A complete, ultra-low input RNA-seq solution for full-length transcriptome analysis and RNA counting

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Abstract

RNA sequencing (RNA-seq) is a powerful way to investigate transcriptional highs and lows, allelic origins, and isoform preferences in the transcriptome that can underlie key biological states. One current limitation of single-cell RNA-seq methodologies is either the absence of unique molecular identifiers (UMIs), or the inability to maintain the yield, sensitivity, and reproducibility when UMIs are employed. To address these problems, we developed a new SMART-Seq® method that includes the use of unique molecular identifiers (UMIs) for RNA counting to allow for allelic and transcript isoform resolution analysis. We benchmarked the new SMART-Seq method against the existing SMART-Seq v4 Ultra® Low Input RNA Kit for Sequencing (SSv4), and the Smart-seq 2 (SS2) homebrew method. The inclusion of UMIs did not compromise data quality and led to superior sensitivity compared to the homebrew SS2 chemistry. In addition, we show that our new SMART-Seq method can enable RNA counting, and while optimized for low RNA input, is compatible with single-cell RNA-seq analysis. Finally, we show that our new SMART-Seq method is compatible with common automation platforms. Our data demonstrate that the new SMART-Seq method leveraging SMART® technology with UMIs for cDNA generation and our unique library preparation protocol, combined with our Cogent™ NGS analysis software, is a complete, robust, and sensitive solution for full-length transcriptome studies.

