Efficient global preamplification via the SuperScript IV Single-Cell/Low-Input cDNA PreAmp Kit

Abstract

Researchers performing single-cell RNA sequencing (scRNA-seq) or quantitative reverse transcription polymerase chain reactions (RT-qPCR) with low-input RNA must often preamplify their samples to obtain enough material for comprehensive and reliable downstream analysis. Traditionally, target-specific preamplification has been preferred for gene expression analysis by RT-qPCR, while exploring the transcriptome via RNA-seq requires a global preamplification approach. Target-specific preamplification is usually performed by highly multiplexed PCR reactions designed to increase the concentration of selected targets in a sample. As such, it is often prone to biases and inaccurate representation of some targets. Furthermore, optimization of primer pools is time consuming, and a new primer pool is required for each new set of target genes. Global preamplification has clear merits over target-specific preamplification when it comes to protocol versatility and uniformity—global preamplification is target-independent and uses the same protocol regardless of the genes studied during downstream analysis. Moreover, preamplified full-length cDNA can be used for both next-generation sequencing (NGS) library preparation or quantification by qPCR, simplifying research workflows. To utilize these advantages and simplify the analysis of single-cell or low-input RNA samples, Thermo Fisher Scientific developed the Invitrogen™ SuperScript™ IV Single-Cell/Low-Input cDNA PreAmp Kit. The SuperScript IV PreAmp kit provides a streamlined solution for global preamplification of high-quality, full-length cDNA compatible with both scRNA-seq and RT-qPCR. In the following study, we investigated the ability of the SuperScript IV PreAmp kit to generate accurate scRNA-seq and RT-qPCR data and compared its performance to a comparable product from another supplier.

Key findings

The SuperScript IV Single-Cell/Low-Input cDNA PreAmp Kit enables:

- Fast one-tube preamplification with minimal sample handling and short reaction times
- High cDNA yields from as little as one cell or 2 pg of RNA input
- The generation of full-length cDNA products, even from long and rare RNA transcripts
- Excellent gene detection and uniform coverage in NGS workflows
- Universal protocol for global preamplification that is compatible with downstream analysis by NGS and RT-qPCR

Introduction

In recent years, mRNA analysis has become increasingly important. To understand complex cellular activities, researchers must go beyond genotypic analysis, thus more and more research teams routinely perform RNA-seq and RT-qPCR for gene detection and expression profiling. Transcriptomics research [1] plays a central role in immunology, oncology, neurology, and stem cell biology fields. Due to natural limitations, researchers must often work with very small amounts of mRNA or study very low abundant transcripts, while still maintaining the utmost levels of experimental reliability, reproducibility, and data accuracy. Unfortunately, low RNA input amounts (<100 pg) can lead to experimental issues and less reliable data in both RNA-seq and RT-qPCR applications [2].

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Perhaps the clearest need for highly sensitive RNA analysis workflows is for single-cell analysis [3]. Conventional methods that analyze cellular populations in bulk to profile transcriptomic information tend to mask cellular heterogeneity that holds physiological and pathological relevance [4]. To discover the importance of gene expression differences in rare cells and subpopulations, researchers turn to single-cell transcriptomics approaches, where cells within a population are individually investigated to uncover information otherwise lost in bulk analysis. For many researchers, it suffices to study specific gene targets in these rare cell populations using RT-qPCR. However, researchers are increasingly adopting scRNA-seq to track gene expression across the totality of a cell's transcriptome [3].

Both scRNA-seq and RT-qPCR rely on the production of cDNA that accurately represents the mRNA nucleotide sequences and their relative abundance. However, this becomes more challenging when starting with very little mRNA. For example, a typical mammalian cell has about 10 pg of total RNA, including about 0.1 pg of mRNA [5]. Notably, certain cell types can have even less; a human T cell contains around 1–2 pg of RNA [6]. Additionally, researchers are often interested in low-copy RNA transcripts within these individual cells, necessitating even greater sensitivity.

To overcome this persistent experimental challenge, scRNA-seq and RT-qPCR with low-input RNA samples requires not only highly sensitive reverse transcription, but also efficient preamplification of the resulting cDNA prior to downstream analysis. Several preamplification protocols have been developed over the years, most of which can be categorized into two approaches: target-specific preamplification and global preamplification (Figure 1). Target-specific preamplification is preferred by RT-qPCR users, while RNA-seq requires a global preamplification approach. Target-specific preamplification is usually performed by a highly multiplexed PCR reaction designed to increase the concentration of selected targets in a sample. Optimization of primer pools may be laborious and time consuming. Researchers must carefully validate the primer sets to ensure that no amplification bias occurs, since over- or under-representation of specific transcripts can negatively impact data analysis. Furthermore, every new application addressing new target genes require a new pool of primers and preamplification from a new sample. Global preamplification can overcome these challenges and is compatible with both RNA-seq and RT-qPCR downstream applications [7]. It uses the same protocol regardless of the genes that will be selected for downstream analysis.

