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SuperScript IV CellsDirect cDNA Synthesis Kit

A more sensitive, reliable, and flexible kit for direct cDNA synthesis

Abstract

Reverse transcriptases play an important role in life science research, and that role continues to grow with the advancement of transcriptomics. Researchers are increasingly eager to investigate biological systems using techniques such as next-generation sequencing (NGS), quantitative PCR (qPCR), and single-cell analysis. In turn, this interest has driven significant demand for high-throughput and reproducible reverse transcription PCR (RT-PCR) workflows with highly sensitive RNA detection and robust reverse transcriptase activity and processivity. In particular, more users want cDNA synthesis kits that can deliver reliable results directly from lysed cell samples and that can be applied flexibly to a wide number of cell types, reaction conditions, and experimental designs. Despite this need and the commercial kits already on the market, researchers have struggled to identify a single kit that clearly distinguishes itself in these ways. To meet this need, we have developed the Invitrogen™ SuperScript™ IV CellsDirect™ cDNA Synthesis Kit, which combines high sensitivity, a faster workflow, and high cDNA yields. In the following study, the technical applications of the SuperScript IV CellsDirect kit are investigated and compared with other commercial kits. Through these evaluations, it is clear that the SuperScript IV CellsDirect kit represents a major step forward for direct first-strand cDNA synthesis.

Key findings

The SuperScript IV CellsDirect kit enables:

- Excellent amplification linearity and RNA detection sensitivity compared to other direct reverse transcription (RT) kits
- High cDNA yield across multiple mammalian cell types, RNA transcripts, and cellular inputs
- Significant time savings and reduced sample handling, compared to conventional approaches
- Reliable experimental reproducibility and compatibility with internal positive control (IPC) RNA transcripts
- Suitable for both endpoint PCR and qPCR

Introduction

In recent decades, few technologies have accelerated biotechnological progress and impacted our understanding of biological networks like RT-PCR. This technology has enabled researchers to rapidly and accurately clone genes and investigate how RNA expression dictates a multitude of important cellular phenotypes. This work only continues to grow as researchers identify newer and more complex problems to study and areas to apply RT-based methods. Furthermore, the growth of RNA sequencing, transcriptomics, and single-cell analysis have rapidly expanded demand for RT kits, especially those with advanced sensitivity and processivity.

Despite their clear importance to the research community, RT workflows are not without challenges. In conventional approaches, it is common to first purify RNA from individual samples to achieve meaningful RNA detection. Then, a reverse transcriptase converts the purified RNA to cDNA for use in subsequent experiments. Loss of yield incurred during the purification process may reduce the amount of RNA available for subsequent applications. It is also well known that RNA is less stable than DNA, which makes sample handling and storage vital in workflow considerations. Generally, the more steps required prior to cDNA synthesis, the higher the likelihood of sample degradation and loss. This instability can impact RNA sample quality, requiring more sensitive RNA detection to achieve meaningful results. The impact of RNA purification is compounded when many samples must be processed and stored simultaneously, making it laborious and time-consuming to perform high-throughput experiments.

For these reasons, there has been significant interest in utilizing workflows that allow RT reactions to be performed directly from unpurified samples. Along with reduced sample handling and increased throughput, this approach comes with the added benefits of increased reproducibility and cDNA yield. However, many existing direct RT kits have struggled to match the RNA detection sensitivity possible through workflows using purified RNA sample. In addition, cDNA yields from these kits can suffer from common RT inhibitors found in lysed cell samples, which negatively impact RT activity and processivity. To maintain strong RNA detection and enzymatic function, researchers should use direct kits with reverse transcriptases that are less affected by a wide variety of common inhibitors. An ideal direct RT kit would replicate sensitivity obtained with purified RNA, and increase sample throughput without sacrificing qPCR accuracy or precision.

To address this long-standing research need, we recently launched the SuperScript IV CellsDirect kit. This direct RT kit is powered by SuperScript IV Reverse Transcriptase, which offers excellent enzymatic performance in the presence of inhibitors, robust sensitivity (even with degraded RNA), and low experimental variability [1]. Furthermore, the processivity of SuperScript IV Reverse Transcriptase (1,500 nt), reaction time (10 min), and temperature stability (up to 65°C) enable cloning and transcript analysis regardless of gene length or PCR conditions [2]. Combining the strengths of SuperScript IV Reverse Transcriptase with CellsDirect lysis reagents, the SuperScript IV CellsDirect kit was designed to speed up RT-PCR workflows and ease experimental difficulty without compromising sensitivity. In this report, the SuperScript IV CellsDirect kit was tested against a number of commercial kits to determine its relative performance in common RT-PCR experiments.

