

# A whole-tissue RNA-seq toolkit for organism-wide studies of gene expression with PME-seq

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**The immune system operates at the scale of the whole organism in mammals. We currently lack experimental approaches to systematically track and study organism-wide molecular processes in mice. Here we describe an integrated toolkit for measuring gene expression in whole tissues, 3-prime mRNA extension sequencing, that is applicable to most mouse organs and any mouse model of interest. Further, the methods of RNA-seq described in this protocol are broadly applicable to other sample types beyond whole organs, such as tissue samples or isolated cell populations. We report procedures to collect, store and lyse a dozen organ types using conditions compatible with the extraction of high-quality RNA. In addition, we detail protocols to perform high-throughput and low-cost RNA extraction and sequencing, as well as downstream data analysis. The protocol takes 5 d to process 384 mouse organs from collecting tissues to obtaining raw sequencing data, with additional time required for data analysis and mining. The protocol is accessible to individuals with basic skills in (i) mouse perfusion and dissection for sample collection and (ii) computation using Unix and R for data analysis. Overall, the methods presented here fill a gap in our toolbox for studying organism-wide processes in immunology and physiology.**

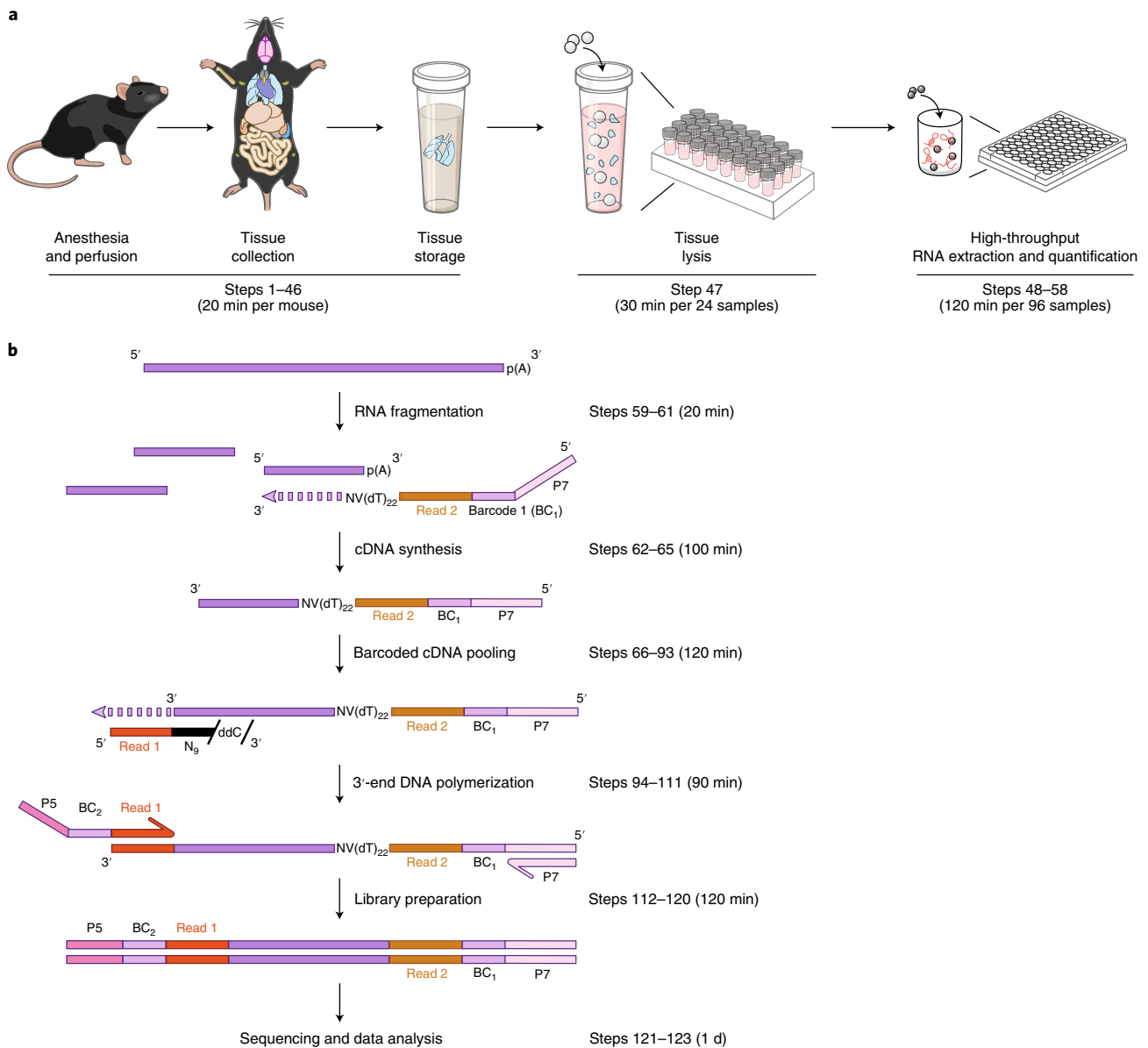
## Introduction

Immune cells are present in every organ of the body, and every cell in the body carries immunological functions. To understand the rules of interdependency between immune cells and processes across multiple organs, it is crucial to study immune responses at the scale of the entire organism. However, we lack experimental approaches to systematically track the organism-wide processes that take place during immunological or other physiological responses in mammals. We recently reported a suite of methods for the quantification of gene expression across most mouse organs (Fig. 1). We demonstrated its use for the study of the molecular and cellular communication conduits that take place between organs during immune responses to vaccination and infection<sup>1,2</sup>.

### Development of the protocol

Our protocol collects whole organs from mice immediately after transcatheter perfusion with saline to remove most blood cells from the body's vasculature. To maximize RNA quality during downstream processing, we optimized procedures for the collection, cleaning and storage in RNA-preserving solution of 14 organ types: bone marrow, brain, colon, female reproductive tract (FRT), heart, kidney, liver, lung, lymph nodes (e.g., brachial, inguinal, mediastinal, mesenteric and popliteal), peripheral blood mononuclear cells (PBMCs), skin, small intestine, spleen and thymus. Given that each tissue type exhibits distinct requirements for lysis due to variations in size and composition, we optimized the lysis conditions for each organ type, including lysis buffer amounts and automated dissociation vessels and settings of shaking speed and time, to allow for the recovery of high-quality total RNA. Whole organs are lysed in appropriate amounts of Trizol buffer using a bead-beating instrument (PowerLyzer 24 Homogenizer) that can process up to 24 samples in parallel in a few minutes for small- to medium-sized organs, or a tissue dissociator (gentleMACS Octo Dissociator) that can process up to 8 samples in parallel for organs requiring a larger volume of Trizol lysis buffer due to their size and/or fat content (small intestine, liver and brain). Next, lysates are transferred to 96-well plates for high-throughput total RNA extraction: (i) chloroform is added to the Trizol lysates and

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**Fig. 1 | Schematic overview of the PME-seq workflow. a**, Tissue collection to RNA extraction. Mice are transcardially perfused, and tissues of interest are collected and stored in an RNA-preserving solution. Tissues are subsequently lysed using Trizol and a bead-beater instrument to process up to 24 samples in parallel (except for liver and small intestine, which require different instrumentation due to their large size). Total RNA is extracted from tissue lysates using a high-throughput, magnetic bead-based protocol. **b**, Highly multiplexed RNA-seq library preparation. Total RNA is heat-fragmented and barcoded during RT with oligo(dT) priming. cDNA samples are then pooled and polymerized in their 3' ends to add the Illumina Read 1 primer sequence. Finally, multiplexed libraries are enriched by PCR, which adds a second barcode, and the resulting dual-indexed libraries are sequenced.

centrifuged for phase separation, and (ii) total RNA is extracted from the aqueous phase using magnetic beads coated with silica-like chemistry and on-bead DNase I treatment to degrade the remaining genomic DNA from the mixture. This custom RNA extraction procedure is rapid and cost effective and allows for the processing of up to 96 samples in parallel either manually, using multichannel pipettes, or automatically, using a liquid handler to increase throughput and reproducibility. Lastly, we created an RNA-seq protocol for multiplexing up to 384 samples in parallel using two polymerase-based steps after reverse transcription (RT). This protocol does not require the use of ligation or transposition steps and offers a simple, fast and low-cost method for high-throughput RNA sequencing.

### Applications of the method

We have used the protocol presented here to study whole-body immunity in response to vaccinating and pathogenic strains of poxviruses<sup>1</sup>, but many other applications of our procedures can be readily envisioned. First, our method is applicable to the study of whole-organ gene expression in virtually any mouse model of interest, including models of vaccination, infection, cancer, allergy, auto-immunity, transplantation or aging, among others. In all of those mouse models, whole-organ expression profiling could be performed systematically for all organs or selectively for a subset of organs, depending on the biological question at hand. Furthermore, while we focused on changes in immune-related genes, other pathways or organismal processes leading to changes in mRNA levels in tissues could be studied.

Second, our protocol can be applied to mice that are naturally exposed to environmental pathogens<sup>3</sup> or mice from various genetic backgrounds, such as those in the Collaborative Cross<sup>4</sup>, to enable the systematic mapping of expression quantitative trait loci similarly to what has recently been demonstrated using dozens of tissue types from hundreds of human donors<sup>5</sup>. One advantage of using our protocol in mice is that whole organs can be analyzed for gene expression studies, avoiding the potential biases that can arise from sampling only pieces of a tissue, as is typically the case for work with human samples. In addition, our protocol could complement the comprehensive phenotyping efforts that have been deployed for the analysis of libraries of mouse knockouts, including mouse models of human Mendelian disorders<sup>6</sup>.

Third, the high-throughput procedures we developed for total RNA extraction and RNA-seq library preparation are broadly applicable to any type of organ or cell type in culture or isolated from tissues, as shown using mouse lymphocytes<sup>7,8</sup>. The all-purpose RNA extraction method detailed here is (i) applicable to low cell numbers (1,000 cells or potentially fewer with optimizations), (ii) readily amenable to automation using liquid handling (see Supplementary Methods), and (iii) compatible with RNA-seq, qPCR or any type of RNA-based measurement. When working with isolated cells as opposed to whole tissues, one can use an alternative lysis buffer, such as RLT buffer (Qiagen) supplemented with 2% (vol/vol) beta-mercaptoethanol and mix with silane-coated magnetic beads directly for RNA extraction from cell lysates without the need for prior phase separation. Further development of the method could lead to the simultaneous extraction of DNA, RNA and proteins from whole organs by processing the three phases obtained after centrifugation of Trizol/chloroform mixtures. Moreover, our RNA-seq procedure is applicable to the detection of both host and exogenous polyadenylated RNAs in whole organs or cells, such as virally infected tissues<sup>1</sup> or humanized mouse models containing mouse and human cells in various proportions across the body<sup>9</sup>.

### Comparison with other methods

We compare and contrast two key aspects of our protocols to currently used methods for: (i) the study of immune processes across multiple organs and (ii) the preparation of multiplexed RNA sequencing libraries.