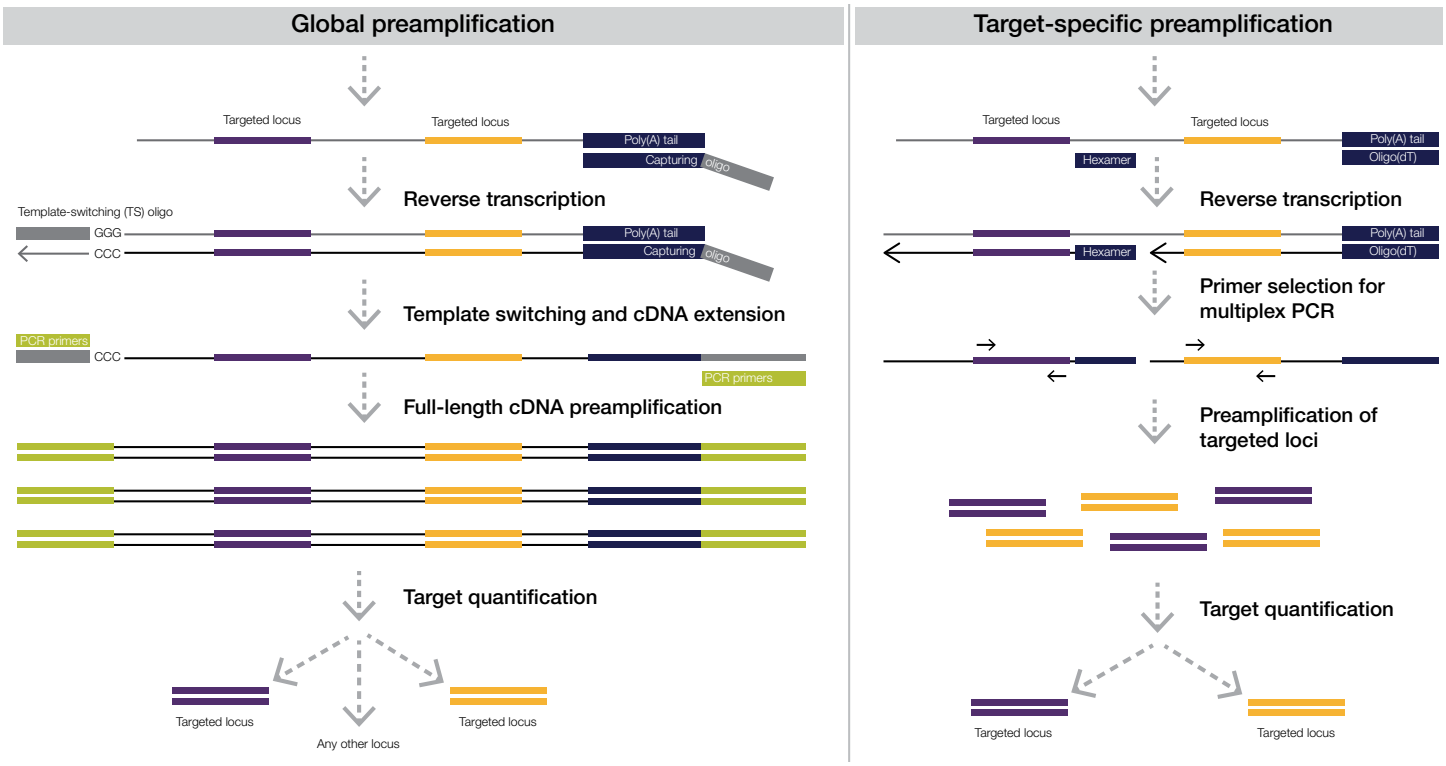

Figure 1. Comparison of global (left) and target-specific (right) preamplification. The SuperScript IV Single Cell/Low-Input cDNA PreAmp Kit uses a global preamplification approach compatible with qPCR and RNA-seq.

The SuperScript IV Single Cell/Low-Input cDNA PreAmp Kit was developed to address the need for a highly efficient yet versatile global preamplification solution. The SuperScript IV PreAmp kit offers a streamlined ready-to-use solution for amplifying global full-length cDNA from intact single cells (1–1,000) or ultra-low RNA input (2pg–10ng). The kit contains all the required components to perform cell lysis, reverse transcription, and PCR amplification in a convenient premixed format. A combination of superior enzymes—Invitrogen™ SuperScript™ IV Reverse Transcriptase and Invitrogen™ Platinum™ SuperFi™ U DNA Polymerase—can achieve high yields, sensitivity, and accuracy.

The SuperScript IV PreAmp kit is based on SMART (switching mechanism at the 5′ end of RNA transcript) technology [8] to achieve robust preamplification. It leverages terminal deoxynucleotidyl transferase (TdT) activity of the SuperScript IV Reverse Transcriptase. Capturing oligonucleotide containing oligo(dT) and adapter sequences is used as a primer for selective first-strand cDNA synthesis from poly(A)-containing RNAs. When the reverse transcriptase reaches the 5′ end of the RNA template, TdT activity adds 1–3 extra nucleotides to the cDNA end, enabling template switching and ligation-free incorporation of adapter sequence to the 3′ end of the resulting cDNA (Figure 2). High sensitivity and template-switching efficiency of the SuperScript IV Reverse Transcriptase allow the capture even of low-abundant targets. Adapter sequences incorporated at both ends of the cDNA serve as primer-binding sites in the subsequent PCR amplification step, allowing global preamplification of full-length templates. The Invitrogen™ Platinum™ SuperFi™ U Preamplification Master Mix includes a superior high-fidelity DNA polymerase that ensures efficient amplification of long templates (up to 10 kb) with minimal risk of PCR-induced bias or errors.

