Complete SMART-Seq cDNA synthesis and library preparation from ultra-low RNA inputs
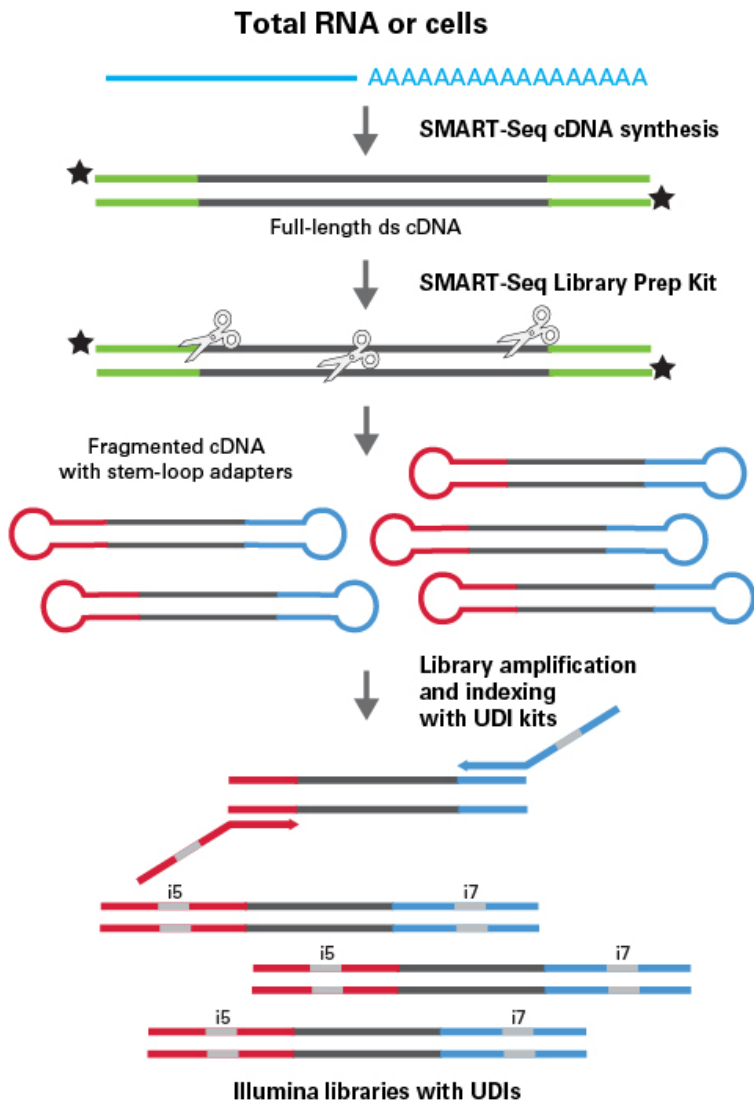
Introduction

The library input quantities (up to 310 µl of 2.5 nM pooled libraries) required to successfully load a high-throughput sequencer, such as the NovaSeq™ system, are much higher than the quantities (5 µl of 4 nM pooled libraries) required for a low-throughput sequencer like the NextSeq® system. For this reason, increased library yield is becoming a very desirable feature for library prep kits. The SMART-Seq v4 PLUS Kit (SSv4 PLUS kit) is designed to synthesize high-quality, full-length cDNA directly from multiple intact cells (up to 1,000 cells) or 10 pg–10 ng of total RNA, and generate a high yield of Illumina-ready sequencing libraries. The kit includes the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing for generating cDNA, the SMART-Seq Library Prep Kit (not sold separately) for fragmenting the cDNA and amplifying the library, and a unique dual index kit for allowing multiplexing of up to 96 samples (Figure 1, Panel A).

For cDNA generation, the SSv4 PLUS kit incorporates our patented SMART (**S**witching **M**echanism **a**t 5′ end of **R**NA **T**emplate) technology. This technology relies on the template-switching activity of reverse transcriptase to enrich for full-length cDNAs and to add defined PCR adapters directly to both ends of the first-strand cDNA (Chenchik et al. 1998). This ensures that the final cDNA libraries contain the entire length of the mRNA, including the 5' end. This strategy maintains a true representation of the original mRNA transcripts, which is a critical factor for gene expression analysis. SMART technology offers unparalleled sensitivity and unbiased amplification of cDNA transcripts, and allows direct cDNA synthesis from intact cells.