There is currently no standard methodology for the study of immune processes at the scale of the whole organism. The integrated toolkit detailed in this protocol provides whole-organ mRNA measurements that are complementary to existing methods for the study of immune responses using imaging and cell-isolation approaches. First, our protocol can complement existing imaging modalities for (i) live animals<sup>10</sup>, (ii) whole-body sections<sup>11,12</sup> and (iii) optically cleared whole animals<sup>13–16</sup> by providing information on the transcriptome of tissues across the entire animal. These imaging methods require costly and complex instrumentation and are not currently amenable to measurements of many molecular entities (proteins and mRNAs) in parallel and at the scale of whole organs and animals. Second, it is extremely challenging, if not infeasible in a single experiment, to isolate multiple immune cell types from dozens of organs extracted from a given animal, let alone from groups of mice. For some applications, our protocol can circumvent the need for cell isolation while providing a dramatic increase in the ability of a single investigator to analyze all or select organs from cohorts of mice. Indeed, the isolation of immune or other cells from organs suffers from sampling errors and biases during tissue dissociation into single-cell suspensions<sup>17</sup>, which can be avoided when processing whole tissues. Of course, in many applications, it is not possible to use whole-tissue profiling instead of isolating cells, which depends on the hypothesis being tested and the expected signal-to-noise ratio that must be measured or estimated on a case-by-case basis. In our experience, transcriptional changes in cells that represent <1% of a tissue (e.g., subcapsular sinus macrophages in a lymph node) can be readily detected if the genes being regulated in that cell type are not widely

expressed by other cell types present in that tissue. In addition, signal amplification across multiple cell types in one tissue can help increase the sensitivity of the method. For example, we could readily detect the activity of a few hundred memory T cells producing interferon- $\gamma$  in whole lung or liver through the broad induction of interferon-stimulated genes<sup>1</sup>. In other cases, our protocol can also serve as a systematic screening method to pinpoint which tissues and cells are likely to play a role in models of disease, therapy or other conditions where a systemic component is hypothesized to play a role but has not been identified yet. For example, results from whole-organ expression studies can help direct in-depth follow-up studies using cell isolation-based readouts<sup>1</sup>.

We report two custom protocols for (i) high-throughput, bead-based RNA extraction and (ii) highly multiplexed RNA-seq library preparation (Fig. 1b). Our total RNA extraction method detailed here is broadly applicable to various types of cell or tissue lysates and is cheaper than commercially available column- or bead-based kits. Our multiplexed RNA-seq library preparation method combines a unique set of features compared to existing methods for bulk or single-cell measurements<sup>18–24</sup>, making this protocol an attractive option for any project requiring bulk RNA-seq analysis on whole tissues or cells. Our method has the following characteristics. (i) It starts with a low input of total RNA: ~1–100 ng, depending on the number of samples being pooled after RT. (ii) It does not require the direct capture of polyadenylated transcripts or the depletion of ribosomal RNAs from total RNA samples before RT. (iii) It allows for early multiplexing of hundreds of samples before library construction, which is only limited by the number of barcodes purchased. (iv) It relies on three simple steps: RT, 3'-end DNA polymerization using the Klenow fragment and PCR enrichment using a standard high-fidelity polymerase, which eliminates the need for ligation or tagmentation steps that can lead to biases<sup>25–28</sup> and increase in cost, respectively. (v) Lastly, our method is fast (8 h from total RNA to a sequencing-ready library), has a low cost and yields gene expression results similar to standard gene expression methods such as qPCR and microarrays.

### Experimental design

This protocol for whole-organ mRNA profiling can be completed in 5 d for 384 samples. Here, we provide a detailed overview of the design of the experiments and suggestions to adapt parts of the protocol to the needs of the experiment (Fig. 1).

### Tissue collection

The procedure starts with the collection of tissues (Steps 1–46) from mice treated as needed and using a group of untreated animals as controls. We recommend the use of three to four replicate animals per experimental group, which should be adjusted depending on the level of reproducibility of the desired effects in mice upon treatment. For time series analyses, we recommend treating animals at time intervals that allow for the collection of multiple groups on the same day. When processing all tissue types from each mouse, we routinely collect tissues from 24 mice per day, although this can vary greatly depending on the number and type of organs to be collected (e.g., small intestine and bone marrow require more processing time than other organs). Each tissue is placed in appropriate amounts of RNA-preserving solution (equivalent to commercially available products such as RNA-later) for storage and extraction of high-quality RNA. An important step is the quality of the transcardial perfusion, which can typically be evaluated visually through the change of coloration of organs, such as lung, liver and kidney, or quantified in tissues of particular interest using intravascular staining<sup>29,30</sup>. Depending on the question at hand, collection of tissues without perfusion can be done, but lysis conditions may need to be optimized on a per-tissue basis to account for the presence of blood in the sample. In addition, without perfusion, downstream RNA-seq measurements are likely to contain more reads mapping to hemoglobin genes. In its current form, the protocol does not lead to the detection of significant levels of reads mapping to hemoglobin transcripts, suggesting that our procedures robustly eliminate most, if not all, red blood cells from tissues, including bone marrow and PBMCs.

### Tissue lysis

Next, mouse organs are lysed in Trizol or an equivalent guanidium thiocyanate-based buffer using volumes and dissociation conditions that were optimized for each tissue type based on their size and composition (Step 47). We reasoned that lysing each tissue in its entirety before isolating RNA from a portion of the lysate would give a more representative picture of whole-tissue mRNA levels than cutting pieces of tissues before lysis. While we describe optimized conditions that rely on the

gentleMACS (Miltenyi Biotec) and PowerLyzer 24 Homogenizer (Qiagen) instruments for high-throughput processing, we obtained similar results using other tissue dissociation platforms such as the TissueLyzer II (Qiagen) or an equivalent bead beater (Retsch). Lysates are transferred to deep-well 96-well plates and should be arranged to keep all samples from a given tissue type on the same plate to avoid potential batch effects during downstream processing and analyses. Various tissue types can be processed on a single 96-well plate, although it is important to carefully group the tissue types on a single plate according to their chloroform volume requirements during RNA extraction. Importantly, as a backup in the event of sample loss during subsequent steps, a portion of each lysate should be stored at  $-80^{\circ}\text{C}$  in deep-well 96-well plates.

### RNA extraction

Tissue lysates are then processed for total RNA extraction from up to 96 samples in parallel (Steps 48–57). First, varying volumes of chloroform are added to the lysates depending on the tissue type being processed, and RNA is enriched through phase separation by centrifugation. Second, the aqueous phase is mixed with silane-coated magnetic beads that bind nucleic acids—RNA and remaining genomic DNA at this step—in the presence of ethanol and RLT Buffer (Qiagen). Third, RNA samples are treated with DNase I to remove remaining DNA and then purified and eluted in water. The concentration of each sample is determined using a fluorescence-based method, which can be done in 96- or 384-well plates (Quant-IT RiboGreen RNA Assay kit), although it can also be done on a spectrophotometer (e.g., NanoDrop). In addition, we randomly select 5–10% of the samples for RNA quality check using a TapeStation (Agilent Technologies) or equivalent (e.g., BioAnalyzer). RNA extraction can be performed manually or automatically using a liquid handling platform (see Supplementary Methods) that can accommodate magnetic bead handling. We provide all protocols and files to use this RNA extraction procedure on the Agilent Bravo platform, which allows the processing of 96 samples in parallel from extraction to quantification to normalization of total RNA.

### Multiplex RNA-seq library construction

The next stage of the procedure consists of preparing multiplexed RNA-seq libraries for Illumina sequencing (Steps 57–120) (Fig. 1b). We routinely start from 100 ng of total RNA from each sample, although this can be modified as needed: decreased when working with low numbers of isolated cells (as low as 1 ng worked well in our hands), or increased when profiling fewer tissues with greater sequencing depth. Modifying the input amounts of RNA may require changes in the heat fragmentation conditions, which can be optimized by varying the length of incubation and examining the resulting RNA fragmentation profiles from varying input amounts on a high-sensitivity RNA kit for TapeStation or Bioanalyzer (Agilent Technologies). The temperature and the composition of the buffer used for fragmentation also impact the length distribution of fragmented RNAs and may be optimized as needed for a specific application. In our experience, however, for most applications, modifying the length of the incubation period is sufficient to obtain a fragmentation profile (350–800 bp) that is compatible with Illumina sequencing. The cations present in the RT buffer used in this procedure are present at a concentration that leads to appropriate RNA fragmentation conditions. Using other RT kits or buffers is compatible with our procedure but will require optimization of the fragmentation conditions.

Fragmented RNAs are then reverse transcribed using a VN-anchored oligo(dT)<sub>22</sub> primer (where V = A, C or G and N = A, C, G or T) containing 5' to 3' sequences for the Illumina P7 adaptor, an 8-bp sample barcode (equivalent to the Illumina i7 barcode) and the Illumina Read 2 primer. The RT primer is added to the fragmentation mixture to favor annealing during cooling of the samples after fragmentation. Equal volumes of cDNA:RNA duplexes are pooled using commercially available binding columns, although magnetic beads (e.g., AMPure from Beckman Coulter) can also be used for this step. The volume of cDNA pooled is determined by the number of samples being processed and the experimental design. For a pool of 96 samples, we routinely use 5  $\mu\text{l}$  of cDNA per sample prepared with a starting amount of total RNA between 50 and 100 ng. The remaining cDNA samples can be stored at  $-80^{\circ}\text{C}$  for a few months in case another pooling scheme is needed to resequence samples of interest.

RNA-seq library construction is performed on pooled cDNA samples, typically from the same tissue type, although pooling different tissue types together can be done. The pooling scheme is flexible and should be adjusted according to the number of samples being analyzed. Remaining RNAs are degraded by alkaline hydrolysis, and resulting single-stranded (ss) cDNAs are polymerized in their 3' ends to add the sequence for the Illumina Read 1 primer. For 3'-end DNA polymerization of

ss cDNA, we use a random nonamer ( $N_9$ ) primer coupled with the sequence for the Illumina Read 1 primer and blocked at the 3' end using a dideoxycytidine (ddC), to avoid the synthesis of a second cDNA strand. DNA synthesis is done using the exo minus Klenow fragment to avoid the removal of the blocking base at the 3' end of the primer. This reaction is carried out in the presence of polyethylene glycol and a high amount of primers (100 pmol) to improve efficiency. Samples are then cleaned up using magnetic beads to remove remaining extension primers in the mixture and enriched by PCR, during which the Illumina P5 adaptor is added by itself or together with a second 8-bp barcode (analogous to the Illumina i5 barcode). Resulting libraries are gel-extracted and quantified, and multiple libraries can be pooled together as needed. For multiplexing based on the scale of the experiment, the user may acquire any desired number of RT primers carrying sample barcodes (e.g., 8, 16, 24, 96 or 384 barcodes), and, optionally, a set of forward primers for the final PCR step containing a second index between the sequences for Read 1 and P5. By using single or double indexing, the desired pooling scheme can be achieved to match any experimental design and desired sequencing depth while minimizing the cost of oligos.

### Sequencing and analysis

Last, RNA-seq libraries are sequenced using any Illumina platform depending on the number of reads needed. It is important to give due consideration to the sequencing depth (i.e., number of reads obtained per sample) that is desired for a specific application. We routinely obtain 3–5 million (M) raw reads per sample by sequencing pools of 80–120 samples using the NextSeq550 platform, which has proven to be sufficient to confidently identify changes in mRNA levels between treated and control samples. The sequencing depth can also be determined by (i) sequencing a few samples at high depth (e.g., >10 M reads per sample) and (ii) performing a down-sampling analysis to pinpoint what is the smallest number of reads that can be used while retaining the ability to discriminate known changes in mRNA levels between conditions. Alternatively, a low sequencing depth can be used during a screening phase (e.g.,  $\leq 1$  M reads per sample), and samples of interest can then be pooled and sequenced at higher depth. For data analysis, from initial run quality checks to read mapping and counting, and normalization and differential expression analysis, we provide an easy-to-use pipeline that draws from well-established, publicly available software and tools. The annotated scripts and files necessary to perform the analysis are available in the following repository: <https://github.com/chevrierlab/PME-seq>.

### Expertise needed to implement the protocol

The procedure requires a good knowledge and level of practice of mouse perfusion and dissection techniques for all organs to be analyzed, which is critical for the rapid isolation of the tissues to be analyzed. When processing all tissues from each animal, two to three experimenters should perform the procedure to minimize delays between the times of perfusion and tissue storage in RNA-preserving solution. Downstream molecular biology techniques used for tissue lysis, RNA extraction and RNA-seq library preparation can all be performed by a single experimenter using standard laboratory equipment. Sequencing is done using the standard Illumina workflow and can thus be done by any laboratory or institutional or commercial facility. A basic knowledge of Unix and R suffices to perform data analysis on a standard computing cluster, and commercially available solutions are also compatible with our data (e.g., CLCbio)<sup>7,8</sup>.

