Unprecedented sensitivity with SMART-Seq® single cell technology

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Abstract

Since the emergence of next-generation sequencing (NGS), the importance of and demand for single-cell analysis have risen rapidly. Extracting meaningful biological information from the small amount of mRNA present in each cell requires an RNA-seq preparation method with exceptional sensitivity and reproducibility. To date, the SMART-Seq v4 kit (SSv4) has been the most sensitive commercial single-cell RNA-seq method, in part due to its incomparable capability to retrieve information from full-length mRNA and not just the 3′ end. However, there is still room for improvement for extremely challenging samples such as cells with very low RNA content or nuclei. To address this need, we have further modified our core technology to create a new chemistry with higher sensitivity—the SMART-Seq Single Cell Kit—that outperforms all current commercial and noncommercial full-length methods, particularly with as little as 2 pg of total RNA. When validating with a B lymphocyte cell line or peripheral blood mononuclear cells from a healthy donor, we were able to detect 50–60% more genes with the new chemistry compared to current methods. The improvement in sensitivity was associated with a clear reduction of the dropout rate as well as an increase in reproducibility. In addition, the new SMART-Seq single cell (SSsc) chemistry generates a high yield of cDNA, which is extremely useful when dealing with difficult cells such as clinical samples that tend to carry very low RNA content.

1 Improved chemistry, same easy workflow

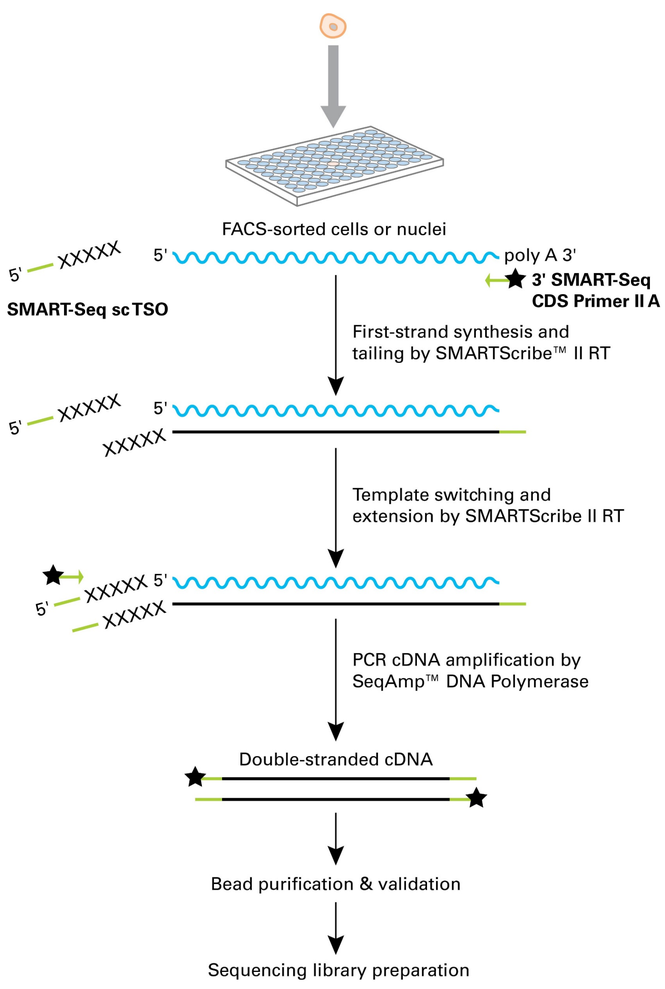


Figure 1. SMART-Seq Single Cell Kit technology and workflow. SMART® technology (Chenchik et al. 1998) is used in a ligation-free workflow to generate full-length cDNA. The reverse transcriptase (RT) adds nontemplated nucleotides (indicated by Xs) that hybridize to the SMART-Seq sc TSO, providing a new template for the RT. Chemical modifications to block ligation during sequencing library preparation are present on some primers (indicated by the black stars). The SMART adapters, added by the oligo(dT) primer (3′ SMART-Seq CDS Primer II A) and SMART-Seq sc TSO, are indicated in green and used for amplification during PCR. The amplified cDNA is then purified, quantified, and used for sequencing library preparation (Illumina® Nextera® XT kit).