Collectively, the SuperScript IV PreAmp kit offers highly sensitive and efficient global preamplification workflows with fast reaction times. The performance of the SuperScript IV PreAmp kit was assessed and benchmarked against a comparable kit from another supplier in low-input RNA experiments, including scRNA-seq and RT-qPCR.

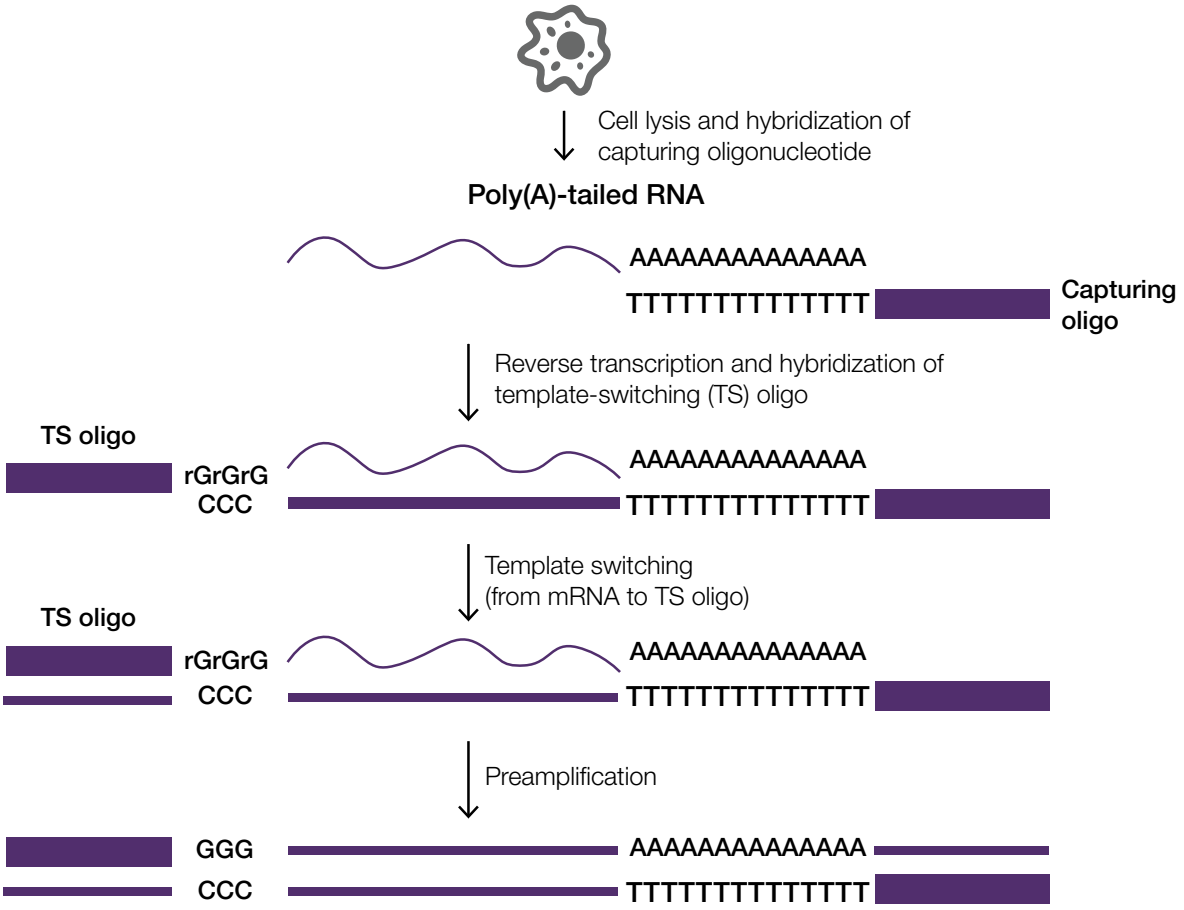

Figure 2. Overview of SMART (switching mechanism at the 5′ end of RNA transcript) technology.

Methods

The SuperScript IV Single-Cell/Low-Input cDNA PreAmp Kit (Cat No. 11752048) workflow is compatible with intact eukaryotic cells and purified total RNA. RNA must be of high quality and must retain the poly-adenylated tail needed for reverse transcription priming by the capturing oligonucleotide.