Materials and methods

Compared kits

- Invitrogen™ SuperScript™ IV CellsDirect™ cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. 11750150)
- Invitrogen™ SuperScript™ III CellsDirect™ cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. 18080200)
- Invitrogen™ SYBR™ Green Fast Advanced Cells-to-C_T™ Kit (Thermo Fisher Scientific, Cat. No. A35381)
- Invitrogen™ TaqMan® Fast Advanced Cells-to-C_T™ Kit (Thermo Fisher Scientific, Cat. No. A35378)
- Direct RT kits from other suppliers for probe-based or SYBR Green assays (“Supplier B”, “Supplier Q”, or “Supplier R”)

Cell culture

Experiments were performed using several different mammalian cell lines (Table 1). Cells were grown according to standard culture protocols for adherent or suspension cells using standard culture vessels. Before starting cell lysis, adherent cells were detached using a trypsin-based dissociation method. Cells were then counted with a hemocytometer and centrifuged at 300 x *g* for 5 min. After aspiration of the medium, cells were washed with 0.5 mL of ice-cold (4°C) PBS per 1 x 10^6 cells and centrifuged at 300 x *g* for 5 min. The PBS was aspirated and discarded without disturbing the pellet. Cells were then resuspended in 0.5 mL of ice-cold PBS per 1 x 10^6 cells. After serial dilution, the cells were recounted to ensure the cell density was 1, 10, 100, 1,000, or 10,000 cells per 5 µL. Aliquots (5 µL) of each cell suspension were distributed to PCR tubes. All procedures with cells were performed on ice.

Table 1. Cell lines utilized in experiments.

Cell line	Culture properties	Organism	Cell type
HeLa	Adherent	*Homo sapiens*	Cervical adenocarcinoma
HeLa S3	Suspension	*H. sapiens*	Cervical adenocarcinoma
Raji	Suspension	*H. sapiens*	B lymphocyte
Jurkat	Suspension	*H. sapiens*	T cell leukemia
K562	Suspension	*H. sapiens*	Bone marrow
iPSC	Adherent	*H. sapiens*	Stem cells
HEK293	Adherent	*H. sapiens*	Kidney

RNA purification

RNA was extracted and purified from HeLa S3 cells using magnetic beads from the Applied Biosystems™ MagMAX™-96 Total RNA Isolation Kit (Thermo Fisher Scientific, Cat. No. AM1830) or columns from the Applied Biosystems™ RNAqueous™-Micro Total RNA Isolation Kit (Thermo Fisher Scientific, Cat. No. AM1931), according to each kit's protocol. Tenfold serial dilutions of cells were made in PBS and used for purification. Eluted samples (32 µL of purified RNA from 1, 10, 100, 1,000, or 10,000 cells per sample) were used for RT using components of the SuperScript IV CellsDirect kit.

Direct cDNA synthesis

Using the SuperScript IV CellsDirect kit, cell solutions were lysed at 4°C using 24 µL of freshly prepared lysis buffer (23.28 µL SuperScript IV CellsDirect Lysis Solution, 0.24 µL Lysis Enhancer (100X), and 0.48 µL DNase I (50X)) and incubated for 7 min at room temperature. Lysis was stopped by adding 3 µL of stop solution and incubating samples for 2 min at room temperature. RT reactions were prepared following the recommendations for the SuperScript IV CellsDirect kit [3]. In brief, 8 µL of SuperScript IV RT Master Mix was added to each sample. These samples were incubated for 10 min at 25°C, 10 min at 50°C, 5 min at 85°C, and then held at 4°C. Samples were used directly or were stored as needed at –20°C until ready for use.

For all other commercial kits, cDNA generation was performed in alignment with the respective manufacturers' recommended protocols.

qPCR

The RT reactions contributed up to 10% of the total qPCR reaction volume. Generally, 2 µL of generated cDNA was used in a 20 µL qPCR reaction with Applied Biosystems™ PowerTrack™ SYBR™ Green Master Mix (Cat. No. A46012) or Applied Biosystems™ TaqMan® Fast Advanced Master Mix (Cat. Co. 4444556) and relevant primers (depending on kit specifications) on the Applied Biosystems™ QuantStudio™ 5 or 7 Flex Real-Time PCR System. Threshold cycle (C_t) values were normalized to the SuperScript IV CellsDirect kit using the formula $2^{(\text{Ct SuperScript IV CellsDirect kit} - \text{Ct alternative kit})}$.