Methods

All cells were labeled with CD81-FITC antibody and 7-AAD (for distinguishing live from dead cells) prior to sorting using a BD FACSJazz cell sorter into a 96-well plate or PCR strips. After sorting, cells were flash frozen on dry ice, and then stored at –80°C until ready to use. Unless otherwise noted, all libraries created with the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing and the SMART-Seq Single Cell Kit were processed at full volume per the user manuals. For the comparison to the Smart-seq2 protocol, cells were sorted and processed as described in Picelli et al. 2014.

For processing the SMART-Seq Single Cell Kit at quarter volume (Section 5), the lysis buffer containing the 3′ SMART-Seq CDS Primer II A oligo was prepared as in the full-volume protocol, but only 3.5 µl was aliquoted in each well prior to cell sorting. A quarter volume of the reverse transcription master mix was added to start the reverse transcription, bringing the volume to slightly over 5 µl. The PCR was started by adding 0.25 µl of PCR Primer, 0.4 µl of SeqAmp DNA Polymerase, 10 µl of SeqAmp CB Buffer, and Nuclease-Free Water up to a 20 µl total volume. PCR cycling and subsequent steps were performed as in the full-volume protocol.

Sequencing libraries were generated using 125 pg of cDNA and the Nextera XT DNA Library Preparation Kit (Illumina) with a quarter of the recommended volume, as described in the SMART-Seq Single Cell Kit User Manual. Libraries were sequenced on a NextSeq® 500 instrument using 2 x 75 bp paired-end reads, and analysis was performed using CLC Genomics Workbench (mapping to the human [hg38] genome with Ensembl annotation). All percentages shown—including the number of reads that map to introns, exons, or intergenic regions—are percentages of mapped reads in each library.

References

Chenchik, A., Zhu, Y., Diatchenko, L., Li., R., Hill, J. & Siebert, P. Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In RT-PCR Methods for Gene Cloning and Analysis. Eds. Siebert, P. & Larrick, J. (BioTechniques Books, MA), pp. 305–319 (1998).

Picelli, S., Faridani, O. R., Bjorklund, A. K., Winberg, G., Sagasser, S. & Sandberg, R. Full-length RNA-Seq from single cells using Smart-seq2. *Nat. Protoc.* **9,** 171–181 (2014).

2 Superior sensitivity and reproducibility with the SMART-Seq Single Cell Kit

Sequencing metrics comparing SMART-Seq v4 kit and SMART-Seq Single Cell Kit						
RNA source	2 pg UHR total RNA					
cDNA synthesis method	**SSv4**			**SSsc**		
Replicate	A	B	C	A	B	C
cDNA yield (ng)	7.8	6.9	5.5	14.8	14.9	9.6
Number of genes with TPM >0.1	7,412	7,522	7,487	8,774	8,614	8,406
Number of genes with TPM >1	8,660	8,868	9,240	10,319	10,276	10,285
Average Pearson/Spearman	0.95/0.59			0.97/0.63		
Proportion of reads mapped (%):						
Genome	92.7	92.5	92.5	80.1	80.9	80.6
Exon	79.3	78.7	76.6	63.4	64.1	62.0
Intron	10.5	10.9	12.5	13.0	12.8	14.0
Intergenic regions	2.9	3.0	3.4	3.7	4.0	4.6
rRNA	0.8	0.7	0.6	6.1	6.0	4.3
Mitochondria	3.5	3.6	3.9	9.3	8.4	10.2

Table I. Increased sensitivity with the SMART-Seq Single Cell Kit. Replicate cDNA libraries were generated from 2 pg of Universal Human Reference (UHR) total RNA using the SMART-Seq v4 kit (SSv4) or the SMART-Seq Single Cell Kit (SSsc); all libraries were processed with 19 PCR cycles. As described in the methods, RNA-seq libraries were generated and sequences analyzed (after normalizing all samples to 1.6 million paired-end reads). SSsc identified about 15% more genes than SSv4, despite showing a higher number of undesirable reads mapping to rRNA and mitochondria.