Compared preamplification kits:

- SuperScript IV Single Cell/Low-Input cDNA PreAmp Kit (Cat No. 11752048)
- NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module (NEB, Cat No. E6421S)

Sample preparation

Invitrogen™ Universal Human Reference RNA (UHRR, Cat. No. QS0639) and Invitrogen™ Human Heart Total RNA (Cat. No. AM7966) were used as template RNA for cDNA synthesis and preamplification. The RNA was diluted to various concentrations depending on the experimental setup. To simulate low-input conditions, the RNA was diluted to 2 pg/µL, and 1 µL of the diluted RNA was used in reactions.

Alternatively, intact single cells listed in Table 1 were used directly in preamplification reactions. Prior to cell isolation and sorting, cells were washed and resuspended in PBS. After FACS sorting in a storage plate (1 cell per well) with lysis reagents, samples were transferred to thin-walled 0.2 mL tubes for subsequent reactions.

Table 1. Cells used for the preamplification of single-cell RNA.

Cell line	Culture property	Organism	Cell type
HeLa-S3	Suspension	*Homo sapiens*	Cervical adenocarcinoma
Daudi	Suspension	*Homo sapiens*	B cell lymphoma
Jurkat	Suspension	*Homo sapiens*	T cell leukemia
Loucy	Suspension	*Homo sapiens*	T cell leukemia

Lysis, reverse transcription, and preamplification

Samples were preamplified using either the SuperScript IV Single-Cell/Low-Input cDNA PreAmp Kit or NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module. All preamplification reaction conditions were applied according to each kit's manual. The number of cycles required during the preamplification step is dependent on the RNA input (Table 2).

Table 2. Number of preamplification cycles required for each RNA input.

RNA input (pg or single cell)	Number of cycles
2 pg RNA	21
HeLa-S3 cell	18
Jurkat cell	20
Daudi cell	20
Loucy cell	20

Purification

After preamplification, Beckman Coulter™ Agencourt AMPure XP beads (Cat. No. A63881) were used to remove excess primers and concentrate the final cDNA product according to the protocol provided in the preamplification kits' manuals. Elution was performed in 10 mM Tris-HCl buffer (pH 8.0).

qPCR

For qPCR experiments, cDNA samples were generated from 1 ng and 10 ng UHRR inputs. After reverse transcription (RT), cDNA samples were split into two aliquots. One aliquot was used in a preamplification reaction, while the other was used as a control (RT only). Preamplified samples were purified and eluted in 50 µL of nuclease-free water. To equalize volumes between control and preamplified samples, control samples were diluted in nuclease-free water to a final volume of 50 µL. Applied Biosystems™ TaqMan® Fast Advanced Master Mix (Cat. No. 4444556) was used for the qPCR reactions, along with the appropriate primer/probe pair for each target (Table 3). Preamplified cDNA or RT-only control (1 µL) was used in a 20 µL qPCR reaction volume.

Table 3. List of qPCR targets.

Target	Relative expression
ACTB	High
GAPDH	High
ANP32B	Average
B2M	Average
MORF4L2	Average
RFE	Average
EIF4E	Average/low
HPRT1	Average/low
GusB	Low
TBP	Low

Endpoint PCR

For the preamplification reactions, 10 ng of total human heart RNA was used as the input. Reaction conditions were applied as recommended in the protocols of the preamplification kits. Following preamplification, 1 µL of cDNA was used in a 25 µL endpoint PCR reaction. Invitrogen™ Platinum™ SuperFi™ II PCR Master Mix (Cat No. 12368050) was used to amplify 10 kb long gene target, according to the manual. In all cases, 35 PCR cycles were performed. PCR products were mixed with Thermo Scientific™ Orange DNA Loading Dye (6X, Cat. No. R0631) and analyzed by electrophoresis of a 1% agarose gel (Thermo Scientific™ TopVision™ Agarose Tablets, Cat. No. R2801) with ethidium bromide. The Thermo Scientific™ GeneRuler™ 1 kb Plus DNA Ladder (Cat. No. SM1333) was run to visualize cDNA length.

Capillary electrophoresis

The Agilent™ 2100 BioAnalyzer System was used to calculate the yields of preamplified cDNA from either 2 pg of UHRR or single cells of Daudi, Jurkat, Loucy and HeLa-S3. The Agilent™ High Sensitivity DNA Kit (Cat No. 5067-4626) was used according to the user manual. Calculations of the yield were based on 300–9,000 bp regions. A minimum of 5 replicates were performed with each preamplification kit and each sample type.

NGS

Preamplified and purified cDNA were used for NGS library preparation. Two types of library preparation approaches were used—ligation-based Invitrogen™ Collibri™ ES DNA Library Prep Kit for Illumina™ Systems with UD indexes (Cat No. A43607024) and transposase-based Nextera™ XT DNA Library Preparation Kit (Cat No. FC-131-1024). All library preparation procedures were performed according to the respective manual. After library preparation, samples were sequenced using the Illumina™ MiSeq™ System.