### Limitations

There are some limitations at various steps of the procedure, which should be taken into consideration for the experimental design. First, when collecting all possible tissues from each mouse (~20), the tissue collection phase of the procedure—including perfusion, dissection, post-collection cleanup and storage—is best performed when two or more experimenters work together for rapid processing. This is crucial when performing time series analysis that require the processing of groups of mice within short time intervals. Second, our procedure for whole-tissue RNA-seq provides information only on the 3' ends of polyadenylated transcripts, which precludes the analyses of splicing events or bacterial RNAs. However, our method allows for higher multiplexing capabilities than full-length approaches because of the fewer number of reads required per sample for standard gene expression analyses. In addition, our RNA-seq library construction protocol in its current form does not contain unique molecule identifiers<sup>31</sup>, although it is possible to simply add this capability to our protocol by using RT primers that contain, for example, a random decamer sequence ( $N_{10}$ ) in

addition to the 8-bp barcode sequence. Third, changes in gene expression identified through whole-tissue RNA-seq can rarely be attributed with high confidence to a specific cell type. One possibility to overcome this limitation is to analyze the data obtained with our procedure using algorithms for the deconvolution of gene expression profiles from tissues or cell mixtures<sup>32</sup>.

## Materials

### Biological materials

- Mice: all mice were adult C57BL/6J female mice from Jackson Laboratories (stock no. 000664). The strain, sex, age and weight of mice should be adjusted according to the experimental design **! CAUTION** Experiments involving mice should be performed in compliance with institutional and governmental guidelines. The procedures in this protocol were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the institutional animal care and use committee at the University of Chicago.

### Reagents

- 2,2,2-Tribromoethanol (Sigma-Aldrich, cat. no. T48402-25G) **! CAUTION** Use of 2,2,2-tribromoethanol as an anesthetic requires permission by institutional authorities. Alternative anesthetics such as ketamine and xylazine can also be used.
- 2-Methyl-2-butanol (Sigma-Aldrich, cat. no. 152463-250ML)
- Ethanol, 200 proof (any supplier)
- 1× PBS (Corning, cat. no. 21-040-cv)
- 0.5 M EDTA (BioExpress, cat. no. E522-100ML)
- FBS (Seradigm, cat. no. 1400-500)
- Sodium citrate (Sigma-Aldrich, cat. no. S1804-500G)
- Ammonium sulfate (Sigma-Aldrich, cat. no. A418-1KG)
- Sulfuric acid (Sigma-Aldrich, cat. no. 339741-100ML)
- Lymphocyte Separation Medium (Cellgro, cat. no. MT25-072-CV)
- RNaseaway (VWR, cat. no. EK-3353)
- Trizol RNA Extraction Reagent (Thermo Fisher Scientific, cat. no. 15-596-018)
- Chloroform (Fisher Scientific, cat. no. BP1145-1)
- Isopropanol (Sigma-Aldrich, cat. no. I9516-500ML)
- RLT buffer (Qiagen, cat. no. 79216) **▲ CRITICAL** Our protocol for PBMC lysis and bead-based RNA extraction is optimized with the RLT buffer from Qiagen.
- ACK lysing buffer (Lonza, cat. no. BW10548E)
- Anti-TER-119 Microbeads, mouse (Miltenyi Biotec, cat. no. 130-049-901)
- LS columns (Miltenyi Biotec, cat. no. 130-042-401)
- Dynabeads MyOne Silane (Thermo Fisher Scientific, cat. no. 37002D) **▲ CRITICAL** Our protocol for bead-based RNA extraction is optimized for the use of Dynabeads MyOne Silane beads. Alternative suppliers can probably be used but would require optimization.
- TURBO DNase (Thermo Fisher Scientific, cat. no. AM2239)
- Nuclease-free water (Thermo Fisher Scientific, cat. no. SH30538FS)
- Primers (order from IDT or alternative; see Reagent setup for details)
- PEG 50% (wt/vol) solution (Fisher Scientific, cat. no. AAJ61495AE)
- AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies, cat. no. 600105) **▲ CRITICAL** Our protocol for RNA fragmentation before cDNA synthesis is optimized for the RT buffer provided in this kit. Using alternative buffers is possible after optimization and testing.
- RiboLock RNase inhibitor (Thermo Scientific, cat. no. EO0381)
- Klenow Fragment (3′–5′ exo-) (New England Biolabs, cat. no. M0212S)
- Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63881)
- RNA Clean & Concentrator-5 (Zymo Research, cat. no. R1013)
- NEBuffer 2 (New England Biolabs, cat. no. B7002S)
- Q5 High Fidelity 2× Master Mix (New England Biolabs, cat. no. M0492S)
- MinElute Gel Extraction Kit (Qiagen, cat. no. 28604)
- E-gel EX Agarose gels, 2% (Thermo Fisher Scientific, cat. no. G401002)
- E-gel 1 Kb Plus DNA ladder (Thermo Fisher Scientific, cat. no. 10488090)
- High Sensitivity D5000 Reagents (Agilent Technologies, cat. no. 5067-5593)
- Qubit double-stranded (ds) DNA HS assay kit (Thermo Fisher Scientific, cat. no. Q32851)

### Equipment

- Miniplus evolution speed control module (Gilson, cat. no. F110701)
- MF1 pump head with 1 cartridge for 1 tubing (Gilson, cat. no. F110705)
- Tubing for MF1 pump heads, Isoversinic 1 MM ID (Gilson, cat. no. F1817743)
- Connector PVDF for 1/2 MM ID (Gilson, cat. no. F1179941)
- Vacutainer Safety-Lok Blood Collection Set (BD, cat. no. 367285)
- FiveEasy pH meter F-20 (Mettler Toledo, cat. no. 30266626)
- Hot plate stirrer (VWR, cat. no. 97042-642)
- Qubit 3.0 fluorimeter (Thermo Fisher Scientific, cat. no. Q32851)
- gentleMACS Octo Dissociator (Miltenyi Biotec, cat. no. 130-095-937)
- PowerLyzer 24 Homogenizer (Qiagen, cat. no. 13155)
- Thermocycler (e.g., Eppendorf Mastercycler X50)
- Benchtop centrifuge (e.g., Eppendorf 5920R)
- Repeat pipetter (e.g., Eppendorf Repeater E3)
- DynaMag-96 Side Magnet (Thermo Fisher Scientific, cat. no. 12331D)
- DynaMag-2 Magnet (Thermo Fisher Scientific, cat. no. 12321D)
- Magnetic separation block for deep-well 96-well plate (V&P Scientific, cat. no. VP771LAZM-1)
- Pipet-Lite Pipette Multi L12-1200XLS (Rainin, cat. no. 17014497)
- Pipet-Lite Pipette Multi L12-200XLS (Rainin, cat. no. 17013810)
- 8-channel adapter for vacuum aspirator (Corning, cat. no. 4931)
- QuadroMACS starting kit (LS) (Miltenyi Biotec, cat. no. 130-09-051)
- Magnetic stir bar (Fisher Scientific, cat. no. 14-513-61SIX)
- 1-l glass beaker (Fisher Scientific, cat. no. 02-555-25F)
- 1.5-ml microcentrifuge tubes (USA Scientific, cat. no. 615-5510)
- 5-ml tubes (VWR, cat. no. 89429-306)
- 15-ml tubes (Fisher Scientific, cat. no. 1495953A)
- 50-ml tubes (Fisher Scientific, cat. no. 1443222)
- Conical-bottom light-protection tubes, 50 ml (Greiner Bio-One, cat. no. 227283)
- Disposable Petri dishes (VWR, cat. no. 25384-342)
- Extra Fine Bonn Scissors (Fine Science Tools, cat. no. 14084-08)
- Fine Scissors-Sharp-Blunt (Fine Science Tools, cat. no. 14028-10)
- Dumont #7 Fine Forceps (Fine Science Tools, cat. no. 11274-20)
- Mini Hippocampal Tool (Fine Science Tools, cat. no. 10099-12)
- 26G needles (BD, cat. no. 305111)
- Plastic syringe, Luer Slip, 5 ml (Grainger, cat. no. 19G339)
- 1-ml tuberculin syringe with needle (BD, cat. no. 309623)
- Absorbent underpads (VWR, cat. no. 56617-016)
- 10-ml serological pipets (VWR, cat. no. 89130-888)
- 5-ml serological pipets (VWR, cat. no. 89130-896)
- Aspiration pipets (VWR, cat. no. 414004-265)
- 1.5-ml storage box (VWR, cat. no. 82021-114)
- 5-ml storage box (USA Scientific, cat. no. 9023-1615)
- Microplate Sealing Film (Axygen Scientific, PCRAS200)
- 25-ml Combitip (VWR, cat. no. 0030089472)
- gentleMACS M tubes (Miltenyi Biotec, cat. no. 130-096-335)
- 0.5-ml 96-well deep-well polypropylene plate (USA Scientific, cat. no. 1896-5000)
- 2.0-ml 96-well deep-well polypropylene plate (USA Scientific, cat. no. 1896-2000)
- Chimney 96-well PCR plates (USA Scientific, cat. no. 1402-9600)
- Skirted, LoBind 96-well PCR Plate (Eppendorf, cat. no. 0030129512)
- Skirted 96-well PCR Plate (VWR, cat. no. 82006-704)
- 2-ml reinforced tubes with screw caps and silicone O-rings (Omni International, cat. no. 19-649)
- 2.8-mm ceramic beads, bulk (Omni International, cat. no. 19-646)
- Illumina sequencer and matching sequencing kit (e.g., HiSeq, NextSeq and NovaSeq models)

### Software

- FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>)
- Bcbio-nextgen (<https://bcbio-nextgen.readthedocs.io/en/latest/index.html>)

- R (<https://www.r-project.org/>)
- EdgeR<sup>33</sup> (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>)
- Limma<sup>34</sup> (<http://bioconductor.org/packages/release/bioc/html/limma.html>)

### Reagent setup

#### 2,2,2-Tribromoethanol anesthetic solution preparation

Add 15.5 ml of 2-methyl-2-butanol directly to the bottle containing 25 g of 2,2,2-tribromoethanol. Keep the bottle on a rotator at room temperature (25 °C) for ~12 h until the tribromoethanol is fully dissolved, producing a 1.6-g/ml stock solution. Prepare a working solution by diluting 500 µl of stock solution in 39.5 ml of 1× PBS to obtain a 40-mg/ml solution. Store at 4 °C protected from light for up to ~10 d. The stock solution is stable at room temperature for a year protected from light.

#### MACS buffer

Prepare a solution of 1× PBS containing 0.5% (vol/vol) FBS and 2 mM EDTA by mixing 500 ml of 1× PBS, 2.5 ml of heat-inactivated FBS and 2 ml of EDTA (0.5 M). Filter through a 0.22-µm filter and store it at 4 °C for ≤2 weeks.

#### Tubes for tissue lysis using the PowerLyzer 24 Homogenizer

Prepare 2-ml reinforced tubes with screw caps by adding six 2.8-mm ceramic beads per tube. Trizol buffer should be added to the tubes shortly before tissue lysis.

#### RNA-preserving solution

Dissolve 29.4 g of sodium citrate into 100 ml of ultrapure water on a stir plate to obtain a 1 M solution, which takes ~10 min (heat to 30 °C while stirring if necessary). Dissolve 350 g of ammonium sulfate in 467.5 ml of ultrapure water on a stir plate. Add 12.5 ml of 1 M sodium citrate and 20 ml of 0.5 M EDTA to the ammonium sulfate solution and adjust the pH to 5.2 by adding H<sub>2</sub>SO<sub>4</sub> (~500 µl per 500-ml solution) and using a pH meter. Store this solution at room temperature for ≤6 months.