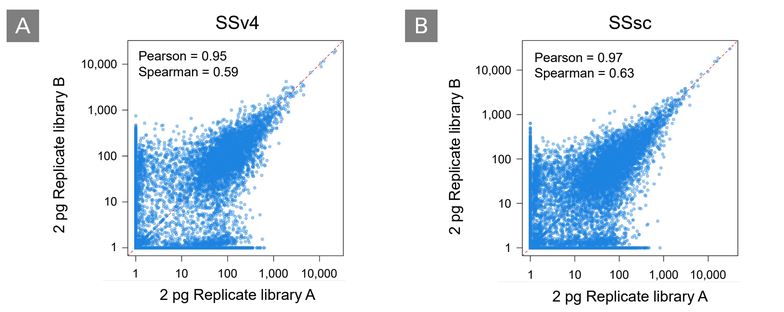


Figure 2. Increased reproducibility with the SMART-Seq Single Cell Kit. Select libraries made from 2 pg of UHR total RNA (**Table I**) were analyzed using scatter plots to visualize the reproducibility between technical replicates (shown are TPM values from all genes with a $\log_{10}$+1 scale). SSv4 (**Panel A**) generated highly reproducible quantification, but SSsc (**Panel B**) produced superior reproducibility, as seen in the increased Pearson and Spearman correlations. In addition, the scatter plots clearly illustrate that SSsc detected additional genes that are expressed at a low level.

3 Superior performance for single cells with low RNA content

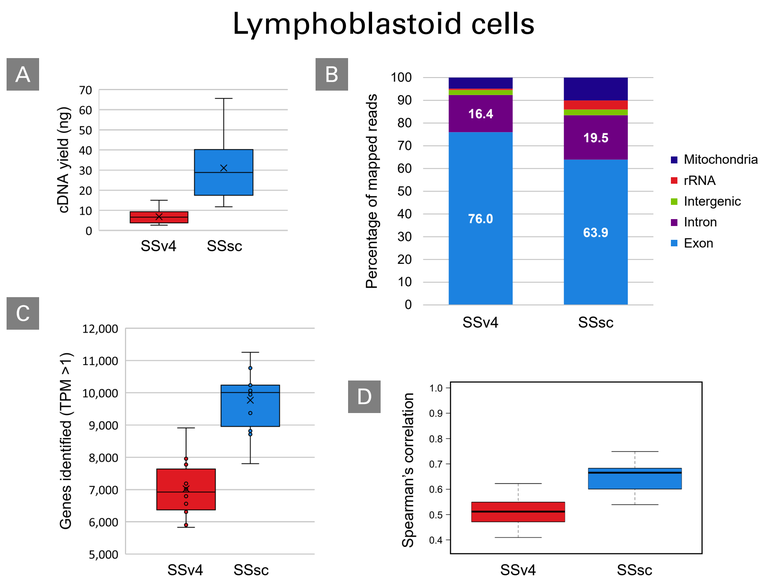


Figure 3. Improved performance for single cells with low RNA content. Twelve single cells from lymphoblastoid cell line GM22601 were processed with SSv4 or SSsc using 19 cycles of PCR. As described in the methods, RNA-seq libraries were generated and sequences analyzed (after normalizing all samples to 1.25 million paired-end reads). **Panel A.** The cDNA yield generated with SSsc is drastically higher than that generated with SSv4. **Panel B.** The read distribution is fairly similar between the two chemistries. **Panel C.** Over 50% more genes are detected in the cells processed with SSsc. **Panel D.** Correlation boxplots showing intragroup Spearman correlation between all cells processed with either method. The higher Spearman correlation among the cells processed with SSsc indicates a higher reproducibility than SSv4, in accordance with the data obtained using UHR total RNA (see Section 2).