1 SSv4+UMIs sequencing workflow

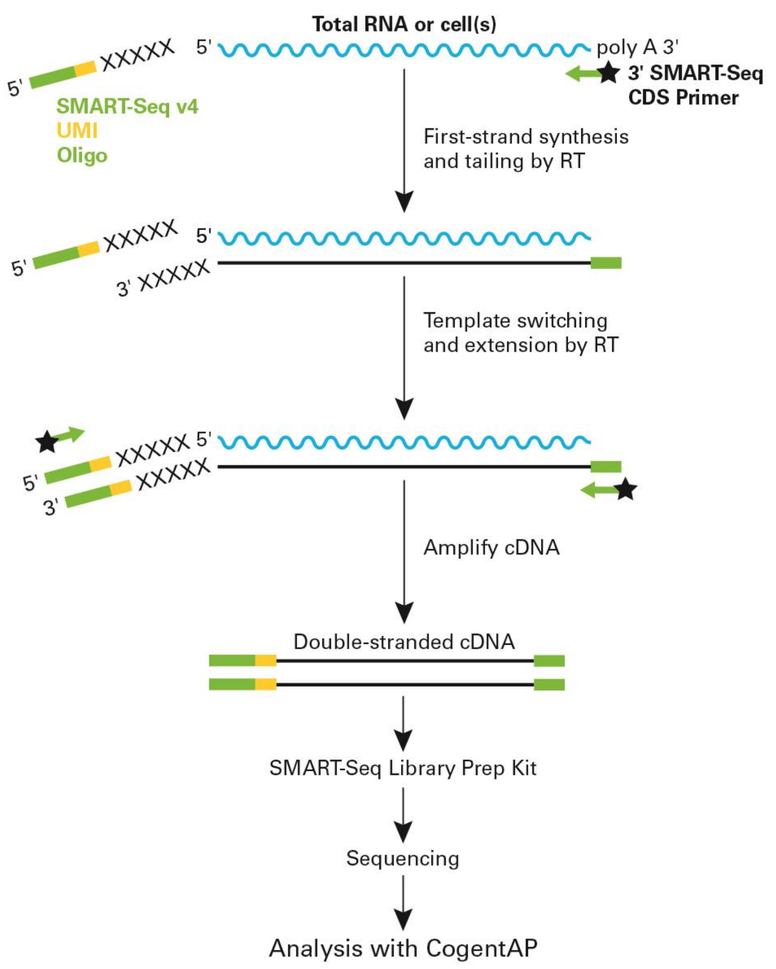


Figure 1. Library preparation and sequencing workflow for the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing using UMIs (Ssv4+UMIs). First-strand cDNA synthesis is primed by the SMART-Seq CDS Primer and performed by an MMLV-derived reverse transcriptase (RT). Upon reaching the 5′ end of each mRNA molecule, the RT adds non-templated nucleotides to the first-strand cDNA. The SMART-Seq v4 Oligonucleotide contains a sequence that is complementary to the non-templated nucleotides added by the RT and the UMI sequence. This primer hybridizes to the first-strand cDNA. In the template-switching step, the RT uses the remainder of the SMART-Seq v4 Oligonucleotide as a template for the incorporation of an additional sequence on the end of the first-strand cDNA. Then, the first-strand cDNA is amplified by PCR. In the second part of the workflow, the SMART-Seq Library Prep Kit is used to generated sequencing ready libraries. After sequencing, data is analyzed using CogentAP software.

2 SSv4+UMIs performance: reproducibility

A

		10 pg		
		Rep1	Rep2	Rep3
10 pg	Rep1	1	0.9575	0.9574
	Rep2	0.9575	1	0.9568
	Rep3	0.9574	0.9568	1

B

		100 pg			1 ng			10 ng		
		Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
100 pg	Rep1	1	0.989	0.985	0.989	0.989	0.961	0.969	0.966	0.962
	Rep2	0.989	1	0.988	0.985	0.986	0.961	0.961	0.959	0.958
	Rep3	0.985	0.988	1	0.983	0.984	0.974	0.96	0.958	0.962
1 ng	Rep1	0.989	0.985	0.983	1	0.994	0.971	0.984	0.982	0.978
	Rep2	0.989	0.986	0.984	0.994	1	0.973	0.984	0.982	0.98
	Rep3	0.961	0.961	0.974	0.971	0.973	1	0.961	0.959	0.97
10 ng	Rep1	0.969	0.961	0.96	0.984	0.984	0.961	1	0.994	0.991
	Rep2	0.966	0.959	0.958	0.982	0.982	0.959	0.994	1	0.991
	Rep3	0.962	0.958	0.962	0.978	0.98	0.97	0.991	0.991	1

0.5 — 1.0

Figure 2. SSv4+UMIs is reproducible across replicates for a broad range of input amounts. The SSv4+UMI workflow was used to create sequencing libraries from 10 pg of K562 RNA (**Panel A**) or three different amounts (100 pg, 1 ng and 10 ng), of universal human reference (UHR) RNA (**Panel B**), in triplicate. Then, the cDNA libraries were sequenced on an Illumina® platform and the RPKM was calculated using CogentAP for each experimental condition. To determine method reproducibility, the R^2 was calculated between all possible replicate pairs.

