Smart-seq2 single-cell RNA-Seq modified method

John J. Trombetta,David Gennert,Diana Lu,Rahul Satija,Alex K. Shalek,Aviv Regev

Abstract

For the past several decades, due to technical limitations, the field of transcriptomics has focused on population-level measurements that can mask significant differences between individual cells. With the advent of single-cell RNA-Seq, it is now possible to profile the responses of individual cells at unprecedented depth and thereby uncover, transcriptome-wide, the heterogeneity that exists within these populations. This unit describes a method that merges several important technologies to produce, in high-throughput, single-cell RNA-Seq libraries. Complementary DNA (cDNA) is made from full-length mRNA transcripts using a reverse transcriptase that has terminal transferase activity. This, when combined with a second “template-switch” primer, allows for cDNAs to be constructed that have two universal priming sequences. Following preamplification from these common sequences, Nextera XT is used to prepare a pool of 96 uniquely indexed samples ready for Illumina sequencing. *Curr. Protoc. Mol. Biol*. 107:4.22.1-4.22.17. © 2014 by John Wiley & Sons, Inc.

In single-cell RNA-Seq, small amounts of sample loss during a number of steps can lead to significant decreases in transcript detection sensitivity. A decrease in assay sensitivity results in data that is only accurate and reproducible for highly expressed genes, limiting the scope and confidence of gene expression analyses. Further complications in assay sensitivity arise from an uneven distribution of sequencing reads along a transcript; usually, in SMARTer, there is a bias towards more reads at the 3′ end of the transcript. Even coverage along a transcript improves the accuracy of analytical tools used to quantify gene expression and transcript isoform abundance. A method published by Picelli et al (Nature Methods, 2013) modified the traditional SMARTer protocol to address this by improving transcript detection, coverage, accuracy, yield, and cost. Following the same strategy as SMARTer library construction, Smart-seq2 uses several alternative reagents to generate whole-transcriptome full-length cDNA libraries.

Avoiding small-volume, bead-based SPRI cleanups of each sample is an effective way of reducing loss and increasing assay sensitivity. Lysing single cells in a guanidine thiocyanate buffer necessitates SPRI cleanup due to the protein denaturing effects of the compound, which will affect downstream reactions, like reverse transcription. Multiple alternative lysis buffers exist that address this. The Ambion Single Cell Lysis buffer (Life technologies, #4458235), often used for single-cell RT-PCR, only requires the addition of a stop solution to inactivate its lytic activity before subsequent reactions. A hypotonic lysis buffer with small amounts of RNase-inhibitor and surfactant, as described in Smart-seq2, is the preferred buffer due to the lack of a need for a post-lysis cleanup or the addition of a stop solution prior to reverse transcription. However, the optimal lysis strategy will depend on the experimental system being analyzed.

Smart-seq2 takes additional steps to minimize sample loss during library construction. The reverse transcription is improved by the addition of betaine and additional magnesium chloride to the reaction mix and by the use of a template-switch oligonucleotide with one locked nucleic acid (LNA) riboguanosine base. These improvements assist in the hybridization between the template-switch oligonucleotide and the cDNA product, thereby increasing the probability of successfully introducing a second PCR adapter onto the cDNA product (see Figure 1). A second key improvement

was made in the preamplification PCR step, which can be heavily biased against either long transcripts or those containing regions with high G/C content. Picelli et al found that the preamplification PCR is improved by using the KAPA HiFi HotStart ReadyMix, which dramatically improved coverage and sensitivity, particularly for GC-rich transcripts.

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Guidelines

Introduction

The development of single-cell RNA-Seq affords new opportunities to study complex cellular systems at unprecedented resolution. While previous RNA-Seq methods were only capable of capturing the average behavior of a population, single-cell methods now enable studies of cellular heterogeneity, as well as the discovery of novel cell types and states. These methods employ a variety of techniques to address the technical challenges posed by single cells – most notably, the ultra-low input quantity of RNA.

Here, we exploit the terminal transferase activity of the SMARTScribe reverse transcriptase in conjunction with a “template-switch” primer to make cDNAs that have PCR priming sites on both ends directly from full length mRNA (Figure 1). First, single cells are lysed in a guanidine thiocyanate solution. Second, a bead cleanup is used to isolate RNA. Third, cDNA is synthesized from the full-length transcripts. Fourth, these cDNA products are amplified by PCR and normalized. Fifth, the amplified cDNA is incubated with a Tn5 transposase to fragment full-length transcripts and append adapters on each molecule. Sixth, each single-cell library is individually barcoded by PCR with index primers, and the sample set is pooled. And finally, the resulting pool is purified by a bead cleanup and is then sent for Illumina sequencing.