For library preparation from the generated full-length cDNA, the SSv4 PLUS kit also incorporates our patented library preparation technology. Unlike other library preparation kits, which are based on ligation of Y-adapters, our approach uses stem-loop adapters to construct high-quality libraries. The easy two-step workflow takes place in a single tube and is completed in about two hours. Sample mix-up, other handling errors, and sample loss are minimized since no intermediate purification steps or sample transfers are necessary (Figure 1, Panel B). The SMART-Seq v4 PLUS Kit also includes unique dual index (UDI) primers for amplification of indexed NGS-ready libraries, allowing the multiplexing of up to 96 samples. Once purified and quantified, the resulting libraries are ready for use on Illumina NGS instruments using standard Illumina sequencing reagents and protocols.
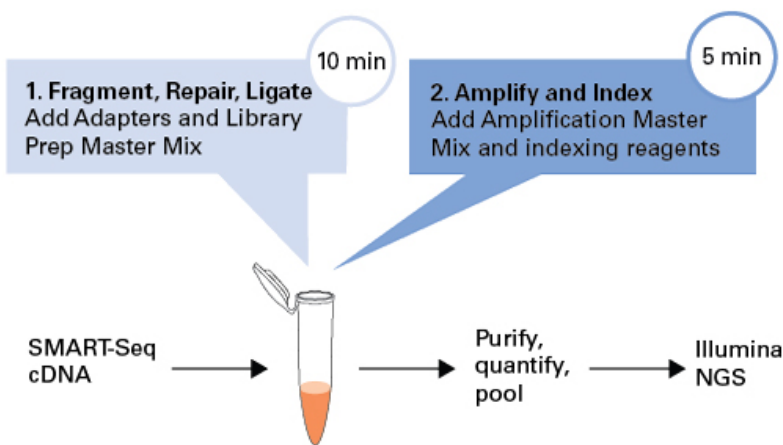
A

Total RNA or cells

AAAAAAAAAAAAAAAAA

SMART-Seq cDNA synthesis

Full-length ds cDNA

SMART-Seq Library Prep Kit

Fragmented cDNA
with stem-loop adapters

Library amplification
and indexing
with UDI kits

i5   i7

i5   i7

i5   i7

Illumina libraries with UDIs

B

10 min

1. Fragment, Repair, Ligate
Add Adapters and Library
Prep Master Mix

5 min

2. Amplify and Index
Add Amplification Master
Mix and indexing reagents

SMART-Seq
cDNA

Purify,
quantify,
pool

Illumina
NGS

Figure 1. SMART-Seq v4 PLUS Kit protocol overview. Panel A. Double-stranded cDNA generated with SMART-Seq v4 chemistry is enzymatically fragmented and ligated to stem-loop adapters in a single step. Libraries are then amplified and indexed, creating Illumina-compatible libraries with unique dual indexes. **Panel B.** The library preparation method features a new streamlined two-step protocol that generates sequencing-ready libraries with minimal hands-on

time.