3 SSv4+UMIs is as sensitive as SSv4

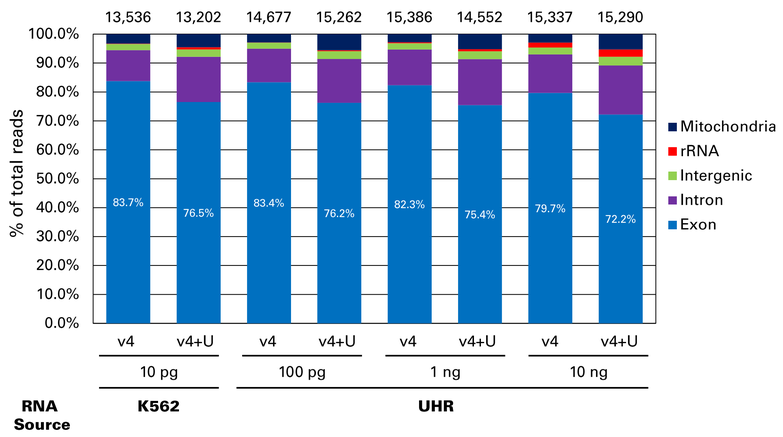
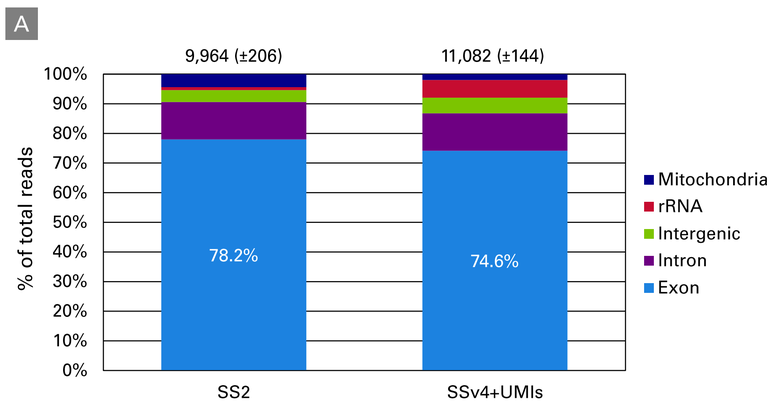


Figure 3. SSv4+UMIs demonstrate sensitivity comparable to SSv4. Gene count and read distribution across major RNA-seq output components for SSv4 (v4) and the SSv4+UMIs (v4+U) workflow. To evaluate the performance of the SSv4+UMI workflow compared to the SSv4 workflow, each method used to create sequencing libraries from K562 RNA (10 pg) or three different amounts of universal human reference (UHR) RNA (100 pg, 1 ng and 10 ng). The resulting cDNA libraries were sequenced on an Illumina platform. After sequencing, data for the 10 pg input samples were downsampled to 1.8 million reads, while data for all other input amounts were downsampled to 1.0 million reads. The average gene count for each condition is shown above the corresponding bar. The percentage of exonic reads is shown. n=3 for all conditions.

4 SSv4+UMIs outperforms SS2 in terms of sensitivity and reproducibility

A



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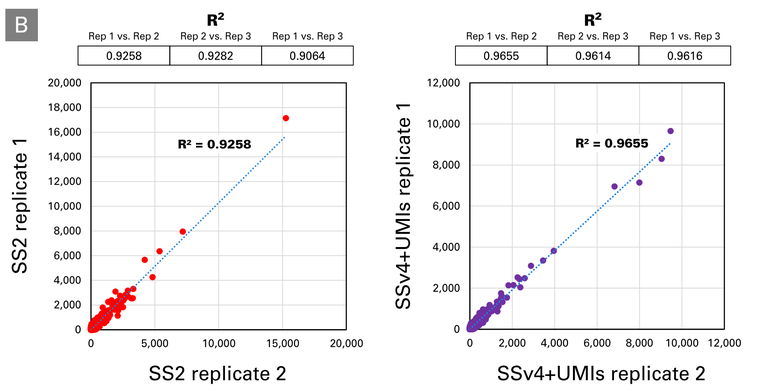


Figure 4. SSv4+UMIs outperforms SS2 in terms of sensitivity and reproducibility. Panel A. Gene count and read distribution across major RNA-seq output components for the SSv4+UMIs workflow and SS2. cDNA was generated from 10 pg UHR RNA using the SSv4+UMIs method or the SS2 method. Then, The SSv4+UMIs cDNA was prepared for sequencing using the SMART-Seq Library Preparation Kit and The SS2 cDNA was prepared for sequencing using the Nextera® XT DNA Library Prep Kit (Illumina). Libraries from both were pooled, sequenced, and downsampled to 1.2 million reads. The average gene count for each condition is shown above the corresponding bar. The percentage of exonic reads is shown. n=3 for all conditions. **Panel B.** Absolute read counts for each gene matrix between each replicate pair for SS2 and SSv4+UMIs were plotted, and regression analysis was performed. The R^2 values were consistently higher for the SSv4+UMIs workflow using the SMART-Seq Library Preparation Kit. An example XY plot for both SS2 and SSv4+UMIs are shown. All data analysis was performed with CogentAP.