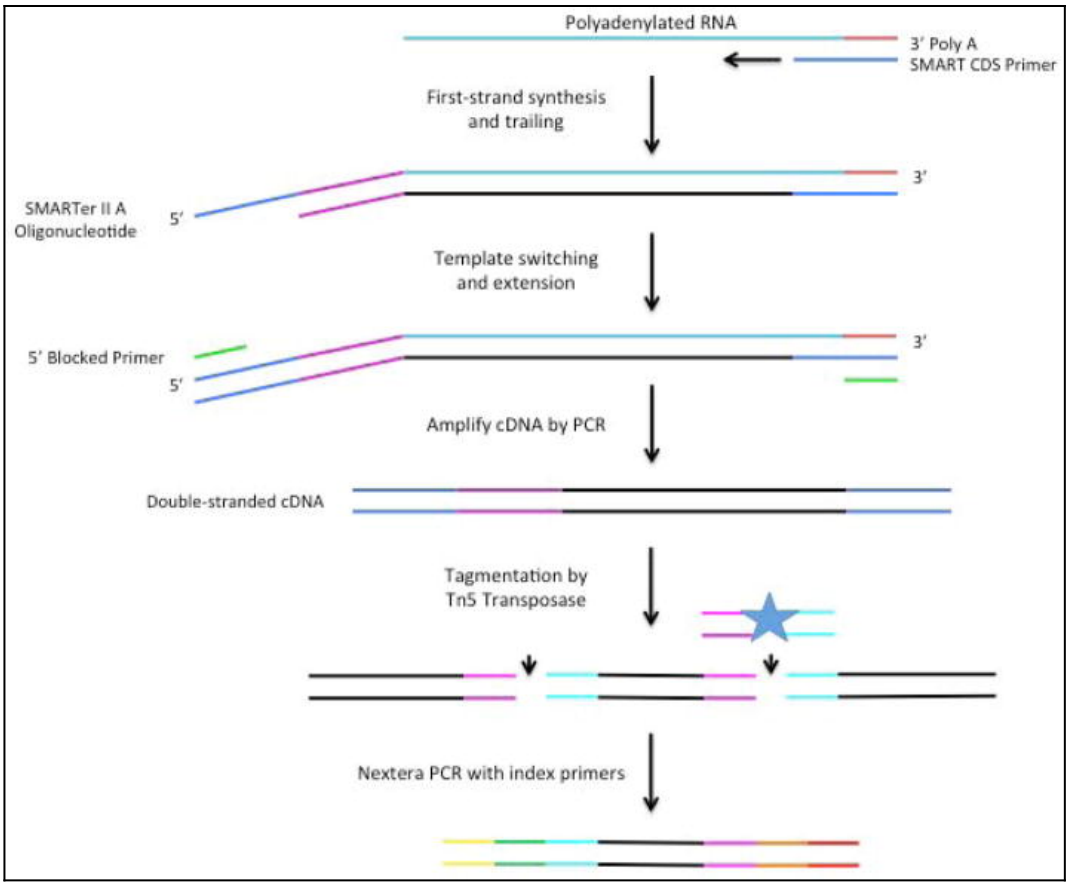

Figure 1. Single-cell RNA-Seq library construction. Illustrated are the steps required to convert mRNA to cDNA with sequencing adapters.

Oligonucleotide Primer Sequences

3′ SMART CDS Primer IIA: 5′ AAGCAGTGGTATCAACGCAGAGTACT(30)VN

SMARTer II A Oligonucleotide: 5′AAGCAGTGGTATCAACGCAGAGTACATrGrGrG

IS PCR Primer: 5′ AAGCAGTGGTATCAACGCAGAGT

TSO: 5′ AAGCAGTGGTATCAACGCAGAGTACATrGrG+G

The protocol workflow is as follows:

Stage I: Preparation of single-cell lysates

Note: In this protocol, we use Microseal F to seal for long-term storage and Microseal B when thermal cycling.

Stage II: RT of mRNA species

Note: These steps can be performed inside a biosafety cabinet or an RNA workstation (if available); otherwise, they can be carefully performed on a standard benchtop.

Stage III: Performing WTA and Post-PCR Cleanup

Stage IV: Nextera XT sequencing-library construction

Stage V: Pooling and DNA SPRI bead cleanup

Background Information

It has long been known that population-level observations of cellular transcription do not always accurately represent transcriptional patterns across the single cells that comprise them (Eberwine et al. 1992; Sandberg 2014; Shapiro et al. 2013). Earlier methods developed to explore this transcriptional heterogeneity in cellular populations first interrogated individual genes of interest in single cells (Eberwine et al. 1992), and later allowed for highly multiplexed qRT-PCR of a up to 96 genes simultaneously across 96 single cells (see, for example, Sanchez-Freire et al. 2012).