For cDNA synthesis and qPCR with an IPC (referred to as "Xeno RNA"), adherent iPSCs were trypsinized, neutralized, and counted using a hemocytometer. Tenfold serial dilutions of cells were then made in PBS. These cell samples were diluted to 5 µL (1, 10, 100, 1,000, or 10,000 cells per condition) and lysed by adding 24 µL of lysis buffer premixed with Xeno RNA control (5 x 10^3 copies in 24 µL of lysis buffer). Six biological replicates were made for each dilution point. Lysates were incubated for 7 min at room temperature, then lysis was stopped by adding 3 µL of stop solution and incubating for 2 min at room temperature. RT reactions were prepared following recommendations for the SuperScript IV CellsDirect kit. cDNA samples (2 µL) were used in 20 µL qPCR reactions with PowerTrack SYBR Green Master Mix (Cat. No. A46012) or TaqMan Fast Advanced Master Mix (Cat. No. 4444556) and Xeno and ACTB control primers.

Endpoint PCR and determination of gDNA removal

The cDNA (10% cDNA sample input from HeLa, HeLa S3, Raji, Jurkat, or K562 samples) was amplified using primers specific to the *GAPDH*, *c-MYC*, *ACTB*, *VIN*, or *LAM* genes using either Invitrogen™ Platinum™ SuperFi™ II PCR Master Mix (Cat. No. 12368010) or Invitrogen™ Platinum™ II Hot-Start Green PCR Master Mix (Cat. No. 14001012) and their respective protocols on an Applied Biosystems™ ProFlex™ PCR System (30 cycles). PCR reactions (7 µL) were resolved by agarose gel (1%) electrophoresis in TAE buffer and visualized by ethidium bromide staining. To determine removal of genomic DNA (gDNA) from Jurkat cell lysates, identical endpoint PCR experiments were performed with and without reverse transcriptase using a range of cell amounts (10,000 to 1). Band sizes were resolved using the Invitrogen™ TrackIt™ 100 bp DNA Ladder (Cat. No. 10488058).

Results and discussion

Determination of amplification linearity and RNA detection sensitivity

Accurate quantitation of transcripts is an essential consideration for RT-qPCR experiments. Researchers must be confident that the C_t values they collect for a given transcript (at a specific sample input quantity) relate accurately to the transcript's abundance in the sample solution. The C_t values should inversely correlate with increasing sample input in a linear fashion. As such, direct RT kits should demonstrate a clear linear range across serial dilutions of a given sample. Additionally, the linear relationship should be independent of a transcript's overall expression (from high to low expression) as well as the qPCR assay protocol utilized.

To this end, the SuperScript IV CellsDirect kit was used to generate cDNA from serially diluted HeLa S3 cells, ranging from 1 to 10,000 cells. Using these cDNA samples, qPCR was performed using two different qPCR assays (SYBR Green and TaqMan assays) on four mRNA targets (*ACTB*, *BCL2*, *PGK1*, *PPIA*) that represent a wide range of cellular transcript abundance. The resulting qPCR C_t values and linearity were then benchmarked to results from other commercially available direct RT kits (Figure 1).

For all qPCR assays, the SuperScript IV CellsDirect kit resulted in the strongest linear correlation across this wide dynamic range. Additionally, C_t values from the SuperScript IV CellsDirect kit were either equal to or lower than those from all other kits at identical cellular inputs, indicating superior cDNA yields. Finally, the SuperScript IV CellsDirect kit was the only product capable of assessing cDNA abundance using one cell, further indicating its advanced RNA detection sensitivity.