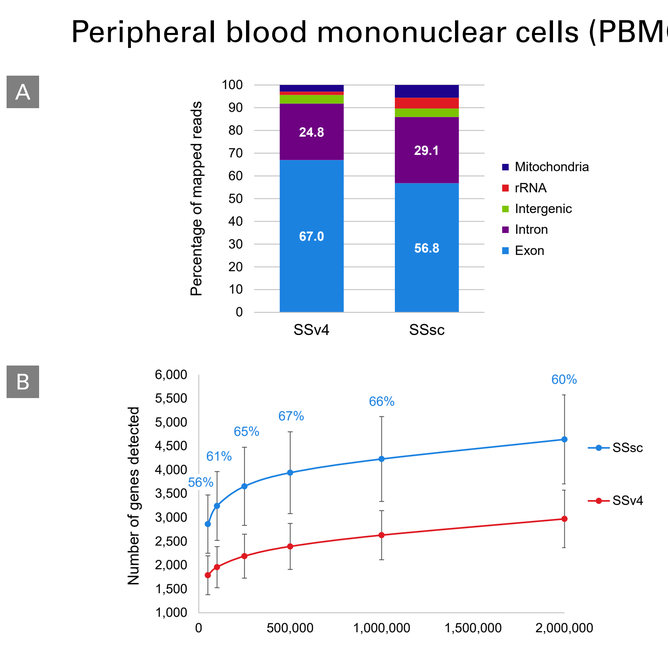
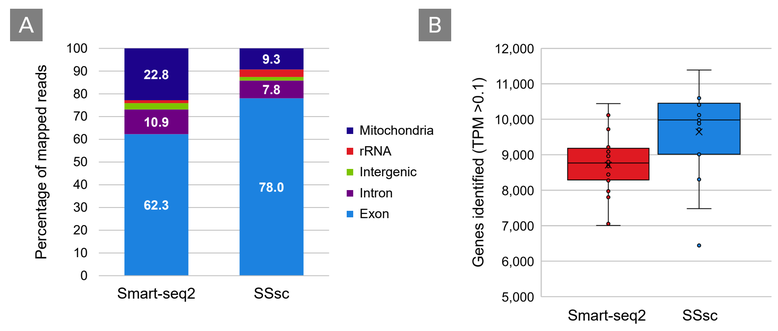


Figure 4. Improved performance with primary samples. About 50 single PBMCs from one donor were processed with SSv4 or SSsc. RNA-seq libraries were generated as described in the methods. **Panel A.** The read distribution is fairly similar between the two chemistries. **Panel B.** About 60% more genes are detected in the cells processed with SSsc, regardless of the number of reads used for the analysis.