Recent technical advances that make high-throughput construction of next-generation sequencing libraries possible from picogram quantities of RNA facilitate studies of whole-transcriptome expression profiles in single cells. Relative to population measurements, single-cell RNA-Seq is able to deconvolute transcriptional profiles of cellular subpopulations and reveal transcriptional profiles of rare cellular subtypes that would be hidden below the detection threshold in population-level analysis.

A key tool that makes the construction of single-cell RNA-Seq cDNA libraries possible is the introduction of uniform adapter sequences to the transcripts of interest, allowing for the amplification and sequencing of all mRNA transcripts. A number of strategies have been developed to efficiently add these adapters. Pioneering work on mRNA-Seq utilized poly(A) tailing of reverse transcribed products followed by poly(T)-primed PCR amplification (Tang et al. 2009). CEL-Seq, a more recently published method, relies on *in vitro* transcription (IVT) to linearly amplify reverse transcribed products, followed by ligation of adapter sequences to the 3′ end of amplified RNA (Hashimshony et al. 2012). Presented here, the SMARTer protocol leverages the terminal transferase activity of a M-MLV-derived reverse transcriptase to reverse transcribe mRNA and then, with a template-switch primer, add an adapter sequence in a single reaction (Zhu et al. 2001). Each method has its own distinct advantages, disadvantages, and biases specific to the biochemical reactions underlying each protocol. For example, CEL-Seq avoids biases introduced by PCR amplification of reverse transcription products by linearly amplifying its reverse transcription products with IVT; this, however, necessitates a cleanup of both reverse transcription products and IVT amplification products prior to subsequent reactions (Hashimshony et al. 2012). With varying levels and tolerances for biases arising in specific experimental contexts, selecting the appropriate method for a given study depends on the system being assayed.

As the throughput and power of single-cell transcriptome profiling increases, these methodologies can be applied to increasingly complex biological questions. Whole-transcriptome sequencing across cell types can reveal transcriptional profiles specific to each type, as well as the

heterogeneity within them. Identification of cell types from a population of cells of unknown composition based solely on transcriptional profiles is now possible and is increasingly easy as more single-cell RNA-Seq profiling data is added to the knowledge base (Shapiro et al. 2013). Meanwhile, primary cells, or rare subtypes of cells, desirable for study, may not appear in extracted populations in sufficient frequency to conduct RNA-Seq at a population level, necessitating single-cell methods. The transcriptional profiles of a patient's tumor cells may elucidate the origin, development, and function of various cell lineages within a tumor. Other questions about cellular response to stimuli (Shalek et al. 2013), transcriptional fluctuation across time (Shapiro et al. 2013), and differential gene expression in disease or pathological tissue (Sandberg 2014) are now being explored; we envision that many more topics will be fruitfully investigated with single-cell RNA-Seq as it becomes a more widely used tool.

Critical Parameters and Troubleshooting

Proper sample preparation is key when preparing viable single-cell RNA-Seq libraries, as degradation and contamination of input material are the most common sources of error in downstream steps. Prior to sorting, sample integrity can be improved by minimizing the time spent preparing cells for sorting, and then freezing lysate plates no more than 10 minutes after sorting has concluded. When preparing for cDNA synthesis, it is vitally important to ensure that all workspaces, equipment, and reagents are RNase free by using products such as RNase-ZAP and RNase inhibitors. Cross contamination is also of great concern throughout this protocol, and care should be taken to avoid sharing tips between samples. Additionally, samples should be covered whenever possible to prevent airborne contamination.

Because sample integrity is a common source of error, and because small quantities of starting material are frequently used in this protocol, several parameters must be assayed following cDNA synthesis, amplification and cleanup to ensure library quality. Average fragment size, cDNA concentration, and the presence of transcripts for housekeeping genes are effective measures of library quality. Measuring, via qPCR, at least two well-established housekeeping genes is particularly important at this stage. In virtually any set of singe cell libraries, there will be samples of low complexity that lack expression of key housekeeping genes, and the results of qPCR measurements can be used to eliminate these samples.

The Tn5 transposase used in Nextera XT library construction fragments dsDNA at a rate proportional to [dsDNA] in your library. Thus, it is crucial to normalize the concentration of your SMARTer single-cell amplified cDNAs prior to Nextera XT library construction. This ensures that dsDNA from each single cell sample will yield tagmentation products with similar distributions of fragment length, and further that after pooling, cleanup, and sequencing the distribution of reads across each sample will be unbiased.