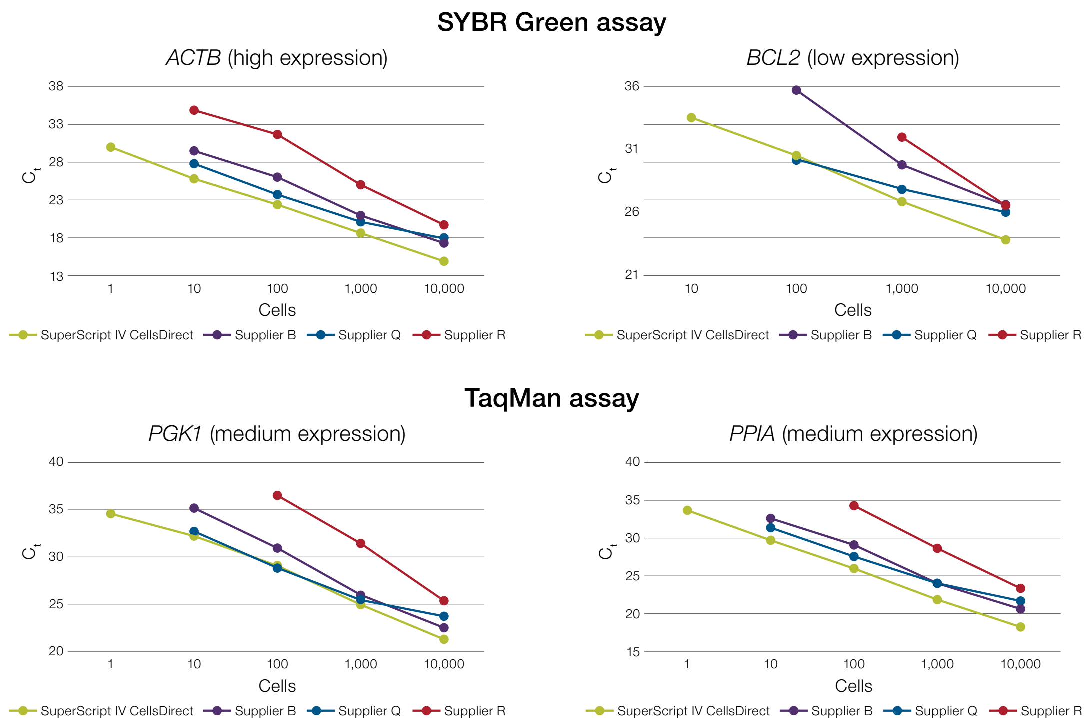


Figure 1. The SuperScript IV CellsDirect kit demonstrates linearity across a wide dynamic range of cellular inputs, and superior RNA detection sensitivity.

cDNA yields across mammalian cell types and a diversity of RNA transcripts

To investigate the RNA detection sensitivity of the SuperScript IV CellsDirect kit in greater detail, samples (1,000 cells each) of three distinct mammalian cell types (Jurkat, K562, and iPSCs) were used for cDNA generation using the SuperScript IV CellsDirect kit and four other commercially available direct RT kits. From the cDNA samples, qPCR was performed to evaluate cDNA yields from 44 different RNA transcripts (Figure 2), representing a wide range of cellular abundance (normalized expression ranging from about one to hundreds of transcripts per cell) [4]. For every transcript across all cell types, the SuperScript IV CellsDirect kit resulted in lower C_t values compared to other direct RT kits, with one exception where the C_t values were equal (*ENO1* gene, iPSCs). Importantly, the observation holds true even when directly compared with an earlier generation of this kit, the SuperScript III CellsDirect kit (Figure 3). The consistently lower C_t values indicate that the SuperScript IV CellsDirect kit produced higher cDNA yields in 99.4% of comparisons and that cDNA production is consistently superior even with low-abundance transcripts (Figure 2).