5 SSv4+UMIs allows for RNA counting

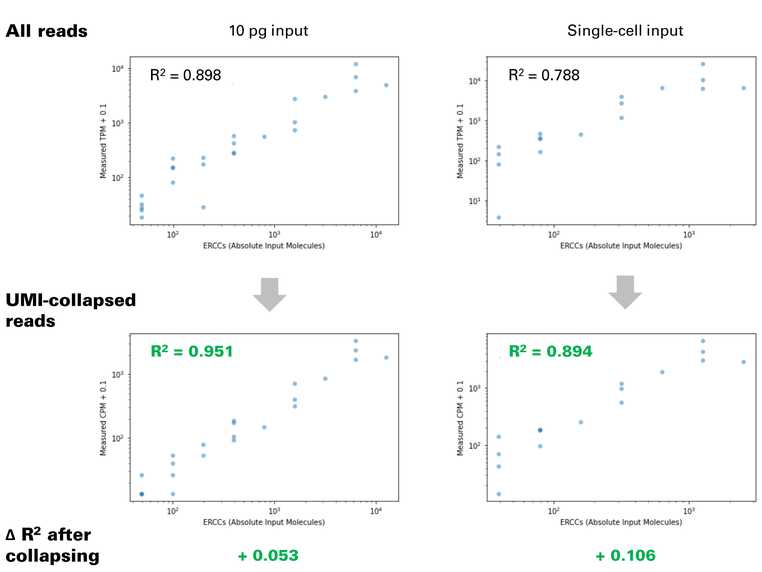
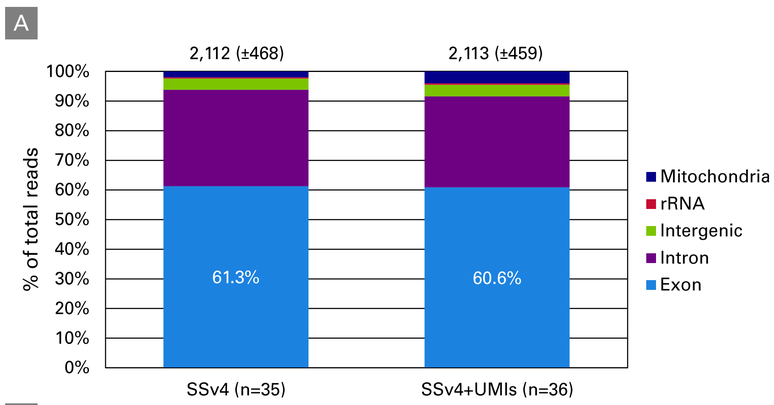


Figure 5. SSv4+UMIs reduce PCR duplications and allows for RNA counting. 10 pg of input RNA or a RNA from a single cell was spiked with ERCC control RNA, and libraries were prepared using the SSv4+UMII method. Libraries were sequenced and then analyzed using CogentAP. The measured TPM or CPM was plotted against the absolute number of input molecules from the ERCC spike-in and regression analysis was performed. There is a significant increase in the R^2 with the collapse of UMIs, indicating addition of UMIs to the SSv4 workflow leads to a reduction of PCR duplications and enables more accurate RNA counting.

