample and the standard curve within the 384-well PicoGreen quantification plate.

Figure 5: Layout of Index Combinations. The combinations of different indexes in each well of a 96-well plate. Shown in this Figure is the Nextera XT Index Kit v2 Set A.

Table 1.

Oligo	Sequence (5' – 3')	Concentration for stock (µM)	Resuspend in	Storage (°C)	Time in storage (months)
TSO	AAGCAGTGGTATCAACGCAGAGTACATrGrG+G	100	1 x TE	-80	6
Oligo-dT30VN	AAGCAGTGGTATCAACGCAGAGTAC(T30)VN	100	1 x TE	-80	6
ISPCR oligo	AAGCAGTGGTATCAACGCAGAGT	100	1 x TE	-20	6

Table 2.

Tube	Fluorophore	Antibody	Clone	Vol (µl)/100µl
Lineage	-	Mouse Hematopoietic Progenitor Isolation Cocktail*	-	1
EPCR	PE	EPCR	RMEPCR1560	0.3
CD48	APC	CD48	HM48-1	0.5
CD150	PE-Cy7	CD150	TC15-12F12.2	0.5
c-kit	APC-Cy7	c-kit	2B8	0.5
Sca 1	BV421	Sca 1	D7	1
CD45	FITC	CD45	30-F11	0.5
Viability	7AAD		-	0.1
			TOTAL	4.3

* Mouse Hematopoietic Progenitor Isolation Cocktail PN (19856C) part of the EasySep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit (Stemcell Technologies).

Table 3.

Fluorophore	Antibody	Clone	Vol (µl)/100 µl	Master mix (500 µl) (see Note 13)
-	Mouse Hematopoietic Progenitor Isolation Cocktail*	-	1	5
PE	EPCR	RMEPCR1560	0.3	1.5
APC	CD48	HM48-1	0.5	2.5
PE-Cy7	CD150	TC15-12F12.2	0.5	2.5
APC-Cy7	c-kit	2B8	0.5	2.5
BV421	Sca 1	D7	1	5
FITC	CD45	30-F11	0.5	2.5
7AAD		-	0.1	
		TOTAL	4.3	

* Mouse Hematopoietic Progenitor Isolation Cocktail PN (19856C) part of the EasySep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit (Stemcell Technologies).

Table 4.

	Well	Concentration (pg/µl)	Volume of 1 x TE (µl)	Volume of diluent (µl)
Std 1	A1	2000	-	-
Std 2	B1	1000	30	30
Std 3	C1	500	30	30
Std 4	D1	250	30	30
Std 5	E1	125	30	30
Std 6	F1	62.5	30	30
Std 7	G1	31.25	30	30
Std 8	H1	15.625	30	30
Std 9	A2	7.813	30	30
Std 10	B2	3.906	30	30*
Blank	C2	-	30	-
Blank	D2	-	30	-
Blank	E2	-	30	-
Blank	F2	-	30	-
Blank	G2	-	30	-
Blank	H2	-	30	-

* remove and discard 30 µl from this well after mixing.