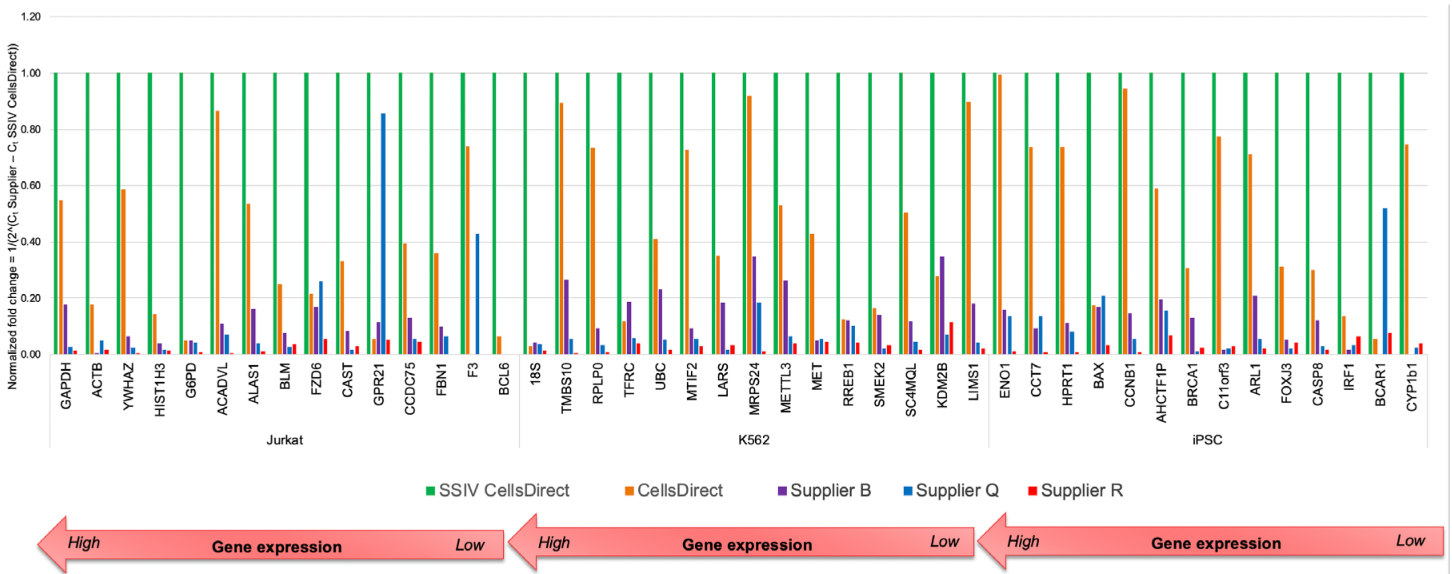


Figure 2. Comparative cDNA yields among direct RT kits for a variety of cellular transcripts.

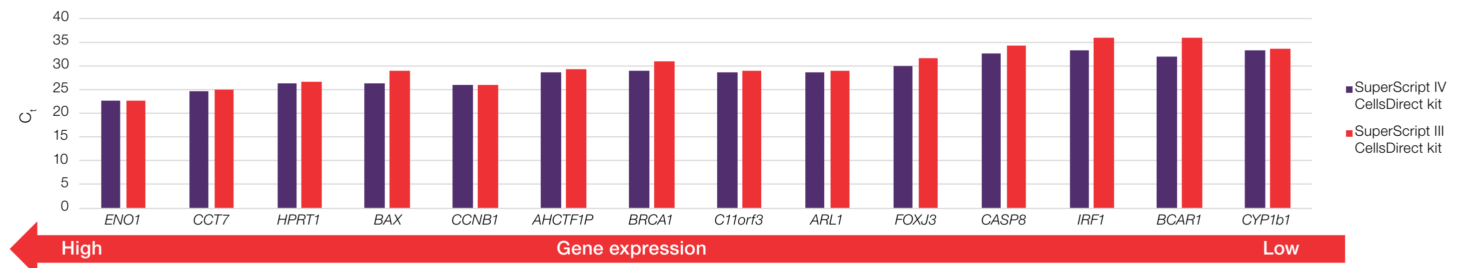


Figure 3. Detection sensitivity of the SuperScript IV CellsDirect kit compared to the SuperScript III CellsDirect kit when using iPSCs.

Assessing reproducibility and compatibility with an IPC

Ensuring high-quality RT-qPCR data and results includes establishing and testing the reproducibility of methods. Unsurprisingly, reproducibility has continued to be an important concern among researchers [5]. Researchers relying on RT-qPCR seek low variability, which means that equivalent sample input loads result in highly similar C_t numbers, regardless of sample dilution. For this reason, researchers are often eager to adopt techniques that increase experimental precision. This is a common explanation for the growing use of manufactured master mixes and kits versus stand-alone enzymes and laboratory-prepared reagents, across the biotechnology and life science research markets. However, master mixes and kits must be reliable to be used for this critical research.

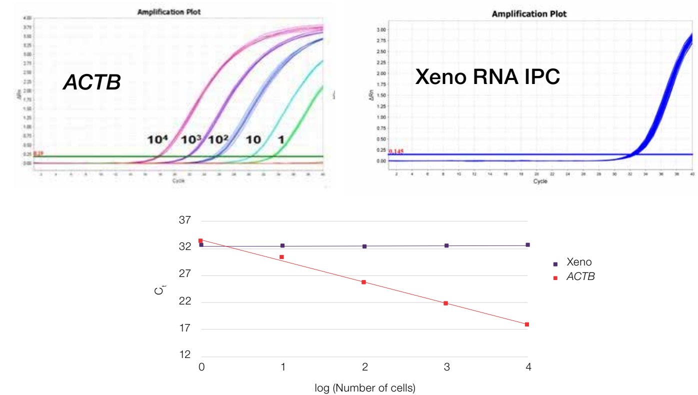
Another common strategy relies on the use of normalization to account for yield variability. Relative quantitation calculations ($\Delta\Delta C_t$ method) [6] will often utilize a highly and consistently expressed endogenous transcript (also referred to as a housekeeping gene) as a normalization control [7,8]. Provided that the expression of the endogenous control is stable across samples, this normalization strategy helps to account for variation in cDNA template amounts. This method allows researchers to reliably evaluate the up- and downregulation of specific transcripts.

Importantly, tracking qPCR experimental consistency using exogenous IPCs has grown into a key strategy for both assessing and increasing reproducibility. In this approach, synthetic nucleic acid sequences lacking homology to known sequences (Xeno RNA) are added in identical amounts to samples at the beginning of sample preparation. The IPC sequences are then simultaneously quantified along with analyte sequences. Variation in IPC C_t values indicates material loss and can act as a red flag for sample treatment and consistency. In addition, these IPCs can be used to determine optimal input cell numbers and monitor lysis, further helping to ensure protocol reproducibility.