#### Oligonucleotides

▲ **CRITICAL** All oligonucleotide sequences are shown in the 5' to 3' direction. Oligonucleotides should be dissolved in water and stored as frozen aliquots to avoid multiple freeze-thaw cycles. ▲ **CRITICAL** P5-BC<sub>2</sub>-R1P and P7 oligonucleotides contain phosphorothioated bases in 3' and should be ordered with HPLC purification. RT-BC<sub>1</sub> primer: CAAGCAGAAGACGGC ATACGAGAT-8-bp barcode BC<sub>1</sub>-GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT(dT)<sub>22</sub>VN (where V = A, C or G and N = A, C, G or T) These oligos are used for oligo(dT) priming during RT and carry the Illumina P7 sequence, an 8-bp sample barcode (BC<sub>1</sub>) and the Illumina Read 2 primer sequence. Depending on the experimental design, order the oligos in tubes or in 96-well plates at a concentration of 100 µM. Dilute stocks to 1-µM working solutions to be divided into aliquots and stored frozen.

R1P-N9 primer: CTACACGACGCTCTTCCGATCTNNNNNNNNN/3ddC/ This oligo contains a random nonamer sequence and a 3' ddC. Dissolve the purified oligo in water to produce a 100-µM solution.

P5-BC<sub>2</sub>-R1P primer: AATGATACGGCGACCACCGAGATCTACAC-8-bp barcode BC<sub>2</sub>-ACACT CTTTCCCTACACGACGCTCTTCCG\*A\*T\*C\*T These oligos contain the Illumina P5 sequence, an 8-bp sample pool barcode (BC<sub>2</sub>) and the Illumina Read 1 primer sequence. These oligos contain phosphorothioated bases (indicated by asterisks in the sequence above) at the 3' end and should be ordered with HPLC purification. Dissolve the purified oligos in water to produce a 100-µM stock solution. Dilute stocks to 10-µM working solutions to be divided into aliquots and stored frozen.

P7 primer : CAAGCAGAAGACGGC ATACG\*A\*G\*A\*T Dissolve the purified oligos in water to produce a 100-µM stock solution. Dilute stocks to 10-µM working solutions to be divided into aliquots and stored frozen.

### Equipment setup

#### Peristaltic pump

Install the tubing in the head of the pump following the manufacturer's instructions. Next, to connect a BD Vacutainer Safety-Lok Blood Collection Set to the pump's tubing, cut off the end of the vacutainer on the opposite side of the butterfly needle and discard it in a sharps collection container.

Use a connector to link the tubing and the vacutainer. The other end of the tubing should be placed in a tube (e.g., 50-ml conical tube) containing the perfusion buffer (Supplementary Fig. 1). Set the pump to 42 r.p.m. and prime the pump until liquid comes out of the vacutainer's butterfly needle. The pump is now ready for perfusion.

Procedure

**Mouse transcardial perfusion and tissue collection ● Timing 20 min per mouse**

**▲ CRITICAL** We recommend performing Steps 1–11 sequentially on batches of multiple mice with two or three investigators working in parallel.

1 Fully anesthetize the mouse with i.p. injection of avertin working solution (assess depth of anesthesia by the lack of response from the mouse to hind limb toe pinch).

**▲ CRITICAL STEP** Optimize the dose of avertin according to the weight of the mouse. The recommended dose is 500 mg/kg body weight.

2 Incise the skin along the midsagittal plane of the abdomen.

3 Cut the abdominal muscle to expose the upper part of the peritoneal cavity without injuring internal organs such as the liver.

4 Expose the chest cavity of the mouse without injuring the lungs, heart or major blood vessels by cutting the diaphragm and the ribs with scissors.

5 If the experiment requires the analysis of blood cells, collect blood by cardiac puncture using a 27G needle with a 1-ml syringe (preferably from the ventricle and slowly to avoid collapsing of the heart).

6 For isolation of PBMCs, transfer ~200 µl of whole blood into a 15-ml tube containing 1.5 ml of 1× PBS supplemented with 2 mM EDTA.

**▲ CRITICAL STEP** Collecting >300 µl of whole blood at this stage can impact the quality of perfusion.

7 Gently mix by inversion the tube with whole blood in PBS/EDTA and maintain on ice until PBMC isolation and processing (see Step 12).

8 Insert a 25G needle from a BD Vacutainer Safety-Lok Blood Collection Set connected to a peristaltic pump into the left ventricle of the heart.

9 Carefully perform a small incision (~1 mm) in the right atrium without damaging the surrounding organs and vessels.

10 Perfuse the mouse with ~15–20 ml of PBS supplemented with 10 mM EDTA using the peristaltic pump set to 42 r.p.m.

**▲ CRITICAL STEP** If perfusion is not performed adequately, organs are likely to be contaminated by blood. Visible indications of an effective perfusion include changes in the color of the lung (from red to pink or white) and the liver (from dark to pale), and a slow and upward movement of the tail towards the end of the perfusion.

**? TROUBLESHOOTING**

11 Collect the tissues of interest into tubes containing the appropriate amounts of RNA-preserving solution as indicated in Table 1, and maintain on ice or at 4 °C.

**▲ CRITICAL** Tissues in Table 1 are listed in the recommended order of dissection (except for lymph nodes, which can be collected as needed), which can be adjusted according to the needs of the experiment. For PBMCs, the small intestine and colon and the bone marrow, detailed processing is described in Steps 12–27, 28–35 and 36–46, respectively.

12 For the isolation and lysis of PBMCs, follow the additional processing Steps 12–27, including a red blood cell (RBC) lysis step followed by the depletion of remaining RBCs using magnetic beads. First, centrifuge diluted whole blood from Step 6 at 2,200g for 3 min at 4 °C.

13 Discard the supernatant and resuspend the pellet by pipetting in 1 ml of RBC lysis buffer and incubate for 3 min at room temperature.

14 Add 10 ml of MACS buffer and centrifuge at 2,200g for 3 min at 4 °C.

15 Discard the supernatant, resuspend cells by pipetting in 1 ml of MACS buffer, add 9 ml of MACS buffer and centrifuge at 2,200g for 3 min at 4 °C.

16 Aspirate and discard the supernatant and resuspend the pellet with 180 µl of MACS buffer.

17 Add 20 µl of anti-Ter-119 microbeads and mix well by pipetting.

18 Incubate for 15 min at 4 °C.

19 Add 3 ml of MACS buffer and centrifuge at 2,200g for 3 min at 4 °C.

**Table 1 | Tissue collection and storage guidelines for optimal RNA preservation**

Tissue	Tube size (ml)	RNA-preserving solution (ml)
Skin	1.5	1
Lymph nodes (all but mesenteric)	1.5	0.1
Heart	1.5	0.5
Thymus	1.5	0.5
Lung	1.5	0.5
Liver	5	3
Spleen	1.5	0.5
Mesenteric lymph node	1.5	0.5
Small intestine	5	3 (see Steps 28–35)
Colon	5	2 (see Steps 28–35)
Kidney	1.5	0.5
FRT	1.5	1
Bone marrow	1.5	1 (see Steps 36–46)
Brain	5	1

- 20 Discard the supernatant and resuspend the pellet in 500  $\mu$ l of MACS buffer and apply to an LS column equilibrated with 3 ml of MACS buffer and set on a QuadroMACS separator magnet and let the cell suspension pass through (1–2 min).
- 21 Collect the flow-through in a 15-ml tube.
- 22 Wash the column with 2 ml of MACS buffer and collect the flow-through in the same 15-ml tube.
- 23 Apply the collected flow-through (~2.5 ml) to a new LS column and collect the flow-through in a new 15-ml tube. Wash the column with 2 ml of MACS buffer and collect the flow-through in the same 15-ml tube.
- 24 Centrifuge the collected flow-through containing PBMCs at 2,200g for 3 min at 4°C.
- 25 Resuspend the cell pellet in 1 ml of PBS and transfer cells to a 1.5-ml tube.
- 26 Centrifuge at 2,200g for 3 min at 4 °C and discard the supernatant.
- 27 Lyse PBMCs with 120  $\mu$ l of RLT buffer supplemented with 1% (vol/vol) 2-mercaptoethanol at room temperature and transfer to a 96-well, non-skirted, chimney-well PCR plate.
  - **PAUSE POINT** PBMC lysates can be processed for RNA extraction (see Step 58B) or stored for several months at –80 °C.
- 28 For storage of the small intestine and colon, follow the additional processing steps detailed below (Steps 29–35).
- 29 Store dissected tissues on ice in a Petri dish (10-cm diameter) containing PBS (use one dish per tissue). Remove any remaining fat as needed by hand or using scissors and forceps.
- 30 Cut the tissue (small intestine or colon) longitudinally. For the colon, push all of the feces out prior to cutting.
- 31 Using the edge of a closed pair of scissors or curved forceps, remove excess feces while transferring the tissue to a new Petri dish containing ~5 ml of PBS.
- 32 Shake vigorously using forceps for ~30 s to wash.
- 33 Transfer the tissue to a 15-ml tube containing ~5 ml of PBS and shake the tube vigorously for ~30 s.
- 34 Transfer the tissue to an empty Petri dish (or a Petri dish lid) and rinse with fresh PBS.
  - ▲ **CRITICAL STEP** Careful washing and removing of feces residues is critical for obtaining good RNA quality downstream.
- 35 Drain excess PBS on a paper towel and transfer to a designated tube containing the appropriate amount of RNA-preserving solution (see Table 1).
- 36 For bone marrow isolation from tibiae and/or femora, follow the additional processing steps detailed below.
- 37 Collect a hind leg, remove muscle tissue and cut bone extremities with a razor blade.
- 38 Flush bone marrow with PBS using a 26G needle and 5-ml syringe and elute into a Petri dish.
- 39 Dissociate bone marrow cell clumps by mixing using a 5-ml serological pipette.
- 40 Transfer the cell suspension to a 15-ml conical tube.
- 41 Centrifuge the tube at 500g for 5 min at 4 °C and discard the supernatant.

**Table 2 | Tissue lysis guidelines**

Tissue	Trizol volume (µl)	Instrument	Cycling conditions*
Lymph nodes	700	PowerLyzer	2 × 5-s cycles at 3,500 r.p.m.
Kidney	1,500	PowerLyzer	2 × 45-s cycles at 3,500 r.p.m.
Spleen	1,500	PowerLyzer	2 × 45-s cycles at 3,500 r.p.m.
Lung	1,500	PowerLyzer	2 × 45-s cycles at 3,500 r.p.m.
Thymus	1,500	PowerLyzer	2 × 45-s cycles at 3,500 r.p.m.
Heart	1,500	PowerLyzer	2 × 45-s cycles at 3,500 r.p.m.
Bone marrow	1,500	PowerLyzer	1 × 5-s cycles at 3,500 r.p.m.
Skin	1,500	PowerLyzer	3 × 45-s cycles at 3,500 r.p.m.
FRT	1,500	PowerLyzer	2 × 45-s cycles at 3,500 r.p.m.
Colon	1,500	PowerLyzer	2 × 45-s cycles at 3,500 r.p.m.
SI	5,000	gentleMACS	4 × program RNA_02.01
Brain	3,000	gentleMACS	1 × program RNA_02.01
Liver	3,000	gentleMACS	1 × program RNA_02.01

\*For all PowerLyzer cycling conditions, a delay time of 30 s is used between cycles.