6 SSv4+UMIs is compatible with single-cell inputs

A



B

Cell #

Cell type	Gene name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Leukocytes	PTPRC (CD45)	1139.9	882.5	1036.7	1330.0	1293.5	1083.0	309.8	1005.8	253.2	153.4	18.2	1031.4	336.5	574.2	1234.5	1019.1	1.6	655.3
T cells (general)	CD3E	873.9	0.0	508.1	0.0	179.0	0.0	67.1	868.4	0.0	94.5	0.0	0.0	1614.7	105.6	426.6	246.7	18.9	181.7
T cells (helper)	CD4	0.0	0.0	0.0	0.0	0.0	171.8	60.3	372.2	0.0	0.0	0.0	367.9	0.0	0.0	0.0	0.0	0.0	0.0
Cytotoxic T cells	CD8A	253.3	0.0	0.0	974.2	848.9	0.0	28.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	30.3	111.9	0.0	0.0
B cells	CD19	0.0	660.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Natural killer cells	NCAM1 (CD56)	0.0	0.0	0.0	0.0	0.0	19.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	32.9	8.7	0.0	3.2	0.0
Dendritic cells	ITGAX (CD11c)	4.3	0.0	0.0	0.0	0.0	660.5	24.3	0.0	19.7	0.0	0.0	114.9	0.0	25.8	1.6	0.0	0.0	0.0
Monocytes/macrophages	CD14	0.0	0.0	0.0	0.0	0.0	13.5	177.0	0.0	0.0	0.0	0.0	0.0	0.0	119.5	0.0	0.0	0.0	0.0
Monocytes/macrophages	CD33	0.0	0.0	0.0	0.0	0.0	0.0	9.6	0.0	0.0	0.0	0.0	0.0	0.0	52.1	0.0	0.0	0.0	0.0

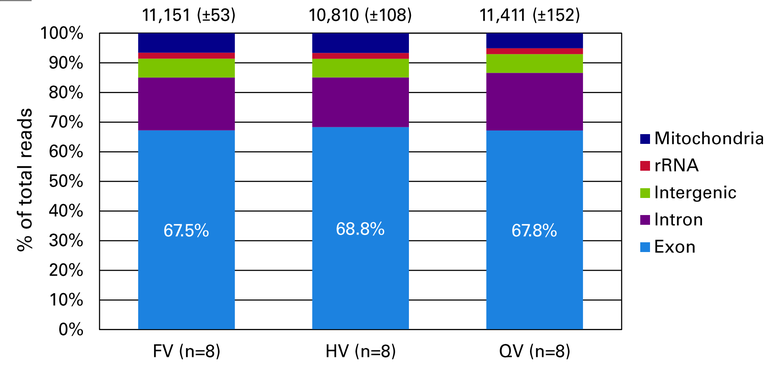
Cell type	Gene name	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Leukocytes	PTPRC (CD45)	958.8	2471.8	1749.3	988.1	764.3	2339.1	1057.3	1304.5	2376.9	796.1	1684.8	274.2	903.0	1380.3	218.0	1233.1	704.1	0.0
T cells (general)	CD3E	500.0	883.6	305.7	623.2	541.6	1662.7	69.5	367.3	93.1	331.4	0.0	203.4	0.0	521.4	0.0	0.0	201.4	10.8
T cells (helper)	CD4	0.0	0.0	60.4	0.0	0.0	0.0	5.4	0.0	0.0	0.0	0.0	166.9	0.0	0.0	0.0	0.0	0.0	0.0
Cytotoxic T cells	CD8A	0.0	0.0	32.6	690.9	195.6	1090.9	0.0	130.1	0.0	0.0	0.0	636.7	0.0	0.0	0.0	0.0	610.1	0.0
B cells	CD19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	281.7	0.0	0.0	0.0	0.0
Natural killer cells	NCAM1 (CD56)	0.0	0.0	0.0	5.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.2	0.0	0.0	0.0
Dendritic cells	ITGAX (CD11c)	0.0	6.2	7.0	10.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	470.4	0.0	239.9	0.0	0.0	0.0
Monocytes/macrophages	CD14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3785.5	0.0	0.0	0.0	0.0	0.0
Monocytes/macrophages	CD33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	21.6	0.0	0.0	0.0	0.0	0.0