Table 1. Troubleshooting for Single-Cell RNA-Seq Library Preparation

Problem	Likely Cause	Solution

Little or no WTA yield	RNA degradation	Start with high-quality RNA; use RNA with RNA integrity number >8; begin with a single RNA sample and work quickly; work in an RNase-free environment
	RNA-SPRI failure	Make sure beads have been fully resuspended prior to use; make sure beads are never exposed to RNases. Use freshly prepared ethanol. Don't overdry beads.
	Reverse transcription failure	Make sure all primers are added to the correct reaction.
	Too few PCR cycles	Increase nul/exZc mber of PCR cycles
	PCR failure	Poor library generation, perhaps due to inactive enzymes/reagents
	Cell sorting failure	Make sure cell sorter dispenses cells accurately to center of each well
Presence of WTA products that are longer than expected (>4 kb)	Too many PCR cycles	Reduce number of PCR cycles
Presence of WTA products that are shorter than expected (<500 bp)	RNA degradation	Start with high-quality RNA; use RNA with RNA integrity number >8; begin with a single RNA sample and work quickly; work in an RNase-free environment
	PCR-SPRI failure or ineffective cleanup	Make sure beads have been fully resuspended prior to use; repeat 0.8X SPRI cleanup
Regularly-spaced spiky peaks on WTA Bioanalyzer trace	Contamination	Make sure to prepare samples and reactions in a clean and sterile environment
Small fragment size after Nextera	Tagmentation reaction ran too long	Reduce reaction time of Nextera tagmentation
	Low tagmentation input cDNA concentration	Make sure tagmentation input cDNA is at the correct concentration; make sure the cDNA Bioanalyzer traces show little primer contamination (<500 bp peaks) after Nextera
Low alignment rate of reads to genome	Contamination	Make sure to prepare samples and reactions in a clean and sterile environment
Low library complexity/ number of genes detected	Too many PCR cycles	Reduce number of PCR cycles
	Loss of material prior to PCR	Make sure loss of material is limited during the initial RNA-SPRI cleanup

Time Considerations

The SMARTer library construction and normalization take 2–3 days, and Nextera XT library construction and pooling takes 1 day.

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Before start

TE Buffer, 1x

- 10 mM Tris-HCl, pH 8.0
- 1 mM EDTA

Materials

✓ RNAse-free Water by Contributed by users

HotStart ReadyMix (KAPA HiFi PCR kit) KK2601 by Kapa Biosystems

Superase-In RNase Inhibitor AM2694 by Thermofisher

Microseal® 'F' Foil MSF-1001 by BioRad Sciences

Microseal® 'B' Adhesive Seals MSB-1001 by BioRad Sciences

✓ 2-mercaptoethanol, ≥ 99.0% by Contributed by users

Buffer TCL 1031576 by Qiagen

Eppendorf twin.tec® PCR 96-well plate, skirted 951020401 by Eppendorf

✓ Dry ice by Contributed by users

✓ RNeasy Micro Kit by Contributed by users

Agencourt RNAClean XP SPRI beads A63987 by Beckman Coulter

RNaseZap® RNAse Decontamination Solution AM9780 by Life Technologies

✓ RNase-free water by Contributed by users

✓ 200 proof ethanol by Contributed by users

SMARTer® Ultra™ Low Input RNA Kit for

Illumina® Sequencing-HV, 96 reactions 634828 by Clontech

Advantage® 2 PCR Kit, 100 reactions 639206 by Clontech

Agencourt AMPure XP SPRI beads A63881 by Beckman Coulter

✓ TE Buffer, 1x (see recipe) by Contributed by users

Agilent High Sensitivity DNA Kit 5067-4626 by Agilent Technologies

Qubit® dsDNA HS assay kit, 100 reactions Q32851 by Life Technologies

Qubit® Assay Tubes Q32856 by Life Technologies

Taqman® Fast Advanced Master Mix, 100 reactions 4444557 by Life Technologies

✓ Taqman® Probe set (Choose housekeeping genes that are appropriate for your system) by Contributed by users

Nextera XT DNA Sample Preparation Kit, 96 samples FC-131-1096 by illumina

Nextera XT Index Kit, 96 indices, 384 samples FC-131-1002 by illumina

TruSeq Index Plate Fixture Kit FC-130-1005 by illumina

✓ Plate centrifuge by Contributed by users

✓ Vortex by Contributed by users

DynaMag™-96 side skirted magnet 12027 by Life Technologies

✓ RNAse and DNAse-free 1.5ml tubes by Contributed by users

✓ Thermal cycler by Contributed by users

Qubit® 2.0 Fluorometer Q32866 by Life Technologies

2100 Electrophoresis Bioanalyzer Instrument G2939AA by Agilent Technologies

DynaMag™-2 Magnet 12321D by Life Technologies

Triton X-100 T8787 by Sigma Aldrich

✓ 10 µM Reverse transcription DNA oligonucleotide primer (custom synthesized by Integrated DNA Technologies, see table for sequence) by Contributed by users

dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10mM) R0192 by Thermo Fisher Scientific