Results

The SuperScript IV PreAmp kit provides uniform preamplification of RNA transcripts

Uniform amplification of RNA transcripts is essential for maintaining accurate representation of mRNA abundance from experimental samples. When done properly, preamplification enables researchers to accurately compare transcript amounts and accurately measure changes in gene expression. To assess whether the SuperScript IV PreAmp kit provides uniform and linear amplification, multiple targets (Table 3) were quantified by qPCR with or without a preamplification reaction. Normalized expression of the targets ranged from 1 (for lowly expressed targets like *GusB*) to 170 (for highly expressed reference genes like *GAPDH* and *ACTB*), covering a wide range of relative abundance. Cycle threshold (C_t) values of samples after preamplification were collected and plotted against C_t values from samples that were not preamplified (Figure 3). The resulting curve showed uniform linear amplification, regardless of the gene target.

This indicates that the SuperScript IV PreAmp kit does not enrich any specific cDNA, regardless of the amount of mRNA. Researchers can rely on the SuperScript IV PreAmp kit to accurately and uniformly preamplify RNA samples and generate reliable qPCR data.

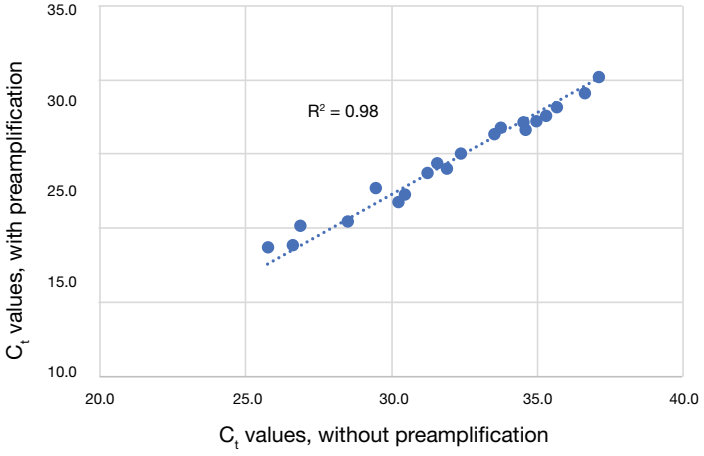

Figure 3. Comparison of C_t values for amplification from preamplified and non-preamplified cDNA. The high correlation between C_t values (R^2 = 0.98) indicates that the SuperScript IV PreAmp kit provides uniform linear preamplification.

The SuperScript IV PreAmp kit generates full-length cDNA

Preamplification kits also need to reliably amplify gene targets regardless of sequence length, particularly for RNA-seq applications. However, long transcripts can be difficult to amplify consistently, especially because they tend to be rather rare. To demonstrate the ability of the SuperScript IV PreAmp kit to generate long cDNA products, endpoint PCR experiments were performed using 10 ng of RNA input, and results were compared to those generated using the NEBNext preamplification kit. The SuperScript IV PreAmp kit successfully generated a full-length preamplification product even for rare long gene target of 10 kb length (Figure 4).

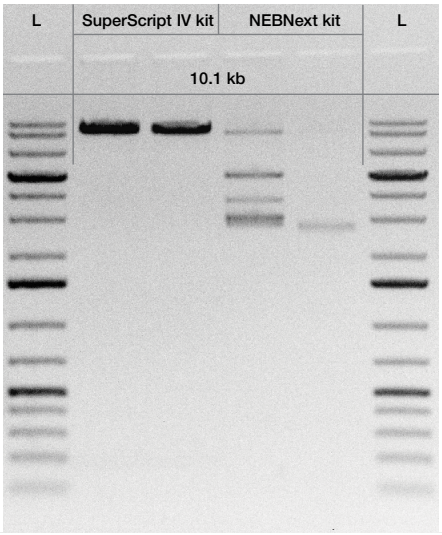

Figure 4. The SuperScript IV PreAmp kit successfully generates full-length preamplification of long gene targets.

The SuperScript IV PreAmp kit generates high cDNA yields from single cells and low-input RNA

For both NGS and qPCR experiments, when starting with single-cell RNA or low-input RNA, it is important to generate high cDNA yields. Therefore, cDNA yields from low-input (2 pg) Universal Human Reference RNA (UHRR) and individual cells were quantified using capillary electrophoresis. These yields were then compared to the yields produced using the NEBNext kit. The SuperScript IV PreAmp kit significantly increased cDNA yield from UHRR samples, nearly doubling the amount produced by the NEBNext kit. Additionally, the SuperScript IV PreAmp kit demonstrated either equivalent or superior cDNA yield when using single cells (Figure 5). It is worth noting that high cDNA yield variation was observed for single cells. It is likely that this variation is due to variability in RNA amounts from these individual cells [7].

The SuperScript IV PreAmp kit detects more gene transcripts than other preamplification kit

When performing RNA-seq, researchers need information on as many distinct gene transcripts as possible in order to sample the widest view of a cell's transcriptome. This allows gene expression measurements across a wide variety of genes. As a result, it is critical to determine how many genes can be detected during scRNA-seq after using the SuperScript IV PreAmp kit.