- 42 Gently resuspend bone marrow cells in 1 ml of RNA-preserving solution.
- 43 Transfer to a 1.5-ml tube and store at 4 °C overnight.
- 44 On the next day, centrifuge samples at 5,000g for 10 min at 4 °C and discard the supernatant.
- 45 Snap-freeze the cell pellet in a dry ice/ethanol bath.
- 46 Store frozen pellets of bone marrow cells at –80 °C until lysis.
  - ▲ **CRITICAL STEP** Once tissue collection is complete, maintain all tissues (except PBMCs) in RNA-preserving solution overnight at 4 °C. The next day, transfer samples to –80 °C for storage (for bone marrow, see additional Steps 44–46 before storage at –80 °C).
  - **PAUSE POINT** Tissues in RNA-preserving solution can be stored before lysis for several days at 4 °C, and for months at –80 °C.

**Tissue lysis**

▲ **CRITICAL** We recommend lysing batches of tissues of the same type to streamline downstream RNA extraction and sequencing while minimizing batch effects. For example, lyse 96 lung samples on the same day and array them into a single deep-well 96-well plate for downstream RNA extraction and processing.

▲ **CRITICAL** We describe below the protocol for tissue lysis using the PowerLyzer 24 instrument, but the procedure can also be performed using a Tissuelyzer or equivalent Rentsch instrument using 2-ml safelock tubes and 5-mm metal beads.

▲ **CRITICAL** Work on ice as much as possible.

! **CAUTION** All steps involving Trizol should be performed in a fume hood.

- 47 Tissue lysis is performed using option A for most tissues (using PowerLyzer 24) or option B for the small intestine and the liver (using GentleMACS).

(A) **Tissue lysis for all tissues except the liver, small intestine and brain** ● **Timing 120 min for 96 samples**

- (i) Thaw tissues stored in RNA-preserving solution on ice.
- (ii) Prepare and label 2-ml reinforced tubes (or equivalent) containing six 2.8-mm ceramic beads according to the number of samples to be processed.
  - ▲ **CRITICAL STEP** Labeling both the tube’s cap and side is encouraged to avoid potential problems with ink loss during lysis.
- (iii) Dispense Trizol or an equivalent buffer to each tube using a repeater pipette and the volumes indicated in Table 2.
- (iv) Use forceps to briefly blot the tissue on a paper towel to drain excess RNA-preserving solution, and to transfer the tissue into the Trizol-containing tube.

▲ **CRITICAL STEP** For the bone marrow, resuspend the cell pellet in Trizol using a P1000 pipette and transfer the sample to the 2-ml reinforced tube.

- (v) Firmly close screw caps and transfer tubes to the PowerLyzer, preferably set up in a cold room ahead of time.
- (vi) Run the PowerLyzer according to the cycling conditions listed in Table 2 for each tissue type.

#### ? TROUBLESHOOTING

- (vii) Cool tubes on ice for 2–3 min.
- (viii) Centrifuge tubes at 12,000g for 5 min at 4 °C to pellet particulates in the tissue lysate.
- (ix) Transfer 350 µl of lysate to a 0.5-ml deep-well 96-well plate.  
**▲ CRITICAL STEP** Plan plate maps carefully at this stage, as each plate position will be maintained for downstream RNA extraction, sample barcoding during RT and sequencing library preparation.
- (x) Transfer the remainder of the lysate to another deep-well 96-well plate (0.5 or 2 ml) as backup.
- (xi) Store lysate plates at –80 °C.  
**■ PAUSE POINT** Lysates can be stored for several months at –80 °C before RNA extraction.

#### (B) Tissue lysis for the liver, small intestine and brain ● Timing 30 min per eight samples

- (i) Thaw tissues stored in RNA-preserving solution on ice.
- (ii) Prepare and label gentleMACS M tubes according to the number of samples to be processed.
- (iii) Dispense 3 ml of Trizol to each tube for the liver and brain or 5 ml for the small intestine.
- (iv) Use forceps to briefly blot the tissue on a paper towel to drain excess RNA-preserving solution, and to transfer the tissue into the Trizol-containing tube.
- (v) Place the tubes on the GentleMACS, preferably in a cold room.
- (vi) Run one (liver and brain) or four (small intestine) cycles of the RNA\_02.01 program.

#### ? TROUBLESHOOTING

- (vii) For the liver, remove ~2.8 ml of the lysate and store it in a 5-ml Eppendorf tube at –80 °C as a crude lysate backup.
- (viii) Place 100 µl of crude liver lysate back into the same gentleMACS M tube and add 1 ml of Trizol.
- (ix) Run two cycles of the RNA\_02.01 program on the gentleMACS to further lyse the crude liver lysates.
- (x) After lysis on the gentleMACS, cool liver, brain and small intestine tubes on ice for 2–3 min.
- (xi) Centrifuge tubes at 3,200g for 5 min at 4 °C to pellet particulates in the tissue lysate.
- (xii) Transfer 350 µl of lysate for liver and brain and 150 µl of lysate for small intestine to a 0.5-ml deep-well 96-well plate. For small intestine samples, dilute the crude lysates by adding an additional 200 µl of Trizol into each well.  
**▲ CRITICAL STEP** Plan plate maps carefully at this stage, as each plate position will be maintained for downstream RNA extraction and sequencing library preparation.  
**▲ CRITICAL STEP** For the brain, transfer lysate while avoiding the fat aggregates that typically form at the top.
- (xiii) Transfer the remainder of the lysates to another deep-well 96-well plate (0.5 or 2 ml) as a backup.
- (xiv) Store lysate plates at –80 °C.  
**■ PAUSE POINT** Lysates can be stored for several months at –80 °C before RNA extraction.

#### High-throughput RNA extraction using magnetic silane beads ● Timing 150 min for 96 samples

**! CAUTION** All steps involving Trizol and chloroform should be performed in a fume hood.

**▲ CRITICAL** This RNA extraction protocol can be performed manually, as described below, or automated on a liquid handler. Detailed automation protocols and scripts for the Bravo Automated Liquid Handling Platform from Agilent are provided in Supplementary Methods and Supplementary Software 1–3.

**▲ CRITICAL** For multiple samples, prepare a 5–10% excess of the reaction mixes described below to ensure that all samples receive the indicated amount of mixture.

**▲ CRITICAL** The 80% (vol/vol) ethanol solution used for washing steps should be made fresh on the day of the procedure.

- 48 Thaw the 0.5-ml deep-well 96-well plate containing 350 µl of Trizol lysates (from Step 47A(xi) or 47B(xiv)) at room temperature, which takes ~1 h.
- 49 Transfer the plate on ice as soon as thawing is complete or near completion.
- 50 Add 70 µl of chloroform using a multichannel pipette to each sample when working with all tissue types except for (i) the skin and small intestine, which require 140 µl of chloroform, and (ii) for the brain and FRT, which require 250 µl of chloroform for adequate phase separation.
- 51 Mix 15–20 times with a multichannel P1000 pipette set to 400 µl.
- 52 Tightly cover the plate with an aluminum seal.
- 53 Incubate at room temperature for 2–3 min.
- 54 Centrifuge the plate at 4,000g for 15 min at 4 °C for phase separation.

**? TROUBLESHOOTING**

- 55 Prepare the following DNase I reaction mix on ice (to be used in Step 56A(xv)):

Component	Volume (µl)	Final concentration
Nuclease-free water	24	-
DNase I Buffer (10×)	3	1×
TURBO DNase I (2 U/µl)	3	0.2 U/µl
Total	30	-

- 56 RNA extraction is performed using option A for most tissues or option B for PBMCs. ●

**(A) RNA extraction for all tissues except PBMCs**

- (i) Prepare the following silane bead mix:

Component	Volume (µl)
Dynabeads MyOne Silane	4
RLT buffer	150
100% (vol/vol) ethanol	200
Total	350

**▲ CRITICAL STEP** Remove the silane bead buffer on a magnet before resuspending the beads in the appropriate amount of RLT buffer and ethanol for the number of samples being processed.

- (ii) Dispense 350 µl of the silane bead mix from Step 58A(i) per well in a new 0.5-mL 96-well plate.
- (iii) Transfer 50 µl of the upper aqueous phase from the Trizol/chloroform plate from Step 54 into the plate from Step 56A(ii) containing 350 µl of silane bead mix.

**▲ CRITICAL STEP** Position tips near the top of each well into the aqueous phase and aspirate slowly to avoid contamination from (i) the interphase (DNA) and/or (ii) the lower red organic phase (proteins and lipids).

- (iv) Mix the aqueous phase and the silane bead solution thoroughly (>20 times).
- (v) Incubate samples at room temperature for 10 min to allow for RNA binding onto the silane beads.
- (vi) Place the plate from Step 56A(v) on the magnetic separation block for deep-well 96-well plates until the supernatant is clear (which takes ~5–8 min).
- ▲ CRITICAL STEP** Gentle pipetting during the incubation on the magnet helps with pelleting beads at the bottom of the well.
- (vii) Remove the supernatant with a multichannel pipette or aspirator without disturbing the bead pellet.
- (viii) Remove the plate from the magnet and resuspend the beads in 150 µl of 80% (vol/vol) ethanol.

**▲ CRITICAL STEP** Bead aggregates are typically seen at this step due to the presence of genomic DNA, which will be removed in subsequent steps.

- (ix) Transfer samples (i.e., beads resuspended in 150  $\mu$ l of 80% ethanol) to a 96-well non-skirted, chimney-well PCR plate.  
**▲ CRITICAL STEP** Using a chimney-well PCR plate is required to accommodate the volumes used in subsequent steps.
- (x) Place the chimney-well PCR plate on the DynaMag-96 side magnet for 1–2 min to pellet the beads.
- (xi) Remove the supernatant with a multichannel pipette without disturbing the bead pellet.
- (xii) Remove the plate from the magnet and resuspend the beads in 150  $\mu$ l of 80% (vol/vol) ethanol.
- (xiii) Repeat Step 56A(x–xii).  
**▲ CRITICAL STEP** To fully discard the supernatant, briefly centrifuge the plate after removing most of the second 80% (vol/vol) ethanol wash and remove the remaining 1–2  $\mu$ l of 80% (vol/vol) ethanol using a P20 multichannel pipette.
- (xiv) Air dry the bead pellet for 1–2 min.  
**▲ CRITICAL STEP** Avoid over-drying by monitoring the change in bead pellet coloration by eye. Overdried beads appear light brown/orange, as opposed to dark brown for appropriately dried beads.
- (xv) Resuspend the dried bead pellets from Step 56A(xiv) in 30  $\mu$ l of DNase I reaction mix from Step 55 by pipetting up and down >10 times to mix.  
**▲ CRITICAL STEP** If bead aggregates were observed in Step 56A(iv), those aggregates should start dissociating during the resuspension of the beads in the DNase I reaction mix.
- (xvi) Incubate the mixture in a preheated thermocycler at 37 °C for 30 min.
- (xvii) Prepare the following RLT/ethanol buffer mix:

Component	Volume ( $\mu$ l)
RLT buffer	90
100% (vol/vol) ethanol	120
Total	210

- (xviii) Add the RLT/ethanol buffer mix to the samples and mix well by pipetting (>20 times).
- (xix) Incubate samples at room temperature for 10 min to allow for RNA binding onto the silane beads.
- (xx) Place the chimney-well PCR plate on the DynaMag-96 side magnet for 3–5 min, or until supernatant is clear, to pellet the beads.  
**▲ CRITICAL STEP** Gentle pipetting during incubation on the magnet helps with pelleting beads at the bottom of the well.
- (xxi) Remove the supernatant with a multichannel pipette or aspirator without disturbing the bead pellet.
- (xxii) Remove the plate from the magnet and resuspend the beads in 150  $\mu$ l of 80% (vol/vol) ethanol followed by transfer to a new chimney-well PCR plate.  
**▲ CRITICAL STEP** Bead aggregates should not appear at this step, indicating that all DNA contaminants have been removed.
- (xxiii) Place the chimney-well PCR plate on the DynaMag-96 side magnet for 1–2 min to pellet the beads.
- (xxiv) Remove the supernatant with a multichannel pipette without disturbing the bead pellet.
- (xxv) Repeat Step 56A(xxii–xxiv).  
**▲ CRITICAL STEP** To fully discard the supernatant, briefly centrifuge the plate after removing most of the second 80% (vol/vol) ethanol wash and remove the remaining 1–2  $\mu$ l of 80% (vol/vol) ethanol using a P20 multichannel pipette.
- (xxvi) Air-dry the bead pellet for 1–2 min.  
**▲ CRITICAL STEP** Avoid over-drying by monitoring the change in bead pellet coloration by eye.
- (xxvii) For RNA elution, resuspend the beads thoroughly in 10–30  $\mu$ l of RNase-free water depending on the desired concentration ranges.
- (xxviii) Incubate at room temperature for 2 min.