Results

Greater sensitivity and reproducibility for cDNA synthesis

The SMART-Seq v4 PLUS kit was designed to provide a streamlined workflow from ultra-low total RNA to Illumina-ready libraries. In this comparison of SSv4 PLUS and a gold-standard library preparation kit, 10 pg of Mouse Brain Total RNA was first processed with SMART-Seq v4 chemistry to generate cDNA in triplicate. Libraries were then produced using either the SMART-Seq Library Prep Kit portion of the SSv4 PLUS kit or Nextera® XT (Figure 2). On average, SSv4 PLUS kit library yields were 5X higher than those obtained when using Nextera XT (Figure 2, Panel B). The libraries were then sequenced on a NextSeq 500, and the number of genes identified over 0.1 transcripts per million (TPM) was reported (Figure 2, Panel C). The SSv4 PLUS kit allowed more genes to be detected than Nextera XT. Both kits showed similar percentages of reads mapping to the exonic regions, intronic regions, intergenic regions, ribosomal RNA (rRNA), and mitochondria (Figure 2, Panel D). Finally, the Pearson and Spearman's correlations calculated from the TPM values were very high for both kits (Figure 2, Panel E), indicating high confidence in the reproducibility of the data.
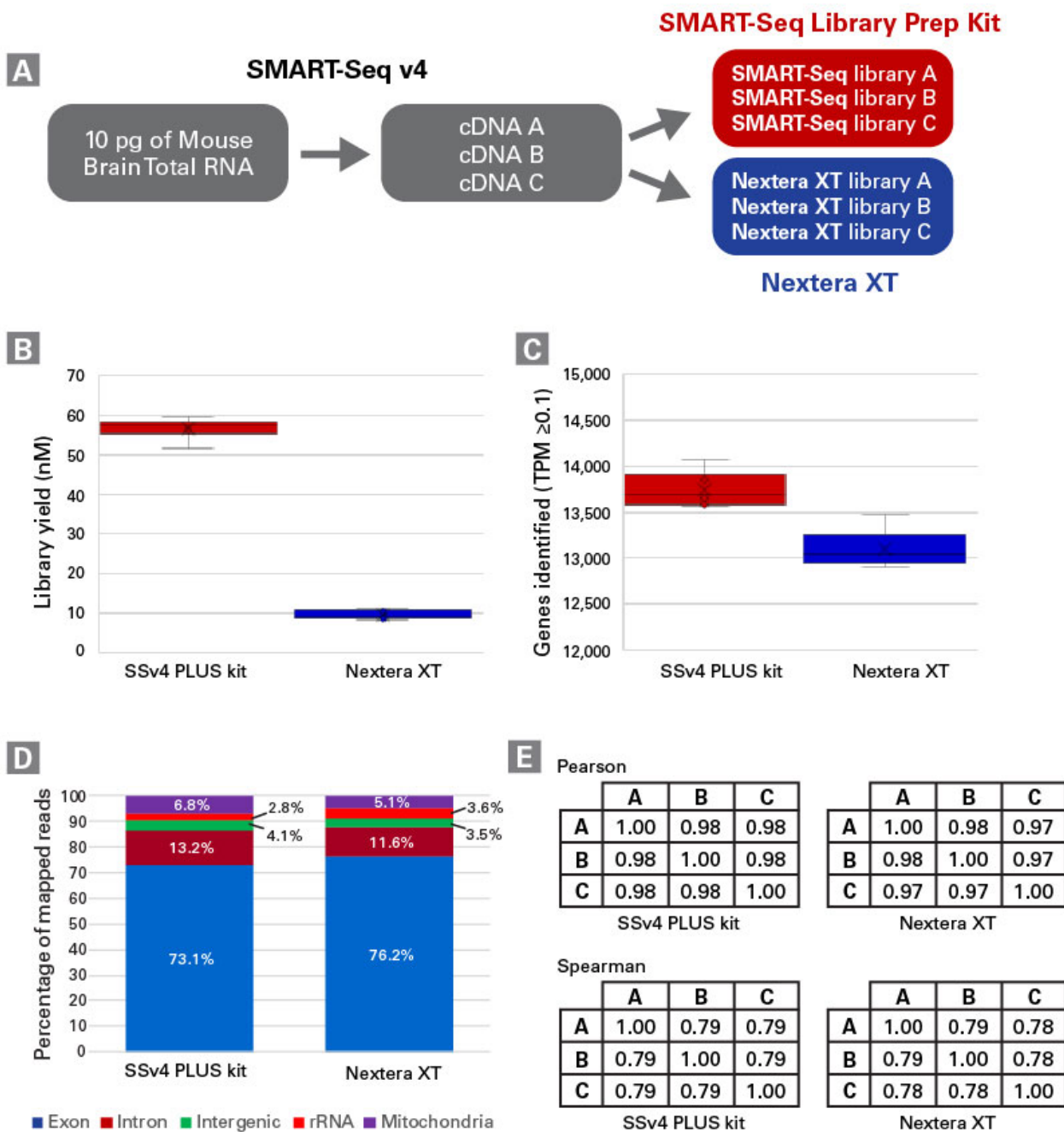
Figure 2. Library preparation with the complete SMART-Seq v4 PLUS Kit increases library yield and genes identified compared to Nextera XT. Panel A. SSv4 chemistry was used to produce cDNA, in triplicate, from 10 pg of Mouse Brain Total RNA. Illumina-compatible libraries were then generated from 1 ng or 125 pg using the SMART-Seq Library Prep Kit portion of the SSv4 PLUS kit or Nextera XT, respectively, and sequenced on a NextSeq 500. The reads were normalized to 4M paired-end reads and analyzed as described in the methods. **Panel B.** The library yield obtained with the SSv4 PLUS kit is higher than the yield obtained when using Nextera XT. **Panel C.** The number of genes identified was also higher for the SSv4 PLUS kit compared to Nextera XT. **Panel D.** As expected, the distribution of the mapped reads is similar between the two library preparation methods. **Panel E.** Pearson and Spearman's correlations were calculated from the TPM values obtained from the triplicate cDNAs (A to C) processed with the SSv4 PLUS or Nextera XT kit.