0 — Highest TPM

Figure 6. SSv4+UMIs outperforms SSv4 at single-cell input levels. Panel A. Single peripheral blood mononuclear cells (PBMCs) were sorted into plates and sequencing libraries were produced using either SSv4 or the SSv4+UMIs workflow. Libraries were then pooled, sequenced, and downsampled to 900,000 reads for analysis. The gene count and read distribution for SSv4 and SSv4+UMIs were comparable. The percentage of exonic reads is shown. **Panel B.** Gene expression profiles of the 36 individual PMBCs were produced using the SSv4+UMIs workflow**.** Transcripts per kilobase million (TPM**)** for genes corresponding to cell surface markers commonly used to do cell-type identification for PBMCs by FACs are shown. Gene expression profiles identified 70% (25/36) of PMBCs as T cells, which is the expected ratio for PBMCs from a normal, healthy donor. In addition, gene expression profiles identified one B cell, one NK cell, one monocyte, and two dendritic cells. Six cells could not be clearly classified.

7 SSv4+UMIs is compatible with automation

A



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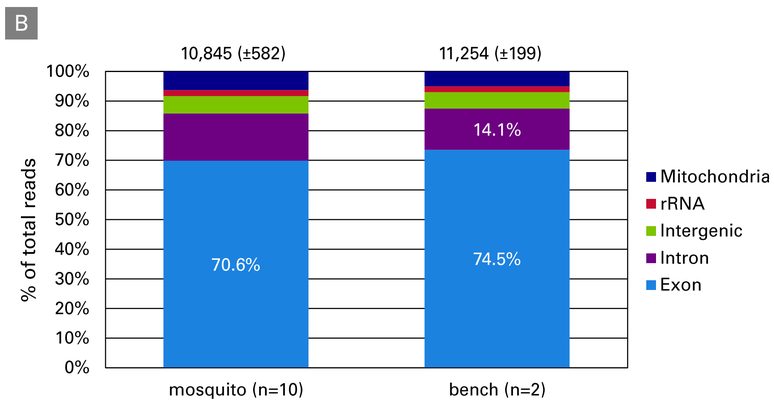

Figure 7. SSv4+UMIs is compatible automation platforms. Panel A. Read count and distribution across major RNA-seq output components using the SSv4+UMIs workflow on the Mantis Liquid Handler (Formulatrix). 10 pg of mouse brain control RNA was used as input for the SSV4+UMIs workflow at Full (FV), half (HV), and quarter (QV) volume reactions on the Mantis Liquid Handler. Libraries were pooled, sequenced, and downsampled to 1.3 million reads for analysis. Analysis was performed using CogentAP. The average gene count for each condition is shown above the corresponding bar. There is a high concordance in read distributions and gene counts across all volumes. **Panel B.** Gene count and read distribution across major RNA-seq output components using the SSv4+UMIs workflow on the mosquito HV liquid handler (SPT Labs) or on the benchtop without the aid of automation. 10 pg of mouse control RNA was used as input for the SSv4+UMIs workflow at 1/8 volume using the mosquito HV or manually on the benchtop. All libraries were prepared with the SMART-Seq Library Preparation Kit at full volume on the bench. The libraries were then pooled, sequenced, and downsampled to 620,000 reads for analysis. Analysis was performed using CogentAP. The average gene count for each condition is shown above the corresponding bar. There is a good concordance in read distributions and gene counts between the mosquito and bench preparations.

Conclusions

- Our new SMART-seq method leveraging SMART technology with UMIs (SSv4+UMIs) for cDNA generation and our unique library preparation protocol, combined with our Cogent NGS analysis software (CogentAP), is a complete, robust, and sensitive solution for full-length transcriptome studies
- The inclusion of UMIs allows for RNA counting without compromising data quality
- While SSv4+UMIs is optimized for ultra-low RNA inputs, it is compatible with single-cell inputs
- SSv4+UMIs is compatible with automation platforms

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