4 Greater sensitivity and reproducibility than the popular full-length method



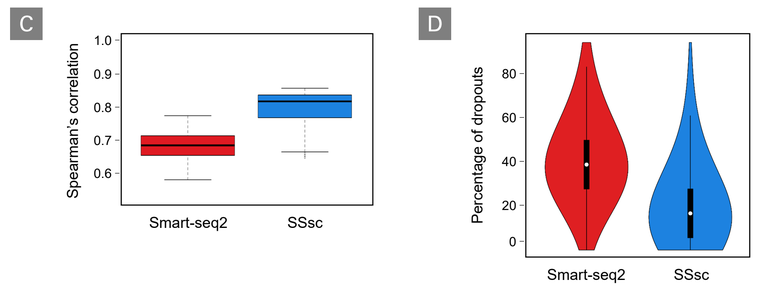


Figure 5. The SMART-Seq Single Cell Kit outperforms the Smart-seq2 protocol. Single cells from the lymphoblastoid cell line GM12878 were processed with SSsc (18 cells) or the Smart-seq2 protocol (20 cells; Picelli et al. 2014) using 19 cycles of PCR. As described in the methods, RNA-seq libraries were generated and sequences analyzed (after normalizing all samples to 1.75 million paired-end reads). **Panel A.** The read distribution is different between the two chemistries, with a drastically greater number of reads mapping to the mitochondrial genome with Smart-seq2 chemistry. This indicates that fewer reads are available for gene identification with the Smart-seq2 method. **Panel B.** More genes are detected in the cells processed with SSsc. **Panel C.** Correlation boxplots showing the intragroup Spearman correlation between all cells processed with either method. The higher Spearman correlation among the cells processed with SSsc indicates a higher reproducibility than the Smart-seq2 method. The higher reproducibility of SSsc is also demonstrated by the lower dropout rate of the genes detected with a TPM >1 (**Panel D**).

5 Uncompromised performance for miniaturization

Sequencing metrics comparing full- and quarter-volume workflows of SMART-Seq Single Cell Kit		
RNA source	Single B cells (GM12878)	
Workflow	**Full volume (20 µl)**	**Quarter volume (5 µl)**
Replicates	n = 18	n = 20
cDNA yield average (ng)	79	61
Number of genes with TPM >0.1	8,660	8,325
Number of genes with TPM >1	9,642	9,270
Proportion of reads mapped (%):		
Genome	87.4	86.5
Exon	78.0	76.9
Intron	7.8	8.2
Intergenic regions	1.6	1.4
rRNA	3.3	3.9
Mitochondria	9.3	9.6

Table II. Similar yield and sensitivity between full-volume and quarter-volume workflows. Single cells from lymphoblastoid cell line GM12878 were sorted in a full volume (12.5 µl) or quarter volume (3.5 µl) of lysis buffer containing the 3′ SMART-Seq CDS Primer II A, and cDNA libraries were generated using the regular SMART-Seq Single Cell Kit protocol (full-volume workflow; reverse transcription in 20 µl) or a quarter-volume workflow (reverse transcription in 5 µl). Pipetting for both workflows was performed using a MANTIS Liquid Handler (Formulatrix), and the PCR step was conducted with 19 cycles. The cDNA yield was similar between the two workflows, generating about 60 to 80 ng of cDNA per cell. As described in the methods, RNA-seq libraries were generated and sequences analyzed (after normalizing all samples to 1.75 million paired-end reads). The full-volume and quarter-volume workflows generated very similar mapping statistics, including the number of genes identified.

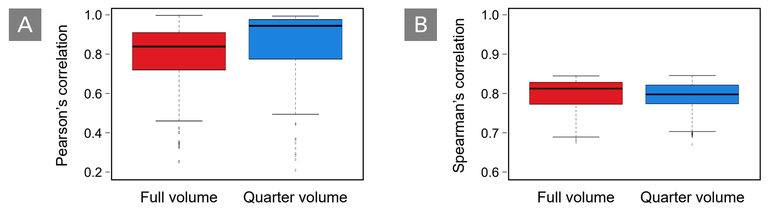


Figure 6. Similar reproducibility among the cells processed with the full-volume and quarter-volume workflows. Correlation boxplots showing intragroup Pearson (**Panel A**) or Spearman (**Panel B**) correlations between all of the cells processed at full volume or quarter volume. Processing the cells with the quarter-volume workflow neither introduced extra variability nor a significant decrease in sensitivity (see Table II), indicating that the SMART-Seq Single Cell Kit is perfectly suitable for miniaturization.

Conclusions

- The new SMART-Seq Single Cell Kit features an easy, plate-based workflow that starts directly from single cells isolated by FACS or other methods.
- It offers unparalleled sensitivity and reproducibility for single-cell, full-length RNA sequencing, particularly for cells with very low RNA content and for nuclei.
- The SMART-Seq Single Cell Kit outperforms the Smart-seq2 method.
- The SMART-Seq Single Cell Kit is compatible with miniaturization at quarter reaction volume without compromising on performance (yield, sensitivity, and reproducibility).

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