- (xxix) Place the plate on the DynaMag-96 side magnet for 1–2 min to pellet the beads.  
**▲ CRITICAL STEP** Gentle pipetting during incubation on the magnet helps with pelleting beads at the bottom of the well.
- (xxx) Transfer the RNA eluates to a 96-well LoBind PCR plate on ice.  
**▲ CRITICAL STEP** Make sure no beads are left in the RNA samples to avoid interference with the RNA fragmentation step downstream.

**(B) RNA extraction for PBMCs**

- (i) Thaw the PBMC lysates on ice.
- (ii) Prepare the following silane bead mix:

Component	Volume (μl)
Dynabeads MyOne Silane	2
100% (vol/vol) ethanol	120

**▲ CRITICAL STEP** Remove the silane bead buffer on a magnet prior to resuspending the beads in the appropriate amount of RLT buffer and ethanol for the number of samples being processed.

- (iii) Add 120 μl of silane bead mix prepared in Step 56B(ii) to the lysates.
  - (iv) Mix the lysates and the silane bead solution thoroughly (by pipetting >20 times).
  - (v) Incubate samples at room temperature for 10 min to allow for RNA binding onto the silane beads.
  - (vi) Place the plate from Step 56B(v) on the DynaMag-96 side magnet until the supernatant is clear.  
**▲ CRITICAL STEP** Gentle pipetting during the incubation on the magnet helps with pelleting beads at the bottom of the well.
  - (vii) Remove the supernatant with a multichannel pipette or aspirator without disturbing the bead pellet.
  - (viii) Follow Step 56A(viii–xxx).
- 57 Quantify RNA concentrations using the Quant-IT Ribogreen RNA assay kit or a spectrophotometer (e.g., NanoDrop) and, optionally, check RNA quality using the Agilent TapeStation or BioAnalyzer.  
**▲ CRITICAL STEP** We routinely check 6–12 random samples for each 96-well plate of RNA samples for a quality control check (see Fig. 2).

**? TROUBLESHOOTING**

- 58 Prepare 100 ng of total RNA from Step 56A(xxx) and 56B(viii) in 7 μl of nuclease-free water for each sample.  
**■ PAUSE POINT** RNA samples can be stored at –80 °C for several months.

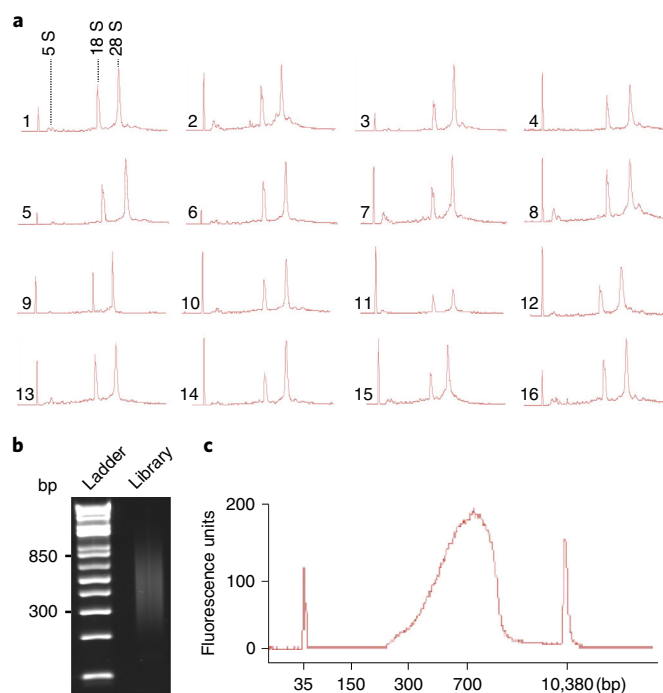
**RNA fragmentation followed by cDNA synthesis ● Timing 120 min for 96 samples**

**▲ CRITICAL** Up to 384 samples can be processed in parallel depending on the number of barcoded RT oligo primers available. If <100 ng of starting total RNA is used, RNA fragmentation conditions in Step 60 should be optimized by the user by varying incubation time and temperature and measuring the resulting distribution of RNA fragment lengths.

- 59 Set up the following reaction for RNA fragmentation on ice:

Component	Volume (μl)
RNA from Step 58	7
10× AffinityScript RT buffer	2
RT-BC <sub>1</sub> primer (1 μM)	1
Total	10

- 60 Incubate the mixture in a preheated thermocycler at 94 °C for 2.5 min (use a heated lid).  
**▲ CRITICAL STEP** Cations in the AffinityScript RT buffer will catalyze RNA fragmentation, although other buffers can be used upon optimization. The composition of the 10× AffinityScript RT buffer is 500 mM Tris-HCl (pH 8.3), 750 mM KCl and 30 mM MgCl<sub>2</sub>.



**Fig. 2 | Anticipated results for RNA quality and library preparation.** **a**, BioAnalyzer electrophoretograms of total RNA extracted from 16 mouse tissues: (1) lung, (2) kidney, (3) colon, (4) thymus, (5) mesenteric lymph node, (6) mediastinal lymph node, (7) brain, (8) liver, (9) PBMCs, (10) inguinal lymph node, (11) heart, (12) female reproductive tract, (13) skin, (14) bone marrow, (15) small intestine and (16) spleen. 5S, 18S and 28S indicate the major ribosomal RNA populations. **b,c**, Gel image (**b**) and BioAnalyzer electrophoretogram (**c**) of a typical RNA-seq library prepared with our protocol, which has an average DNA amplicon size of ~475 bp.

- 61 Immediately place samples on ice to cool for 5 min and briefly centrifuge at 4 °C.  
 62 Add 10 µl of the following RT mix to each sample:

Component	Volume (µl)	Final concentration
100 mM dNTP mix	0.8	4 mM
100 mM DTT	2	10 mM
RiboLock RNase inhibitor (40 U/µl)	0.5	1 U/µl
AffinityScript Multiple Temperature RT enzyme	0.8	-
Nuclease-free water	5.9	-
Total	10	

- 63 Incubate the mixture in a preheated thermocycler at 42 °C for 90 min.  
 64 Optionally, take 1 µl of each sample and dilute 1:50–100 in nuclease-free water for quality check by qPCR using primers targeting the 3' end of housekeeping genes or other genes of interest.  
 65 Proceed to Step 66 directly or store cDNA samples at –80 °C.  
**■ PAUSE POINT** cDNA can be stored at –80 °C for several months.

### Pooling and cleanup of barcoded cDNA samples ● Timing 120 min for 96 samples

**▲ CRITICAL** The maximum binding capacity of the Zymo-Spin columns is 10 µg; do not exceed when pooling samples. Alternatively, solid phase reversible immobilization (SPRI) beads can be used instead of columns for this step, depending on the experimental setup and scale.

- 66 Pool 5 µl of cDNA from each sample from Step 65 into a LoBind 1.5-ml Eppendorf tube. The amount of cDNA used for pooling can be adjusted depending on the experimental design. For example, we routinely pool from 8 to 96 samples per column, depending on the barcoding scheme used during the RT and library construction steps.

- 67 Add 1 volume of RNA Binding Buffer from the RNA Clean & Concentrator-5 kit and 1 volume of 100% (vol/vol) ethanol, which amounts to 480  $\mu$ l for each buffer when processing 96 samples in parallel.
- 68 Transfer up to 650  $\mu$ l of the sample to a Zymo-Spin column (use a vacuum manifold when pooling large volumes).
- 69 Centrifuge the column for 30 s at >14,000g at room temperature and discard the flow-through.  
▲ **CRITICAL STEP** Make sure there is no residual buffer left in the column after each centrifugation step. If necessary, lengthen the centrifugation time.
- 70 Add 400  $\mu$ l of RNA Prep Buffer to the column, centrifuge the column for 30 s at >14,000g at room temperature and discard the flow-through.
- 71 Add 650  $\mu$ l of RNA Wash Buffer to the column, centrifuge the column for 30 s at >14,000g at room temperature and discard the flow-through.
- 72 Add 500  $\mu$ l of RNA Wash Buffer to the column, centrifuge the column for 30 s at >14,000g at room temperature and discard the flow-through.
- 73 Centrifuge the column for 2 min at >14,000g at room temperature to dry the column matrix.
- 74 Transfer the column to a LoBind 1.5-ml Eppendorf tube.
- 75 Add 20  $\mu$ l of nuclease-free water directly to the column matrix and let sit for 2 min.
- 76 Centrifuge the column for 1 min at >14,000g at room temperature.
- 77 Re-apply the eluate to the column and repeat Step 76 to improve recovery.
- 78 Transfer the sample to a PCR strip or plate as needed.
- 79 Add 5  $\mu$ l of the following exonuclease I mix to each sample to degrade remaining RT primers:

Component	Volume ( $\mu$ l)
Exonuclease I reaction buffer (10 $\times$ )	2.5
Exonuclease I	1
Nuclease-free water	1.5
Total	5

- 80 Incubate the mixture in a preheated thermocycler at 37  $^{\circ}$ C for 30 min and 80  $^{\circ}$ C for 20 min.
- 81 Place samples on ice and add 10  $\mu$ l of 0.5 M EDTA and 10  $\mu$ l of 1 M NaOH and mix.
- 82 Incubate the mixture in a preheated thermocycler at 65  $^{\circ}$ C for 15 min for the alkaline hydrolysis of RNA.
- 83 Add 100  $\mu$ l of Oligo Binding Buffer from the Oligo Clean & Concentrator kit and mix well (>10 times). The Oligo Binding Buffer is sufficient for neutralizing the reaction.
- 84 Transfer the sample (~150  $\mu$ l) to a LoBind 1.5-ml Eppendorf tube and add 200  $\mu$ l of 100% (vol/vol) ethanol.
- 85 Mix well (by pipetting >10 times) and transfer the mixture to a Zymo-Spin column.
- 86 Centrifuge the column for 30 s at >14,000g at room temperature and re-apply the flow-through to the column to maximize recovery.
- 87 Centrifuge the column for 30 s at >14,000g at room temperature and discard the flow-through.
- 88 Add 650  $\mu$ l of DNA Wash Buffer to the column, centrifuge the column for 30 s at >14,000g at room temperature and discard the flow-through.
- 89 Centrifuge the column for 2 min at >14,000g at room temperature to dry the column matrix.
- 90 Transfer the column to a LoBind 1.5-ml Eppendorf tube.
- 91 Add 7  $\mu$ l of nuclease-free water directly to the column matrix and let sit for 2 min.
- 92 Centrifuge the column for 1 min at >14,000g at room temperature.
- 93 Re-apply the eluate to the column and repeat Step 92 to improve recovery.  
■ **PAUSE POINT** ss cDNA can be stored at  $-80^{\circ}$  C for several months.

**3'-end DNA polymerization of ss cDNA using the Klenow fragment ● Timing 90 min for a 96-sample pool**

▲ **CRITICAL** The 80% (vol/vol) ethanol solution used for washing steps should be made fresh on the day of the procedure.

▲ **CRITICAL** Gentle pipetting during incubation on the magnet helps with pelleting magnetic beads at the bottom of the well.