To this end, it is critical to ensure that master mixes and kits are able to generate reproducible C_t values while maintaining compatibility with IPCs. This is particularly important for workflows that do not include RNA purification, given that chemical inhibition of the reverse transcriptase is more likely in complex mixtures such as lysed cell solutions.

Lysed iPSC samples (with 0 to 10,000 cells) were spiked with identical amounts of the Xeno RNA IPC. Using both TaqMan Fast Advanced and PowerTrack SYBR Green Master Mixes, expression of β-actin (*ACTB*), a commonly used endogenous control gene, was assessed, while simultaneously tracking Xeno RNA C_t values for sample variation across six replicates (Figure 4, top panel). In both cases, *ACTB* C_t values inversely correlated with the number of cells in each sample in a highly linear fashion (Figure 4, bottom panels) indicating complete lysis and no RT-qPCR inhibition. Conversely, all samples (regardless of cellular load) generated equivalent C_t values for the Xeno RNA IPC. A constant IPC C_t across all samples also points to complete sample lysis and no RT-qPCR inhibition. In addition, very little technical variation across all six replicates was observed for both *ACTB* and the Xeno RNA IPC. Collectively, these results indicate that the SuperScript IV CellsDirect kit reproducibly generates cDNA for precise quantitation of a key housekeeping gene, while also maintaining compatibility with a Xeno RNA IPC. Being able to utilize this kit for both approaches makes it well suited for highly reproducible results.

TaqMan Fast Advanced Master Mix



PowerTrack SYBR qPCR Master Mix

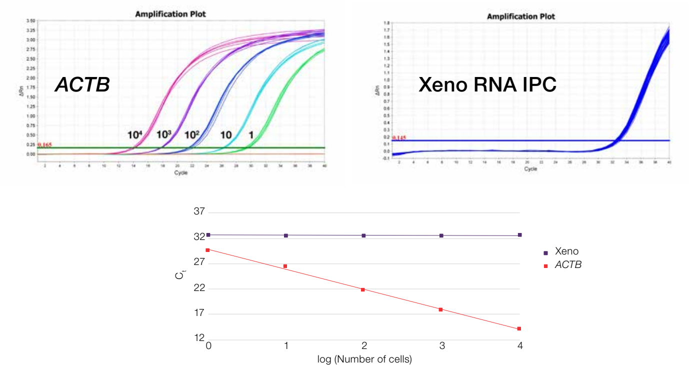
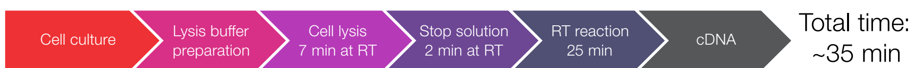


Figure 4. Quantitation of *ACTB* transcript in iPSCs with Xeno RNA IPC.

Comparison of sensitivity and cDNA yield using Cells-to-C_T kits

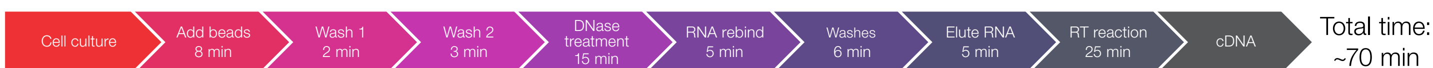
The SuperScript IV CellsDirect kit offers significant time savings and reduces sample handling compared to workflows that require a discrete RNA purification step (Figure 5). Given that the SuperScript IV CellsDirect kit offers excellent RNA detection sensitivity even compared with strategies that require RNA purification, the additional time savings offer users an opportunity to increase experimental throughput and reduce RNA sample handling without sacrificing sensitivity and quality of results.

SuperScript IV CellsDirect cDNA Synthesis Kit



Total time: ~35 min

Traditional methods



Total time: ~70 min

Figure 5. Comparison of workflows of SuperScript IV CellsDirect kit vs. RNA isolation followed by cDNA synthesis.

Other commercial kits such as the SYBR Green and TaqMan Fast Advanced Cells-to-C_T Kits include reagents to take samples from lysis all the way through qPCR. Given the popularity of these kits, we investigated how their performance compared to the SuperScript IV CellsDirect kit using identical amounts of cells and PCR primers. Samples of K562, HeLa S3, and Raji cells (1,000 cells each) were processed according to protocols for the SuperScript IV CellsDirect kit or Cells-to-C_T kits. In this process, seven distinct transcripts were quantified for comparison (Figure 6). For all transcripts and cell types, C_t values obtained from the SuperScript IV CellsDirect kit were lower than those obtained from Cells-to-C_T kits. These results suggest that the SuperScript IV CellsDirect kit yields more cDNA than Cells-to-C_T kits do. However, Cells-to-C_T kits are useful to users who primarily perform RT-qPCR, since the kits include qPCR reagents.