Switch with confidence

Next, we set out to evaluate any bias introduced by the library preparation step. To do this, the same SSv4-generated cDNA was processed in duplicate with the SMART-Seq Library Prep Kit portion of the SSv4 PLUS kit or Nextera XT (Figure 3, Panel A). The TPM values were plotted and used to calculate correlations. The correlations obtained for the SSv4 PLUS kit libraries when using the same starting cDNA (P: 0.999 & S: 0.90 for cDNA A1 vs. cDNA A2; Figure 3, Panel B, left) were higher than those obtained with different input cDNAs (P: 0.98 & S: 0.79 cDNA A vs. cDNA B; Figure 2, Panel E). We, therefore, concluded that the main source of variation in the TPM values was coming from the cDNA generation.

Lastly, we aimed to assess the genes identified with the SSv4 PLUS kit and Nextera XT. Here, we found that the Spearman's correlation for the same cDNA replicate processed with the two different kits (S: 0.90 for SSv4 PLUS cDNA A1 vs. Nextera XT cDNA A1; Figure 3, Panel B, right) was greatly superior to the correlations for different cDNA replicates prepared with the same kit (0.79 [SSv4 PLUS] & 0.78/0.79 [Nextera XT]; Figure 2, Panel E), demonstrating the similarity in gene-identification performance between the two library prep technologies. Finally, the number of genes identified in Figure 3, Panel C, for the SSv4 PLUS replicates (12,309 genes) was higher compared to those found with the Nextera XT replicates (11,973 genes). Most of the genes identified that did not overlap between replicates showed low TPM values, as depicted by the dots overlying the X- and Y-axis of the plots (Figure 3, Panel B). Both methods detected the same 12,097 genes at similar TPM values, as depicted by correlation plot (Panel B) and the Venn diagram (Panel C). Therefore, existing Nextera XT users should see minimal effects on their data when using the new SMART-Seq v4 PLUS Kit.
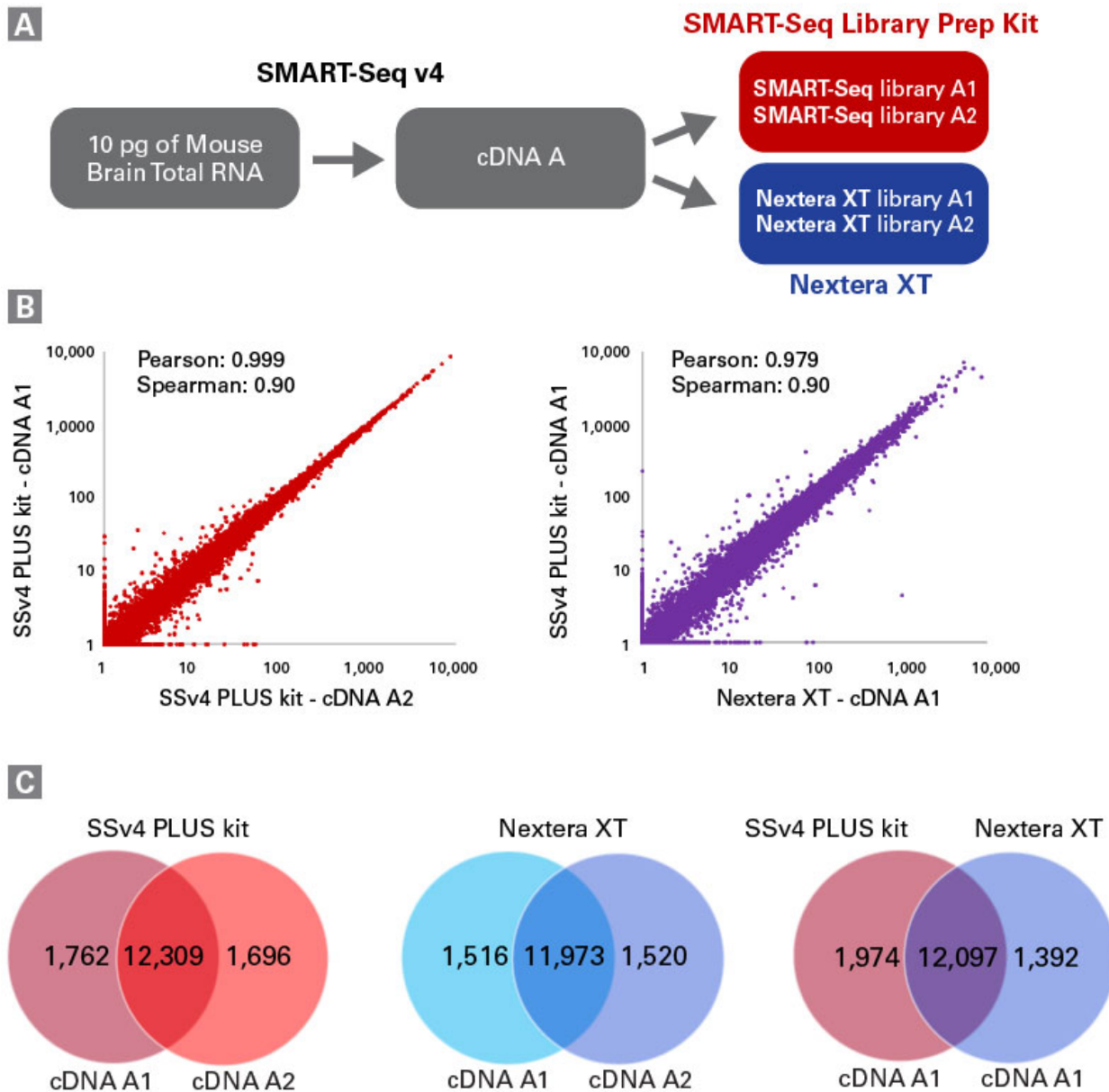
Figure 3. Comparable data between SMART-Seq library prep and Nextera XT kits enable existing users to easily make the switch. Panel A. Libraries were prepared in duplicate from the same SSv4 cDNA using either the SMART-Seq Library Prep Kit portion of the SSv4 PLUS kit or Nextera XT. **Panel B.** Correlation plots between libraries made with SSv4 PLUS and Nextera XT kits. Pearson and Spearman's correlations were calculated from the TPM values obtained. Scatter plots showed the TPM values from all genes with a log10+1 scale. **Panel C.** Venn diagram showing the number of genes identified from the processed cDNA, in duplicate, with the SSv4 PLUS kit, Nextera XT, or between both chemistries.