SuperScript™ II Reverse Transcriptase Kit 18064-014 by Life Technologies

✓ 5 M betaine by Contributed by users

✓ 100 mM MgCl2 by Contributed by users

✓ 10 µM SMARTer TSO primer (custom synthesized by Exiqon, see table for sequence) by Contributed by users

✓ 10 µM PCR oligonucleotide primer (custom synthesized by Integrated DNA Technologies, see table for sequence) by Contributed by users

Protocol

Stage I: Preparation of single-cell lysates

Step 1.

Prepare a mild hypotonic lysis buffer of 0.2% Triton X-100 and 2 U/µL RNase-Inhibitor and distribute 4 µL of this solution into each well of a skirted-side 96-well PCR plate, and 350 µL into a 1.5 mL RNase-free centrifuge tube. Cover this plate with Microseal F and keep at room temperature until ready for single-cell isolation.

NOTES

Anita Bröllochs 31 Mar 2018

In this protocol, we use Microseal F to seal for long-term storage and Microseal B when thermal cycling.

Stage I: Preparation of single-cell lysates

Step 2.

Prepare a cell suspension in complete media and use a FACS machine to sort a single cell into each well of the abovementioned 96-well plate containing Buffer TCL, and an additional sample of ≥10,000 cells into the 1.5 mL centrifuge tube to use as a population control.

NOTES

Anita Bröllochs 31 Mar 2018

To improve yield, we recommend sorting single cells on both the presence of a positive viability indicator (e.g., Calcein AM, Life Technologies) and the absence of a cell death marker (e.g., the membrane-impermeant DNA stain EthD-1, Life Technologies).

Stage I: Preparation of single-cell lysates

Step 3.

Once sorting is completed seal plate with Microseal F and centrifuge (800g, 1 min).

Stage I: Preparation of single-cell lysates

Step 4.

Immediately freeze plate and population control on dry ice and keep at −80°C until ready for lysate cleanup.

TEMPERATURE

-80 °C Additional info:
Freezing

Stage II: RT of mRNA species

Step 5.

Bring RNA-SPRI beads (Agencourt RNAClean XP SPRI beads) to room temperature (allow 30 minutes) and use RNAseZap to clean workbench and all equipment used to process RNA.

Stage II: RT of mRNA species

Step 6.

Add 1 µL of 10 µM anchored oligo-dT RT primer and 1 µL of 10 mM dNTP mix to each lysate well, seal plate with Microseal 'B' and centrifuge (800g, 1 min).

AMOUNT

1 µl Additional info: 10 µM
anchored oligo-dT RT
primer

AMOUNT

1 µl Additional info: 10 mM
dNTP mix

Stage II: RT of mRNA species

Step 7.

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

TEMPERATURE

72 °C Additional info:
Incubation

Stage II: RT of mRNA species

Step 8.

Centrifuge plate (800g, 1 min), unseal, and add the following as a mastermix:

- 2 µL SuperScriptII first strand buffer
- 0.25 µL 100 mM DTT
- 2 µL 5M betaine
- 0.9 µL 100 mM $MgCl_2$
- 1 µL 10 µM template switching oligonucleotide
- 0.1 µL H_2O

- 0.25 µL 40 U/µL RNase-Inhibitor
- 0.5 µL 200 U/µL SuperScriptII RT

AMOUNT

2 µl Additional info: SuperScriptII first strand buffer

AMOUNT

0.25 µl Additional info: 100 mM DTT

AMOUNT

2 µl Additional info: 5M betaine

AMOUNT

0.9 µl Additional info: 100 mM MgCl2

AMOUNT

1 µl Additional info: 10 µM template switching oligonucleotide

AMOUNT

0.1 µl Additional info: H2O

AMOUNT

0.25 µl Additional info: 40 U/µL RNase-Inhibitor

AMOUNT

0.5 µl Additional info: 200 U/µL SuperScriptII RT

Stage II: RT of mRNA species

Step 9.

Mix well by pipetting, seal, and centrifuge (800g, 1 min).

Stage II: RT of mRNA species

Step 10.

Carry out the following RT in a thermal cycler using the following conditions:

Initial step:	90 min	42°C
10 cycles:	2 min 2 min	50°C 42°C
Inactivation:	15 min	70°C
Cool:	Hold	4°C

Stage III: Performing WTA and Post-PCR Cleanup

Step 11.