94 Set up the following reaction in a PCR strip on ice:

Component	Volume ( $\mu$ l)
ss cDNA from Step 90	6
R1P-N9 primer (100 $\mu$ M)	1

95 Incubate the mixture in a preheated thermocycler at 95 °C for 3 min.

96 Immediately place samples on ice to cool for 5 min and briefly centrifuge at 4 °C.

97 Add the following components to each sample:

Component	Volume ( $\mu$ l)	Final concentration
NEBuffer 2 (10 $\times$ )	2	1 $\times$
dNTPs (100 mM)	0.8	4 mM
Klenow fragment (3'-5' exo-)	1 (5 U/ $\mu$ l)	0.25 U/ $\mu$ l
PEG 8000 50% (wt/vol)	10	25%
Total	13.8	-

**▲ CRITICAL STEP** PEG 8000 50% (wt/vol) is viscous and must be added carefully. For long-term storage, keep frozen in small aliquots.

98 Mix well by pipetting followed by flicking.

99 Incubate the mixture in a preheated thermocycler at 37 °C for 60 min and 65 °C for 5 min.

100 Place samples on ice to cool for 2–3 min.

101 Add 30  $\mu$ l of nuclease-free water and 40  $\mu$ l (0.8 $\times$ ) of AMPure XP beads and mix well (by pipetting >20 times).

102 Incubate samples at room temperature for 7–10 min to allow for DNA binding onto the beads.

103 Place the sample on the DynaMag-96 side magnet for 5 min to pellet the beads.

104 Remove the supernatant without disturbing the bead pellet.

105 Remove the sample from the magnet and resuspend the beads in 150  $\mu$ l of 80% (vol/vol) ethanol.

106 Place the sample on the magnet to pellet the beads and remove the supernatant without disturbing the bead pellet.

107 Repeat Steps 105 and 106.

**▲ CRITICAL STEP** To fully discard the supernatant, briefly centrifuge the sample after removing most of the second 80% (vol/vol) ethanol wash and remove the remaining 1–2  $\mu$ l of 80% (vol/vol) ethanol using a P20 pipette.

108 Air-dry the bead pellet for 1–2 min.

**▲ CRITICAL STEP** Avoid over-drying by monitoring the change in bead pellet coloration by eye.

109 Resuspend the beads thoroughly in 20  $\mu$ l of nuclease-free water for elution.

110 Incubate at room temperature for 2 min and place the sample on the dynaMag-96 magnet for 1–2 min to pellet the beads.

111 Elute ss cDNA samples and store at –80 °C.

**■ PAUSE POINT** ss cDNA can be stored at –80 °C for several months.

### Library preparation and sequencing ● Timing 120 min

112 Prepare the following PCR reaction mix on ice:

Component	Volume ( $\mu$ l)	Final concentration
ss cDNA from Step 111	20	-
P5-BC <sub>2</sub> -R1P primer (10 $\mu$ M)	2.5	0.5 $\mu$ M
P7 primer (10 $\mu$ M)	2.5	0.5 $\mu$ M
Q5 High-Fidelity 2 $\times$ Master Mix	25	1 $\times$
Total	50	-

**▲ CRITICAL STEP** Thaw the Q5 master mix at room temperature and mix well to dissolve all precipitates.

113 Incubate the samples in a thermal cycler using the following cycling conditions:

Cycle	Denature	Anneal	Extend	Hold
1	98 °C, 30 s	-	-	-
2–11	98 °C, 10 s	60 °C, 30 s	72 °C, 20 s	-
12	-	-	72 °C, 5 min	-
13	-	-	-	4 °C

**▲ CRITICAL STEP** Adjust the number of cycles according to the initial sample input. Using 10–12 cycles works well for a pool of 24–96 samples, starting from 100 ng of total RNA from each sample.

114 Add 40 µl (0.8×) of AMPure XP beads and mix well by pipetting (>20 times).

115 Repeat Steps 102–111.

116 Run the DNA eluate (20 µl) on a 2% E-Gel EX Agarose gel.

117 Excise the smear between 300 and 850 bp (Fig. 2b).

118 Extract the DNA using the MinElute Gel Extraction kit.

**▲ CRITICAL STEP** Add three volumes of QG Buffer (300 µl) and dissolve the gel on a thermomixer or vortex for ~45 min or until fully dissolved at room temperature; then, follow the manufacturer’s instructions.

119 Quantify the library concentration using the Qubit dsDNA HS assay kit and, optionally, check the size distribution of the library using the Agilent High Sensitivity D5000 kit on the TapeStation (Fig. 2c).

**? TROUBLESHOOTING**

120 Calculate the molarity using the measured concentration from the Qubit fluorometer and the average molecular length from the Bioanalyzer as follows:  $Molarity (nM) = Concentration (ng/\mu l) \times 10^6 / (Average\ length (bp) \times 660\ g \cdot mol^{-1})$ , where the average length is typically ~475 bp.

121 Sequence the library using 75 cycles in Read 1, 8 cycles in Index Read 1 and 8 cycles in Index Read 2 using, for example, a 75-cycle kit on an Illumina NextSeq550 sequencer according to the manufacturer’s instructions.

**▲ CRITICAL STEP** The choice of the Illumina instrument and kit to be used depends on the number of samples and desired sequencing depth. Typically, ≤5 M raw reads per sample is sufficient for most differential expression analyses.

**Raw data processing and differential expression analysis ● Timing 1 d**

**▲ CRITICAL** Detailed scripts for (i) data preprocessing, (ii) differential expression analysis, and (iii) comparative analyses to existing public datasets (e.g., GTEx and ENCODE) are available here: <https://github.com/chevrierlab/PME-seq>.

122 Process the fastq files generated in Step 121 using the pipeline from the bcbio-nextgen pipeline (<https://github.com/bcbio/bcbio-nextgen>), which includes steps for quality control, adapter trimming, alignment, variant calling, transcriptome reconstruction and post-alignment quantitation at the level of the gene and isoform.

**▲ CRITICAL STEP** The bcbio-nextgen pipeline also creates plots using the FastQC and QualiMap<sup>35</sup> tools for quality check, which should be examined to ensure the quality of the experiment and sequencing run (e.g., ensure acceptable read quality per base and high enough fraction of aligned reads).

**? TROUBLESHOOTING**

123 Use the edgeR<sup>33,36</sup> and limma<sup>34</sup> packages in R to perform differential expression analysis. For example, to perform tissue-specific gene expression analyses, use limma in R to model gene expression differences between each tissue and all the other tissues, and filter tissue-specific genes using the resulting log fold change and *P* values. For principal component analysis, use the prcomp function in R to visualize variation in transcriptional states across the tissues.

## Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3   Troubleshooting table			
Step	Problem	Possible reason	Solution
10	Too much blood remaining in tissues after perfusion	Poor perfusion due to incorrect needle insertion, damaging of internal organs or vessels, or peristaltic pump problems	Ensure that perfusion is performed adequately by careful preparation of the animal while exposing the chest cavity and correct needle insertion and nicking of the heart atrium
47A(vi) and 47B(vi)	Suboptimal lysis	Poor lysis due to incorrect Trizol volume or tube	Carefully follow the lysis conditions delineated in Table 2 for each tissue type If using a tissue type not described in this protocol, optimize lysis conditions by varying Trizol buffer volumes and lysing conditions
	Lysis buffer leakage	Tubes not closed properly or damaged during lysis	Ensure that tubes are tightly closed before each cycle on the PowerLyzer For gentleMACS, do not remove tubes between each lysis cycle
54	Improper phase separation of Trizol/chloroform mixture	Aqueous and organic phases were mixed during plate removal from the centrifuge, or an insufficient amount of chloroform was used.	Centrifuge plate one more time Increase the amount of chloroform added to the Trizol lysate
57	Poor RNA quality	Silane bead contamination in RNA eluate	Ensure proper magnetic separation and use a higher volume of water for elution (e.g., elute in 22 $\mu$ l and transfer 20 $\mu$ l)
		Poor RNA quality (poor RNA integrity number (RIN) value)	Ensure that all of the work is performed in RNase-free conditions Repeat the extraction using Trizol lysates backup samples stored at $-80^{\circ}\text{C}$
		Insufficient lysis	Carefully follow the steps for lysis for each tissue type Increase the volume of Trizol
119	Incorrect library size distribution (e.g., fragments shorter than the 350–800 bp range)	Over-fragmentation of total RNA	Ensure that the correct buffer is used during the fragmentation step
		Use too much or too little input RNA	Optimize fragmentation conditions according to the amount of input RNA
	Low library yield	Insufficient input RNA	Increase starting RNA amounts or number of PCR cycles during the final enrichment of the library
		Sample loss during cleanup or gel extraction steps	Ensure proper use of columns and magnetic beads, use freshly made 80% ethanol and fully melt agarose during gel extraction step
122	Low read alignment rate (<70%)	Contaminated or low-quality primers	Ensure proper handling and storage at $-80^{\circ}\text{C}$ in single-use aliquots
		Wrong reference file	Ensure that the correct reference genome and/or transcriptome is used
		Poor library preparation	Ensure that all steps are performed adequately, which can be assessed by intermediate qPCR checks along the procedure
		Poor sequencing quality	Ensure that there were no technical issues with the sequencer.

## Timing

Steps 1–46, mouse transcardial perfusion and tissue collection: 20 min per mouse, depending on the number of organs harvested and the skill level and number of investigators working in parallel; this does not include subsequent isolation of PBMCs using density gradient centrifugation

Step 47A, tissue lysis for all tissues except the brain, liver and the small intestine: 120 min for 96 samples

Step 47B, tissue lysis for the brain, liver and the small intestine: 30 min for 8 samples

Steps 48–58, high-throughput RNA extraction using magnetic silane beads: 240 min for 96 samples

Steps 59–65, RNA fragmentation followed by cDNA synthesis: 120 min for 96 samples  
 Steps 66–93, pooling and cleanup of barcoded cDNA samples: 120 min for 96 samples  
 Steps 94–111, 3'-end DNA polymerization of pooled, ss cDNA: 90 min  
 Steps 112–121, library preparation and Illumina sequencer loading (this does not include sequencer run time): 120 min  
 Steps 122 and 123, raw data processing and differential expression analysis: 1 d