Conclusions

Having a sensitive and reproducible end-to-end approach is crucial to efficiently and easily analyze samples and extract meaningful biological information from the sequencing data. The SMART-Seq v4 PLUS Kit combines the proven SMART-Seq cDNA generation approach and user-friendly library preparation chemistry to generate Illumina-compatible libraries ready for sequencing. By using the SMART-Seq v4 PLUS Kit, researchers can produce about 5X higher library yields than traditional methods. An increase in the number of genes identified is also observed, which is extremely useful when dealing with difficult samples containing low RNA levels, including clinical research samples.

Methods

cDNA and Illumina libraries were generated from 10 pg of Mouse Brain Total RNA using the SMART-Seq v4 PLUS Kit. For comparison, sequencing libraries were generated using 125 pg of cDNA and the Nextera XT DNA Library Preparation Kit (Illumina) with a quarter of the recommended volumes. The cDNA and sequencing libraries were quantified using Bioanalyzer HS reagents and Qubit, respectively.

Libraries were sequenced on a NextSeq 500 instrument using 2 x 75 bp paired-end reads, and analysis was performed using CLC Genomics Workbench. Reads were downsampled to 4M reads and mapped to the mouse [GRCm38] genome with Ensembl annotations. All percentages shown—including the number of reads that map to introns, exons, or intergenic regions—are percentages of mapped reads in each library. TPMs were reported using CLC.

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).

Related Products

Cat. #	Product	Size	License	Quantity	Details
R400752	SMART-Seq® v4 PLUS Kit	48 Rxns	🔗	*	🔼

The SMART-Seq v4 PLUS Kit is a complete kit designed to first generate high-quality cDNA from ultra-low amounts of total RNA (10 pg–10 ng) and then high-quality Illumina sequencing-ready libraries. Indexes are added using a unique dual index kit (Cat. # R400744 or R400745). This kit supports up to 48 reactions.

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Documents	Components	Image Data

Cat. #	Product	Size	License	Quantity	Details
R400753	SMART-Seq® v4 PLUS Kit	96 Rxns	🔗	*	🔼

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