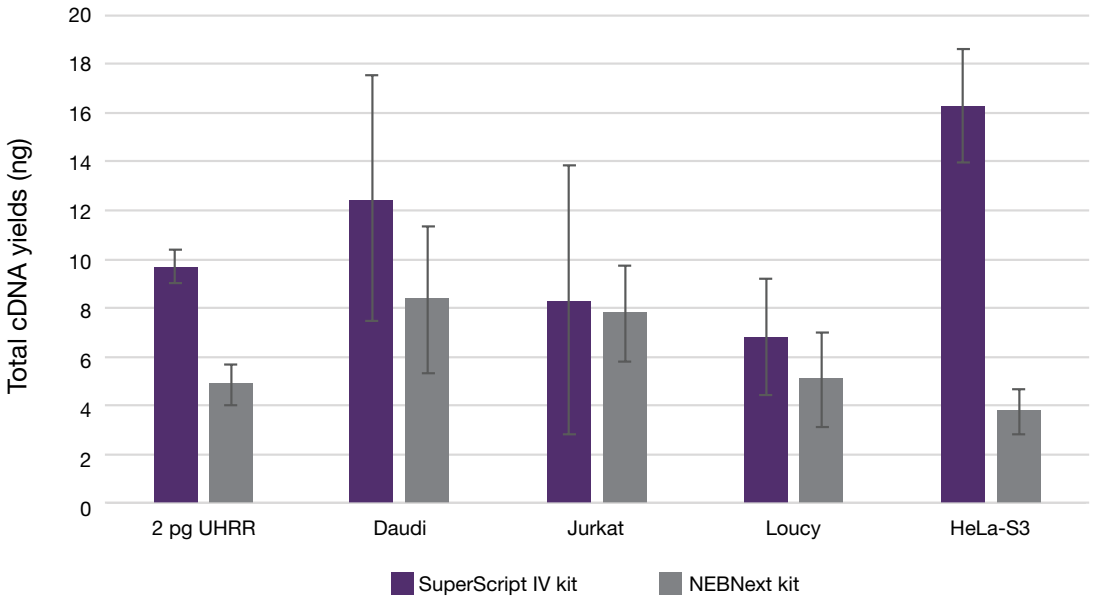

Figure 5. The SuperScript IV PreAmp kit produces excellent cDNA yield, compared to the NEBNext preamplification kit. Error bars represent standard deviation among at least 5 replicates.

To test how many genes can be detected, RNA-seq was performed following preamplification by either the SuperScript IV PreAmp kit or the NEBNext kit (Figure 6). To simulate scRNA-seq conditions, only 2 pg of UHRR input was preamplified. Library preparations were performed using two different platforms to ensure independence of results across both ligation-based (Collibri ES DNA Library Prep Kit) and transposase-based (Nextera XT DNA Library Preparation Kit) NGS library preparation workflows. Regardless of the workflow used, the SuperScript IV PreAmp kit led to the detection of more unique gene transcripts compared to workflows using the NEBNext kit. This indicates the SuperScript IV PreAmp kit enables superior transcriptome coverage in NGS experiments.

The SuperScript IV PreAmp kit helps generate NGS libraries with low sequence bias

Avoiding biases in NGS library preparation is crucial for collecting reliable RNA-seq results, since biases can lead to undue over- or under-representation in critical data sets. For instance, biases can occur toward poly(dA/dT) sequences during library preparation [9]. To assess this possibility, sequence coverage was normalized along transcript lengths to measure the level of bias for samples preamplified using the SuperScript IV PreAmp kit. Preamplification was performed using single cells (HeLa-S3, Jurkat, and Daudi). Libraries prepared from cDNA generated with the SuperScript IV PreAmp kit resulted in low bias across most of the normalized transcript distance (Figure 7), indicating that the SuperScript IV PreAmp kit uniformly preamplifies transcripts for reliable scRNA-seq data collection.

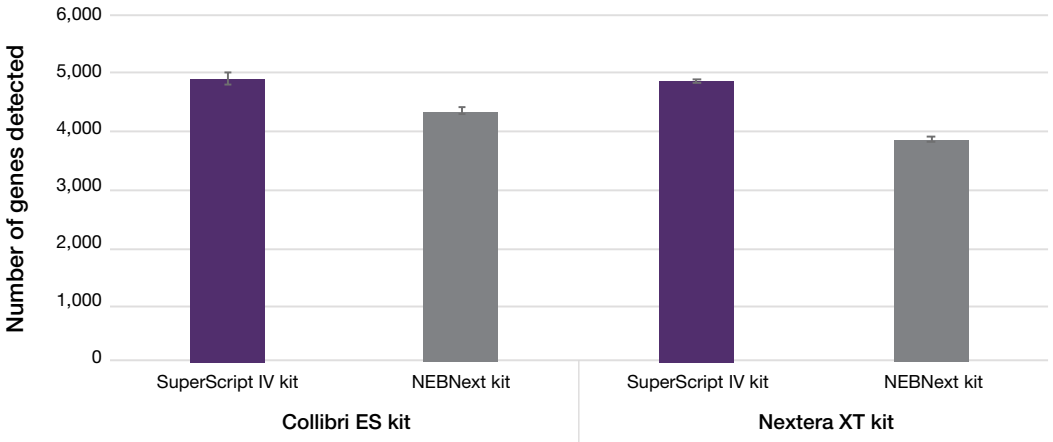

Figure 6. The average number of genes detected was higher in samples preamplified with the SuperScript IV Single-Cell/Low-Input cDNA PreAmp kit compared to the number of genes detected using the NEBNext kit.