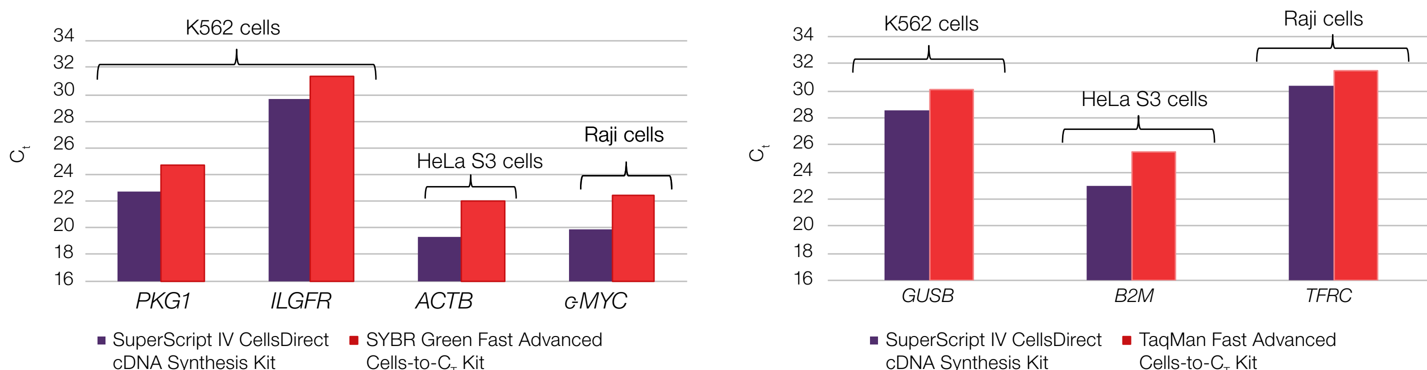


Figure 6. The SuperScript IV CellsDirect kit outperforms Cells-to-C_T kits in cDNA yield when lysing the same amount of cells.

Performance of SuperScript IV CellsDirect kit in endpoint PCR

While researchers continue to incorporate RT-qPCR into their research, many users also rely on conventional RT-PCR strategies, like endpoint and semiquantitative PCR. Unlike qPCR, these methods rely on conventional PCR and band assessment on agarose gels. These approaches are particularly important for cloning RNA transcripts and for cheaper, rapid assessment of gene expression. For some projects, it is common for researchers to first assess expression of key genes using endpoint strategies, prior to committing to larger and more expensive qPCR, RNA-Seq, and microarray experiments. There can be significant value to adopting workflows that are compatible with both endpoint PCR and qPCR.

The SuperScript IV CellsDirect kit generates cDNA that can be used in a variety of ways following completion of the protocol. To assess the compatibility of the SuperScript IV CellsDirect kit with endpoint PCR, *GAPDH* and *c-MYC* targets in cDNA generated from Raji, K562, and HeLa cells were amplified by traditional PCR and run on agarose gels (Figure 7). Band intensities of both transcripts in all cell types correlated with increasing cell number inputs (1 to 10,000 cells, left to right). In the case of *c-MYC* in K562 cells, sample loading normalization made this correlation clear, given the observed decrease in band intensity between 100 and 1,000 cells.