Add the following as a mastermix:

- 13 µL KAPA HiFi HotStart ReadyMix
- 1 µL 10 µM IS PCR primer

AMOUNT

13 µl Additional info: KAPA HiFi HotStart ReadyMix

AMOUNT

1 µl Additional info: 10 µM IS PCR primer

Stage III: Performing WTA and Post-PCR Cleanup

Step 12.

Carry out the amplification in a thermal cycler using the following conditions:

Initial step:	3 min	98°C
20 cycles:	15 sec 20 sec 6 min	98°C 67°C 72°C
Extension:	5 min	72°C
Cool	Hold	4°C

NOTES

Anita Bröllochs 08 Apr 2018

Following this step, the product can be stored at 4°C overnight.

Stage III: Performing WTA and Post-PCR Cleanup

Step 13.

Bring DNA SPRI beads (Agencourt AMPure XP) to room temperature.

Stage III: Performing WTA and Post-PCR Cleanup

Step 14.

Centrifuge WTA plate (800g, 1 min).

Stage III: Performing WTA and Post-PCR Cleanup

Step 15.

Unseal and add 0.8 volumes of DNA SPRI beads to each well and mix well by pipetting.

Stage III: Performing WTA and Post-PCR Cleanup

Step 16.

Cover with a clean lid from a pipette tip box and incubate bead suspension for 5 min on bench.

Stage III: Performing WTA and Post-PCR Cleanup

Step 17.

Place plate on 96-well plate magnet and incubate for another 5 minutes.

Stage III: Performing WTA and Post-PCR Cleanup

Step 18.

Wash beads by adding 100 µL 80% ethanol to each well and move the plate left and right on the magnet to move the beads from side to side in each well. Continue shifting plate on magnet for approximately 30 sec. (1/3)

AMOUNT

100 µl Additional info:
80% ethanol

Stage III: Performing WTA and Post-PCR Cleanup

Step 19.

Aspirate ethanol. (1/3)

Stage III: Performing WTA and Post-PCR Cleanup

Step 20.

Wash beads by adding 100 µL 80% ethanol to each well and move the plate left and right on the magnet to move the beads from side to side in each well. Continue shifting plate on magnet for approximately 30 sec. (2/3)

AMOUNT

100 µl Additional info:
80% ethanol

Stage III: Performing WTA and Post-PCR Cleanup

Step 21.

Aspirate ethanol. (2/3)

Stage III: Performing WTA and Post-PCR Cleanup

Step 22.

Wash beads by adding 100 µL 80% ethanol to each well and move the plate left and right on the magnet to move the beads from side to side in each well. Continue shifting plate on magnet for approximately 30 sec. (3/3)

AMOUNT

100 µl Additional info:

80% ethanol

Stage III: Performing WTA and Post-PCR Cleanup

Step 23.

Aspirate final ethanol wash. Leave the plate on the magnet and allow beads to dry at room temperature for approximately 10 minutes. Keep plate covered with the lid of a pipette tip box (left slightly ajar) to prevent dust and debris from falling into samples.

Stage III: Performing WTA and Post-PCR Cleanup

Step 24.

Once bead pellet has dried, remove plate from magnet and elute DNA from beads by resuspending dried beads with 20 µL TE buffer. Transfer eluent to a new 96-well plate.

AMOUNT

20 µl Additional info: TE
buffer

NOTES

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Following this step, the new product plate can be sealed and stored at 4°C overnight or at −20°C for months.

Stage III: Performing WTA and Post-PCR Cleanup

Step 25.

Use 1 µL of the purified PCR product to measure the fragment size distribution using the Agilent HS DNA BioAnalyzer or similar instrumentation (as per the manufacturer's recommendations) and 1 µL to estimate the library concentration using the Qubit® dsDNA HS Assay kit with Qubit® assay tubes and the Qubit® fluorometer or similar instrumentation (as per the manufacturer's recommendations).

AMOUNT

2 µl Additional info:
Purified PCR product

Stage III: Performing WTA and Post-PCR Cleanup

Step 26.

Use 1 µL of the purified PCR product to measure single-cell viability with the Taqman qPCR assay.

AMOUNT

1 µl Additional info:
Purified PCR product

NOTES

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*In most single-cell sample sets, a small subset of libraries will have low complexity and will lack expression of highly expressed housekeeping genes. Since there are, in most systems, a set of housekeeping genes that should be expressed in every cell (*Shalek et al., 2013*), we use qPCR of two housekeeping genes (usually ACTB and B2M) to identify viable samples. Samples with low or no expression of these genes can be removed from Nextera library construction to reduce cost.*

Stage III: Performing WTA and Post-PCR Cleanup

Step 27.