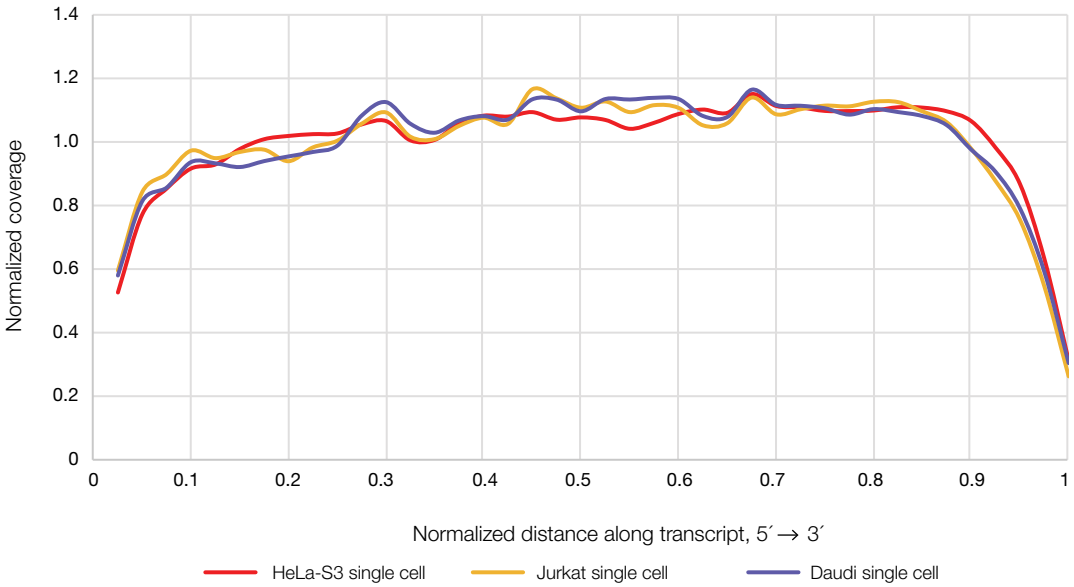

Figure 7. Normalized transcript coverage across several different input types. Single-cell RNA was reverse transcribed, and obtained cDNA was preamplified using the recommended number of PCR cycles. The Collibri ES kit was used to prepare libraries for sequencing using the MiSeq Sequencing system.

The SuperScript IV PreAmp kit provides high reproducibility of data

Reproducibility of results is paramount. Researchers require workflows that produce consistent data across technical replicates, especially when RNA inputs are low. To demonstrate the reproducibility of preamplification kits, NGS results were compared between replicates from the SuperScript IV PreAmp kit and the NEBNext kit. Plotting their correlation demonstrates that replicates using the SuperScript IV PreAmp kit resulted in higher correlation percentages (>66%) compared to replicates using the NEBNext kit (<59%) (Figure 8). These results indicate that the SuperScript IV PreAmp kit offers a reproducible workflow for highly sensitive analysis.