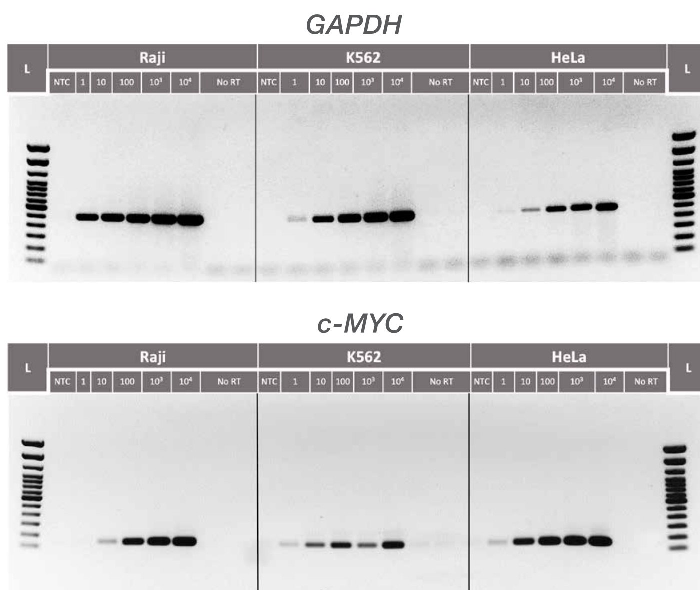


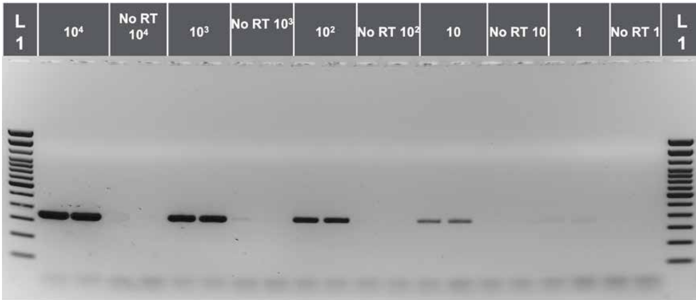
Figure 7. The SuperScript IV CellsDirect kit is compatible with endpoint PCR.

Additionally, the endpoint PCR results with the SuperScript IV CellsDirect kit were comparable to results collected using discrete RNA purification steps or the SuperScript III CellsDirect kit (data not shown). These results were further confirmed by assessing additional transcripts (*ACTB*, *VIN*, *LAM*) of varying lengths and expression levels (data not shown). The Superscript IV CellsDirect kit was also compatible with two popular endpoint PCR master mixes, the Invitrogen™ Platinum™ SuperFi™ II Master Mix and Platinum™ Green Hot Start PCR Master Mix (data not shown). Collectively, these results indicate that the SuperScript IV CellsDirect kit is fully compatible with endpoint PCR procedures, allowing researchers to readily use this kit for conventional PCR in addition to qPCR.

Evaluation of gDNA removal using the SuperScript IV CellsDirect kit

Removal of gDNA is an important step towards achieving RT-PCR success and accuracy. Persistence of gDNA can lead to overestimation of the amount of cDNA in a given sample. Additionally, it is possible for some RT-PCR primers to amplify gDNA sequences in addition to their intended cDNA targets, thereby interfering with gene expression analysis and cloning [9]. It is possible to design primers that are specific to cDNA as opposed to gDNA. This additional verification may be time-consuming or impractical for researchers, especially if they already have reliable primers for their gene of interest. As such, RT-PCR users generally remove gDNA from their samples to eliminate the possibility of gDNA sequence amplification and improve target nucleic acid quantitation.

The SuperScript IV CellsDirect kit includes a lysis enhancer and DNase I to help ensure efficient gDNA removal. To confirm gDNA elimination, Jurkat cells (input ranging from 1 to 10,000 cells) were lysed according to the standard protocol, and then the cell lysate was mixed with either the SuperScript IV RT Master Mix or SuperScript IV No RT Control included with the kit. Endpoint PCR of *ACTB* was performed with the respective mixtures, and the PCR products were run on an agarose gel to assess the presence or absence of gDNA (Figure 8). Given that the *ACTB* primers used amplify both cDNA and gDNA, the presence of a 350 bp band in the conditions without RT enzyme would indicate the persistence of gDNA. Even at high cellular loads (10,000 cells), bands only appeared when the RT enzyme was present in the workflow, indicating efficient removal of gDNA prior to PCR.



L1 - TrackIt 100 bp DNA Ladder

Figure 8. The SuperScript IV CellsDirect kit readily eliminates gDNA from samples.

Conclusion

RT-PCR will continue to be a mainstay of cutting-edge life science research in the future. However, the continued demand for RT-PCR in more complex methods like RNA-Seq, coupled with a need to analyze greater sample numbers, puts a significant burden on researchers. While researchers are eager for kits and workflows that reduce these challenges, they also need reliable protocols to maximize experimental efficiency. With excellent reliability, sensitivity, and cDNA yields, the SuperScript IV CellsDirect kit was designed to meet these needs. Furthermore, data comparisons using other commercially available kits highlight its superior performance and flexibility. With the SuperScript IV CellsDirect kit, researchers can be more confident that their critical qPCR and cloning experiments can proceed with minimal obstacles and maximum data quality.

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Ordering information

Description	Quantity	Cat. No.
SuperScript IV CellsDirect cDNA Synthesis Kit	50 reactions	11750150
	500 reactions	11750350
SuperScript IV CellsDirect Lysis Reagents	500 reactions	11750550

Find out more at **thermofisher.com/cellsdirect**

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