For each sample, dilute the purified PCR product to a concentration of between 0.1 and 0.2 ng/µL with buffer TE in preparation for Nextera XT library construction.

Stage IV: Nextera XT sequencing-library construction

Step 28.

Before beginning library construction, thaw index primers and mix by brief vortexing followed by centrifugation. Arrange Nextera index tubes in the TruSeq Index Plate Fixture, such each slot in the fixture holds one index tube. The 12 index 1 (i7) primers, which have orange caps, should be arranged in order horizontally, such that each tube corresponds to a column (Figure 2). The column 1 slot should hold N701 and the column 12 slot should hold N712. Similarly, the index 2 (i5) primers should be arranged in order vertically, such that S501 is in the slot for row A and S508 is in the slot for row H.

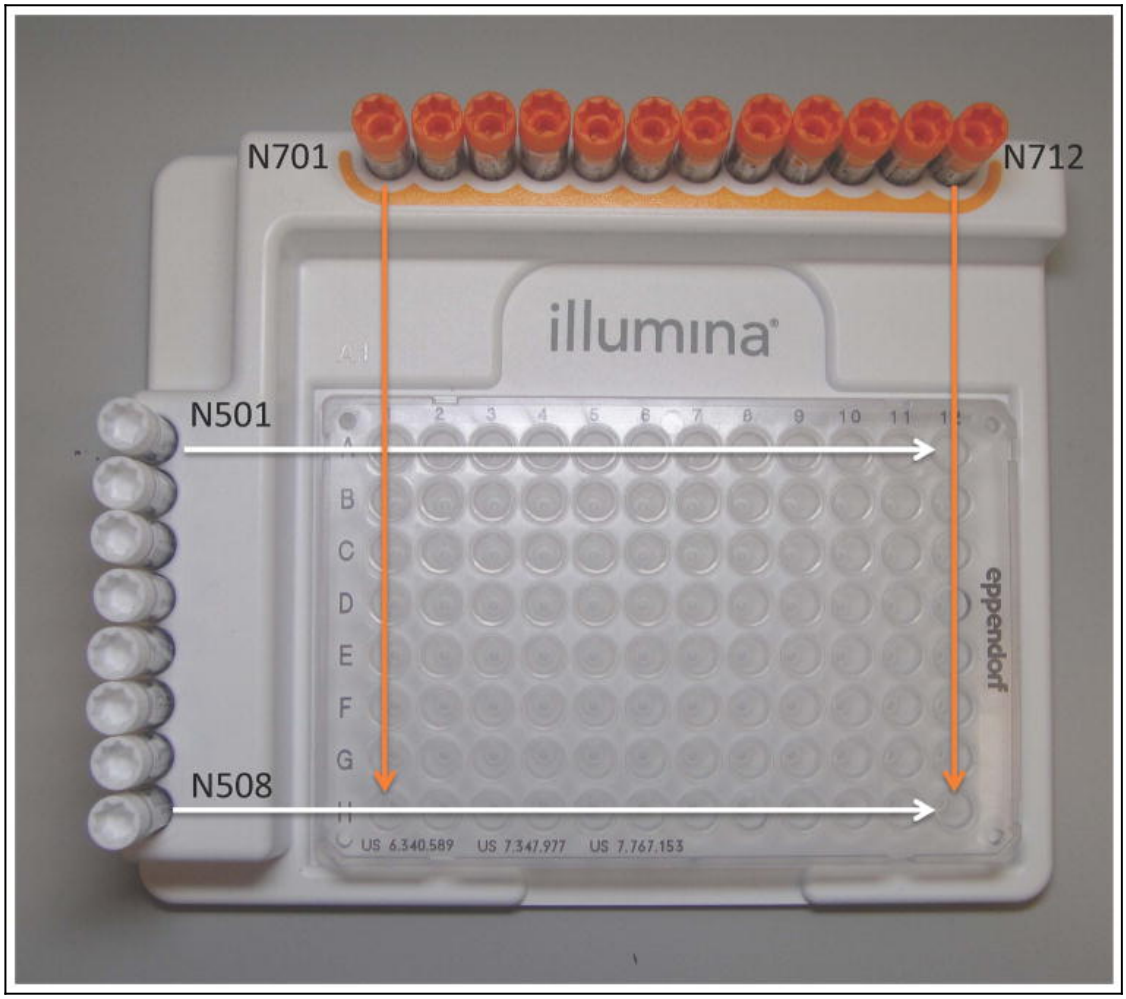

Figure 2

Layout to prepare the combined dual-index primer plate that is used in Nextera XT library construction.