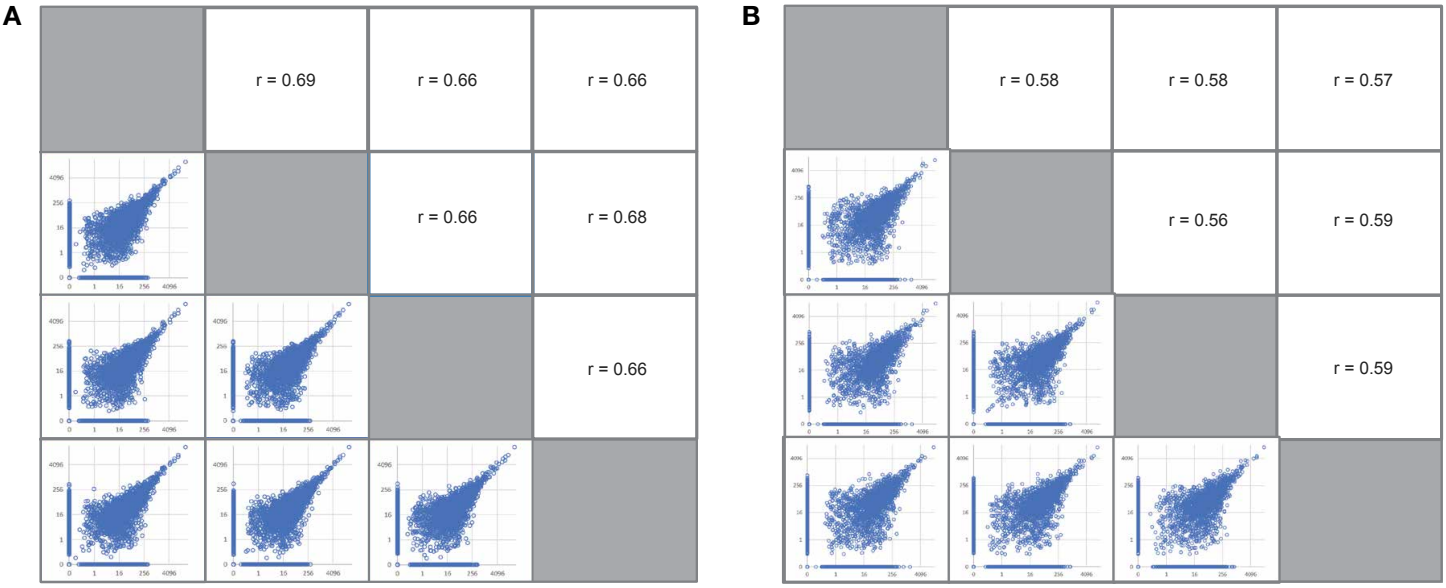

Figure 8. Correlation of technical replicates produced by (A) the SuperScript IV PreAmp kit and (B) the NEBNext PreAmp kit. The SuperScript IV PreAmp kit shows higher correlation between technical replicates, demonstrating increased reproducibility.

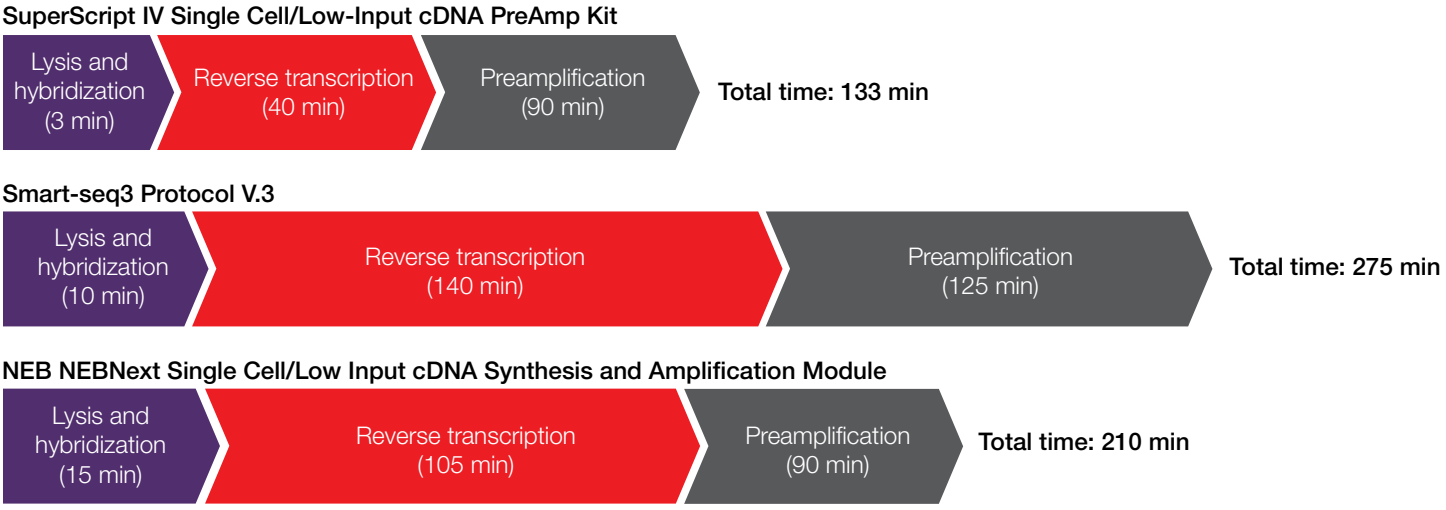

Figure 9. Workflows and timelines for the SuperScript IV Single Cell/Low-Input cDNA PreAmp Kit in comparison to the Smart-seq3 Protocol V.3 workflow [10] and the NEBNext kit workflow.

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Conclusions

In the pursuit of groundbreaking scientific insights, it has become necessary to derive crucial information from biological samples that may have low RNA input, including single cells. The SuperScript IV Single-Cell/Low-Input cDNA PreAmp Kit solves existing preamplification challenges for scRNA-seq and low-input RT-qPCR applications and produces reliable and accurate results. The combination of SuperScript IV Reverse Transcriptase and Platinum SuperFi U DNA Polymerase demonstrates superior sensitivity, higher cDNA yields, a greater number of detected genes, low sequence bias, uniform amplification, and good reproducibility. Moreover, compared to alternative preamplification workflows, the SuperScript IV Single-Cell/Low-Input cDNA PreAmp Kit provides a faster protocol with less total and less hands-on time (Figure 9). The kit provides full-length transcript information with uniform coverage and is suitable for accurate quantification by qPCR or NGS library preparation. With the SuperScript IV Single-Cell/Low-Input cDNA PreAmp Kit, researchers can collect high-quality data with less cost, time, and, most importantly, RNA.

References

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Ordering information

Product	Number of reactions	Cat. No.
SuperScript IV Single-Cell/Low-Input cDNA PreAmp Kit	48	11752048
	96	11752096
	192	11752192
	384	11752384
	480	11752480

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