**Anticipated results**

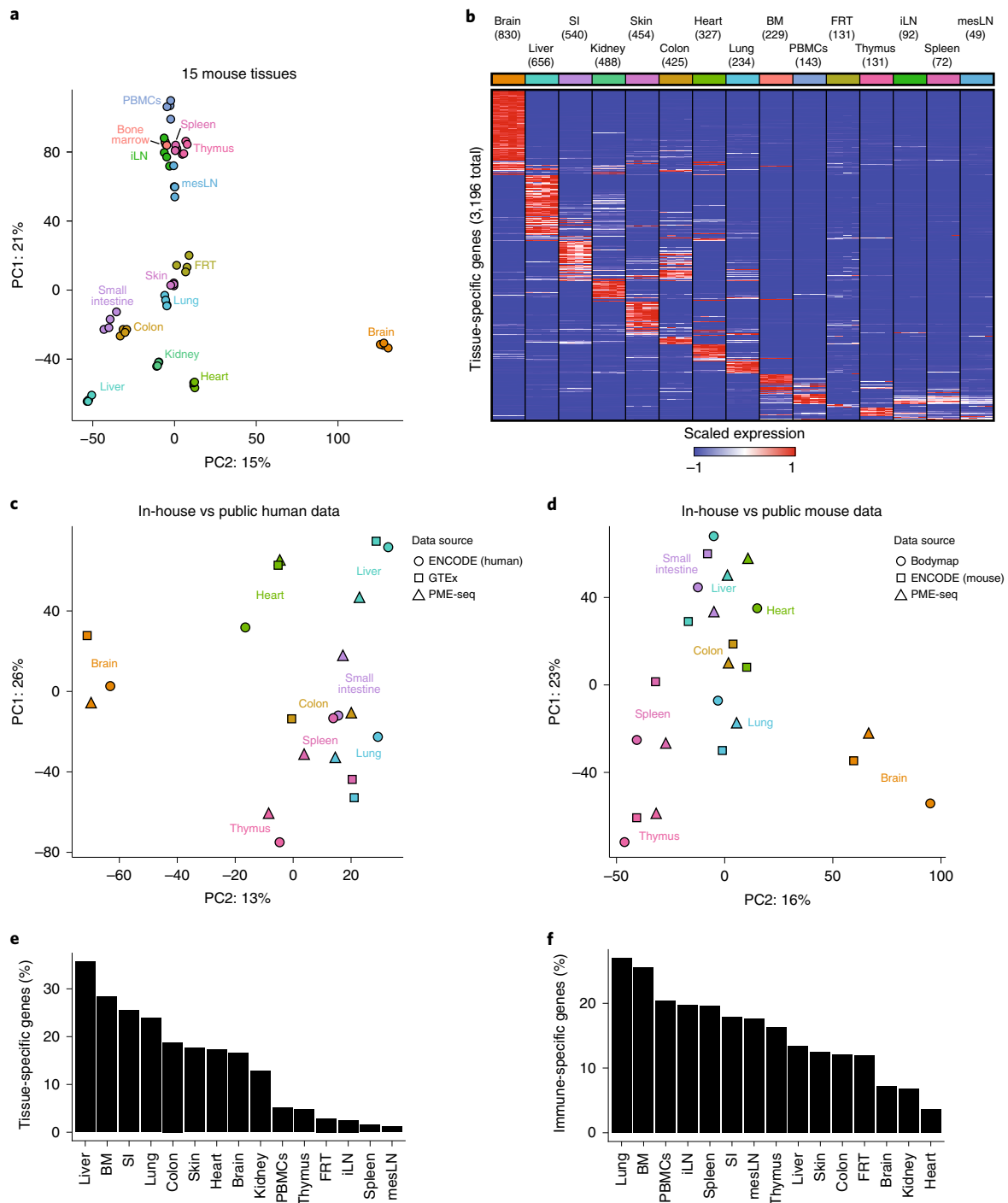
We previously demonstrated that this procedure can provide high-quality whole-tissue RNA-seq profiles from hundreds of samples processed in parallel<sup>1</sup>. The first part of the protocol—from tissue collection and lysis to RNA extraction with magnetic beads (Fig. 1a)—yields high-quality total RNA samples from all mouse tissue types tested (Fig. 2a and Table 4). Second, RNA-seq libraries prepared with our procedure will result in DNA fragment sizes ranging from 300 to 850 bp (Fig. 2b,c). Third, we typically obtain ~85–90% mapping rates when aligning raw sequencing reads to the reference genome. Overall, using manual procedures from beginning to end, we have observed a sample failure rate of <4% (28/702 tissues, whereby failure is defined as sample loss, poor RNA quality or lack of sufficient sequencing reads), and an even lower failure rate when using an automated liquid handler. Importantly, the built-in stopping points in the protocol to backup samples (e.g., tissue lysates, total RNA and barcoded cDNA) have allowed us to successfully reprocess failed samples in all cases. Lastly, the results of the differential expression analyses will depend entirely on the nature of the mouse model under study, but they will closely mirror measurements obtained by approaches such as qPCR or microarrays.

Using whole-tissue profiles from 15 mouse organ types, we show that our protocol recapitulates known variations in transcript levels across organs (Fig. 3a,b), in agreement with recent reports in humans<sup>37</sup>. To go further, we compared the mouse tissue profiles obtained with our protocol to mouse and human tissue profiles reported by public consortium studies, namely ENCODE<sup>38</sup> and GTEx<sup>5</sup>, and by an independent study referred to as BodyMap<sup>39</sup>. We found that the gene expression profiles we obtained closely matched those previously reported for the eight tissue types that could be cross-referenced across all five data sources from mouse and human samples (Fig. 3c,d). In addition, we report the typical proportions of sequencing reads mapping to tissue- and immune-specific genes using our protocol (Fig. 3e,f). Importantly, the proportions of tissue- and immune-specific transcripts were calculated in tissue expression profiles from wild-type adult mice and may vary according to the physiological and immunological states of the animal under study.

**Table 4 | RNA yield estimates per tissue type**

Tissue	RNA yield (ng/mg of tissue*)
Mesenteric LN (mesLN)	~125
Colon	~450
Lung	~250
Heart	~350
Thymus	~475
Kidney	~1,500
Inguinal LN (iLN)	~100
Liver	~3,000
Popliteal LN (pLN)	~10
SI	~500
Skin	~50
Brain	~200
FRT	~450
Spleen	~2,400
PBMCs	~50 (per 10 <sup>5</sup> cells)
BM	~150 (per 10 <sup>7</sup> cells)

\*Weights indicated for perfused tissues stored in RNA-preserving solution.



**Fig. 3 | Patterns of mRNA expression and tissue-specific genes are similar between our protocol and existing mouse and human consortium data sets.** **a**, Principal component (PC) analysis of whole-tissue mRNA profiles for 15 tissues in quadruplicates (45 samples total). Colors indicate various tissues. **b**, Heatmaps of differentially expressed, tissue-specific genes (rows) from whole-tissue mRNA profiles (columns), ordered by hierarchical clustering (Pearson’s correlation) and tissue type (colors). Shown are normalized read count values for all tissue-specific genes (false discovery rate (FDR)-adjusted  $P$  value < 0.01,  $\log_2$ (fold change) > 4,  $n = 4$ ). Numbers in parentheses on top indicate the number of tissue-specific genes in each tissue type. **c** and **d**, Principal component analysis of whole-tissue mRNA profiles for eight tissues from human (**c**) and mouse (**d**). Colors denote different tissues, symbols indicate the data source and axes show the percentage of variance. **e,f**, Proportion of tissue- (**e**) and immune-specific (**f**) genes found in indicated tissue types. Tissue-specific genes were identified as indicated in **b**. Genes annotated as part of ‘immune process’ in the Gene Ontology knowledgebase (2,792 genes in total for *Mus musculus*) were used to calculate proportions of immune-specific genes (**f**). BM, bone marrow; iLN, inguinal lymph node; mesLN, mesenteric lymph node; SI, small intestine; PME-seq, 3-prime mRNA extension sequencing.

### Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

### Data availability

The example datasets used in this protocol are available in the Gene Expression Omnibus under accession number [GSE138103](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138103).

### Code availability

All scripts are available at <https://github.com/chevrierlab/PME-seq>.

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### Author contributions

Conceptualization, N.C.; methodology and investigation, S.P., M.T., M.Z., K.C. and N.C.; formal analysis, A.G. and N.C.; writing, S.P., A.G. and N.C.; supervision and funding acquisition, N.C.

### Competing interests

The authors declare no competing interests.

### Additional information

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#### Key references using this protocol

Kadoki, M. et al. *Cell* **171**, 398–413 (2017): <https://doi.org/10.1016/j.cell.2017.08.024>

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Data collection

<https://github.com/chevrierlab/PME-seq>

Data analysis

<https://github.com/chevrierlab/PME-seq>

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

### Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data is publicly available in the NCBI GEO under the accession number GSE138103

## Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences       Behavioural & social sciences       Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

## Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Describe how sample size was determined, detailing any statistical methods used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.
Data exclusions	Describe any data exclusions. If no data were excluded from the analyses, state so OR if data were excluded, describe the exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.
Replication	Describe the measures taken to verify the reproducibility of the experimental findings. If all attempts at replication were successful, confirm this OR if there are any findings that were not replicated or cannot be reproduced, note this and describe why.
Randomization	Describe how samples/organisms/participants were allocated into experimental groups. If allocation was not random, describe how covariates were controlled OR if this is not relevant to your study, explain why.
Blinding	Describe whether the investigators were blinded to group allocation during data collection and/or analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

n/a	Included in the study
<input type="checkbox"/>	<input type="checkbox"/> Antibodies
<input type="checkbox"/>	<input type="checkbox"/> Eukaryotic cell lines
<input type="checkbox"/>	<input type="checkbox"/> Palaeontology
<input type="checkbox"/>	<input checked="" type="checkbox"/> Animals and other organisms
<input type="checkbox"/>	<input type="checkbox"/> Human research participants
<input type="checkbox"/>	<input type="checkbox"/> Clinical data

### Methods

n/a	Included in the study
<input type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

## Antibodies

Antibodies used  Describe all antibodies used in the study; as applicable, provide supplier name, catalog number, clone name, and lot number.

Validation  Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer's website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.

## Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)  State the source of each cell line used.

Authentication  Describe the authentication procedures for each cell line used OR declare that none of the cell lines used were authenticated.

Mycoplasma contamination  Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines (See [ICLAC](#) register)  Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

## Palaeontology

Specimen provenance  Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).

Specimen deposition  Indicate where the specimens have been deposited to permit free access by other researchers.

Dating methods  If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement).

## Dating methods

where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

## Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

## Laboratory animals

Mus musculus strain C57BL/6J between 6 and 8 weeks of age were used in this study.

## Wild animals

Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.

## Field-collected samples

For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

## Ethics oversight

Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Human research participants

Policy information about [studies involving human research participants](#)

## Population characteristics

Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

## Recruitment

Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

## Ethics oversight

Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Clinical data

Policy information about [clinical studies](#)

All manuscripts should comply with the ICMJE [guidelines for publication of clinical research](#) and a completed [CONSORT checklist](#) must be included with all submissions.

## Clinical trial registration

Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.

## Study protocol

Note where the full trial protocol can be accessed OR if not available, explain why.

## Data collection

Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.

## Outcomes

Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

## ChIP-seq

### Data deposition

Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).

Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

## Data access links

May remain private before publication.

For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

## Files in database submission

Provide a list of all files available in the database submission.

## Genome browser session

(e.g. [UCSC](#))

Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

### Methodology

## Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth	<i>Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.</i>
Antibodies	<i>Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.</i>
Peak calling parameters	<i>Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.</i>
Data quality	<i>Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.</i>
Software	<i>Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.</i>

## Flow Cytometry

### Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

Sample preparation	<i>Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.</i>
Instrument	<i>Identify the instrument used for data collection, specifying make and model number.</i>
Software	<i>Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.</i>
Cell population abundance	<i>Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.</i>
Gating strategy	<i>Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.</i>

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

## Magnetic resonance imaging

### Experimental design

Design type	<i>Indicate task or resting state; event-related or block design.</i>
Design specifications	<i>Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.</i>
Behavioral performance measures	<i>State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).</i>

### Acquisition

Imaging type(s)	<i>Specify: functional, structural, diffusion, perfusion.</i>
Field strength	<i>Specify in Tesla</i>
Sequence & imaging parameters	<i>Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.</i>
Area of acquisition	<i>State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.</i>
Diffusion MRI	<input type="checkbox"/> Used <input type="checkbox"/> Not used

## Preprocessing

Preprocessing software	<i>Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).</i>
Normalization	<i>If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.</i>
Normalization template	<i>Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.</i>
Noise and artifact removal	<i>Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).</i>
Volume censoring	<i>Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.</i>

## Statistical modeling & inference

Model type and settings	<i>Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).</i>
Effect(s) tested	<i>Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.</i>
Specify type of analysis:	<input type="checkbox"/> Whole brain <input type="checkbox"/> ROI-based <input type="checkbox"/> Both
Statistic type for inference (See <a href="#">Eklund et al. 2016</a> )	<i>Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.</i>
Correction	<i>Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).</i>

## Models & analysis

n/a	Involvement in the study	
<input type="checkbox"/>	<input type="checkbox"/> Functional and/or effective connectivity	
<input type="checkbox"/>	<input type="checkbox"/> Graph analysis	
<input type="checkbox"/>	<input type="checkbox"/> Multivariate modeling or predictive analysis	
Functional and/or effective connectivity		<i>Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).</i>
Graph analysis		<i>Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).</i>
Multivariate modeling and predictive analysis		<i>Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.</i>