Using a multichannel pipette, distribute 10 µL of each index into the corresponding row/column. (i7 primers will be distributed down rows, while i5 primers will be distributed across columns.)
Seal, vortex mildly to mix, and then centrifuge (800g, 1 min). These mixed index primers can be used immediately, or stored long-term at −20°C.

NOTES

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There is enough combined index primer solution in each well to generate 8 × 96 single-cell libraries.

Stage IV: Nextera XT sequencing-library construction

Step 29.

Using a new 96-well plate, add to each well:
2.5 µL TD Buffer
1.25 µL ATM
1.25 µL Diluted PCR product from SMART RT-PCR

Mix well by pipetting, seal, and centrifuge (800g, 1 min).

AMOUNT

2.5 µl Additional info: TD Buffer

AMOUNT

1.25 µl Additional info: ATM

AMOUNT

2.35 µl Additional info: Diluted PCR product from SMART RT-PCR

Stage IV: Nextera XT sequencing-library construction

Step 30.

Carry out tagmentation using the following conditions:

Initial step:	10 min	55°C
Cool:	Hold	10°C

Stage IV: Nextera XT sequencing-library construction

Step 31.

Unseal and immediately add 1.25 µL NT Buffer to each well and mix well by pipetting.

Stage IV: Nextera XT sequencing-library construction

Step 32.

Wait 5 minutes at room temperature.

Stage IV: Nextera XT sequencing-library construction

Step 33.
Add 3.75 µL NPM and 2.5 µL combined Index primer solution to each well. Mix well by pipetting, seal, and centrifuge (800g, 1 min).

Stage IV: Nextera XT sequencing-library construction

Step 34.
Carry out the amplification in a thermal cycler using the following conditions:

Initial step:	3 min	72°C (initial annealing)
1 cycle:	30 sec	95°C (denaturation)
12 cycles:	10 sec 30 sec 60 sec	95°C 50°C 72°C
1 cycle:	5 min	72°C
Cool:	Hold	4°C

NOTES

Anita Bröllochs 08 Apr 2018

Following this step, the product can be stored at 4°C overnight.

Stage V: Pooling and DNA SPRI bead cleanup

Step 35.
Bring DNA SPRI beads (Agencourt AMPure XP) to room temperature.

Stage V: Pooling and DNA SPRI bead cleanup

Step 36.
Spin down plate (800g, 1 min), unseal, and pool 2.5 µL from each well into a single 1.5 mL tube. Measure total volume of resulting pool and add 0.9 volumes SPRI beads to tube and mix well by pipetting.

Stage V: Pooling and DNA SPRI bead cleanup

Step 37.
Incubate bead suspension for 5 min on bench.

Stage V: Pooling and DNA SPRI bead cleanup

Step 38.
Place tube on Dynamag™-2 magnet, and incubate for 5 min.

Stage V: Pooling and DNA SPRI bead cleanup

Step 39.
Wash beads by adding 500 µL 80% ethanol to the tube and rotate it on the magnet to move the beads from front to back across the tube. (1/2)

AMOUNT

500 µl Additional info:
80% ethanol

Stage V: Pooling and DNA SPRI bead cleanup

Step 40.

Aspirate ethanol.

Stage V: Pooling and DNA SPRI bead cleanup

Step 41.

Wash beads by adding 500 µL 80% ethanol to the tube and rotate it on the magnet to move the beads from front to back across the tube. (2/2)

AMOUNT

500 µl Additional info:
80% ethanol

Stage V: Pooling and DNA SPRI bead cleanup

Step 42.

Aspirate final ethanol wash. Leave the tube on the magnet and allow beads to dry at room temperature for approximately 10 minutes. Keep the tube covered to prevent dust and debris from falling into your sample.

Stage V: Pooling and DNA SPRI bead cleanup

Step 43.

Once bead pellet has dried, remove the tube from the magnet and elute DNA from beads using 30 µL TE buffer (consider smaller volumes if working with fewer than 96 samples). Transfer eluent to a new 1.5 mL tube and repeat 0.9X SPRI bead cleanup once.

AMOUNT

30 µl Additional info: TE
buffer

GOTO

Repeating 0.9X SPRI bead cleanup -> go to step #53

Stage V: Pooling and DNA SPRI bead cleanup

Step 44.

Use 1 µL of the purified PCR product to measure the fragment size distribution using the Agilent HS DNA BioAnalyzer or similar instrumentation (as per the manufacturer's recommendations) and 1 µL to estimate the library concentration using the Qubit® dsDNA HS Assay kit with Qubit® assay tubes and the Qubit® fluorometer or similar instrumentation (as per the manufacturer's recommendations).

AMOUNT

2 µl Additional info:

Purified PCR product

Warnings

Please refer to the SDS (Saftey Data Sheet) for safety warning and hazard information.