

First-Strand cDNA Synthesis Kit for Real-Time PCR



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**50 reaction size
(20 µl reaction volume)**

STORAGE

Store at -15°C to -30°C.

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.



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COMPONENTS

All reagents have been extensively tested and carefully prepared to meet USB® standards. USB recommends the reagents be used as directed in order to achieve the best possible results. This kit contains reagents sufficient for 50 reverse transcription reactions in a 20 µl volume. In addition, this kit includes HeLa cell total RNA and control qPCR primers that can be used to verify that the First-Strand cDNA Synthesis Kit for Real-Time PCR components, the equipment used, and the user-supplied real-time PCR (qPCR) reagents are working properly.

The following components are included with each kit:

Component	Amount
10X RT Buffer, PN 75781	100 µl
10mM dNTPs, PN 77212	50 µl
RNase Inhibitor (10 units/µl), PN 75782	50 µl
RNase-Free, DEPC-treated Water, PN 70783	1 ml
M-MLV RT, PN 75784	50 µl
Anchored Oligo(dT) ₂₃ VN (50µM), PN 75785	100 µl
Random Hexamers (75µM), PN 75786	100 µl
10X Primer Mix, PN 75787	100 µl
HeLa Total RNA (100 ng/µl), PN 75788	10 µl
Control Primer Mix for qPCR, PN 75789	10 µl

The enclosed reagents should be stored at -15°C to -30°C (NOT in a frost-free freezer). HeLa total RNA should be stored at -80°C. After thawing for use, keep reagents on ice.

QUALITY CONTROL

The First-Strand cDNA Synthesis Kit for Real-Time PCR is a Tested User Friendly™ product assuring reliable results. This kit is functionally tested for first-strand cDNA synthesis using HeLa cell total RNA (supplied in the kit) followed by real-time PCR amplification of a GAPDH target (for assay dynamic range) and a Clathrin target (for assay sensitivity). All components were tested for contaminating ssDNA and dsDNA endonucleases, ssDNA and dsDNA exonucleases, and ribonucleases.

SAFETY WARNINGS AND PRECAUTIONS

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Caution: All chemicals should be considered as potentially hazardous. We, therefore, recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as lab coat, safety glasses, and gloves. Care should be taken to avoid contact with skin and eyes. In the case of contact with skin or eyes, wash immediately with water. See MSDS (Material Safety Data Sheet) for specific advice.

DESCRIPTION

RT-PCR, or reverse transcription-polymerase chain reaction, is a method for converting and amplifying a single-stranded RNA target into a double-stranded DNA product, generally from a complex pool of cellular transcripts. RT-PCR is used in a variety of applications, such as qualitative and quantitative analyses of gene expression or other RNA targets, characterization of RNA splice variants, and the generation and cloning of cDNAs. This versatile kit is useful for all of these applications.

In the initial step, the conversion of RNA into single-stranded cDNA involves a complex, enzyme-catalyzed reaction known as reverse transcription. Reverse transcription (RT) followed by real-time or quantitative PCR (qPCR) amplification is the most sensitive technique for mRNA detection and quantification currently available⁽¹⁾.

This kit is specifically tailored to convert RNA into first-strand cDNA that is suitable as template for real-time PCR applications (Fig. 1). The kit provides reagents for global first-strand cDNA synthesis from total RNA or poly(A)⁺ mRNA samples by reverse transcription using either anchored oligo(dT)₂₃VN (V: A/G/C; N: A/G/C/T), random hexamers (N₆), or an optimized combination of both (Primer Mix). As shown in Fig. 2, the Primer Mix overcomes 5' and/or 3' amplification biases by providing first-strand cDNA synthesis over the entire, full-length transcript. This mixed priming strategy minimizes experimental variability observed between priming strategies that utilize a single RT primer⁽²⁾.

One reaction is suitable for the detection of multiple user-defined targets in subsequent amplification reactions. Using this kit and following this protocol, transcripts over 10 kb (e.g. Utrophin) can easily be reverse transcribed, and amplified by real-time PCR (Fig. 3). Since the reaction produces cDNA strands which are complementary to the mRNA pool, this kit can be used in front of the USB line of qPCR products for either SYBR[®] Green or fluorescent, probe-based detection, as well as our line of end-point PCR products (Fig. 4). See the Related Products section for relevant product numbers.

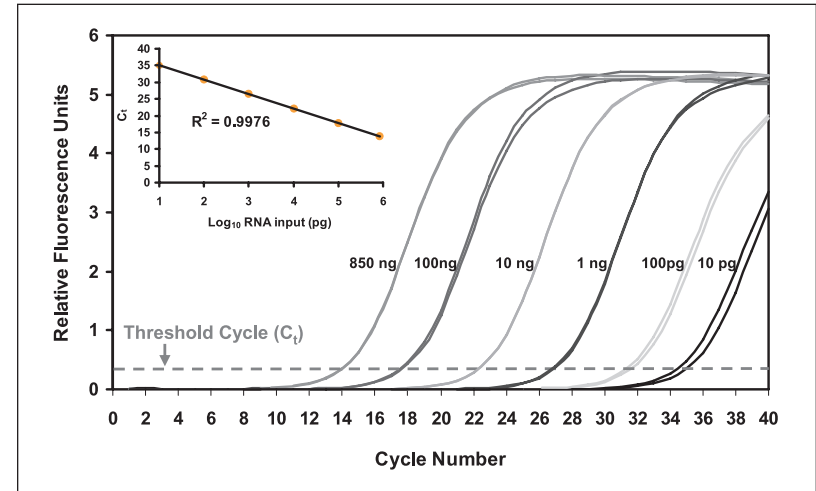


Figure 1. Assay Sensitivity and Dynamic Range. Various amounts of HeLa cell total RNA, ranging from 850 ng down to 10 pg, were reverse transcribed using anchored oligo(dT)₂₃VN as the primer. 1 μ l of each reverse transcription reaction was then used in 20 μ l real-time PCR reactions (in duplicate) to amplify a 122 bp GAPDH amplicon (on an ABI 7500 Fast instrument), using HotStart-IT[®] SYBR[®] Green qPCR Master Mix (PN 75762). GAPDH amplification and linear correlation curve above show the wide dynamic range and sensitivity of the First-Strand cDNA Synthesis Kit for Real-Time PCR.

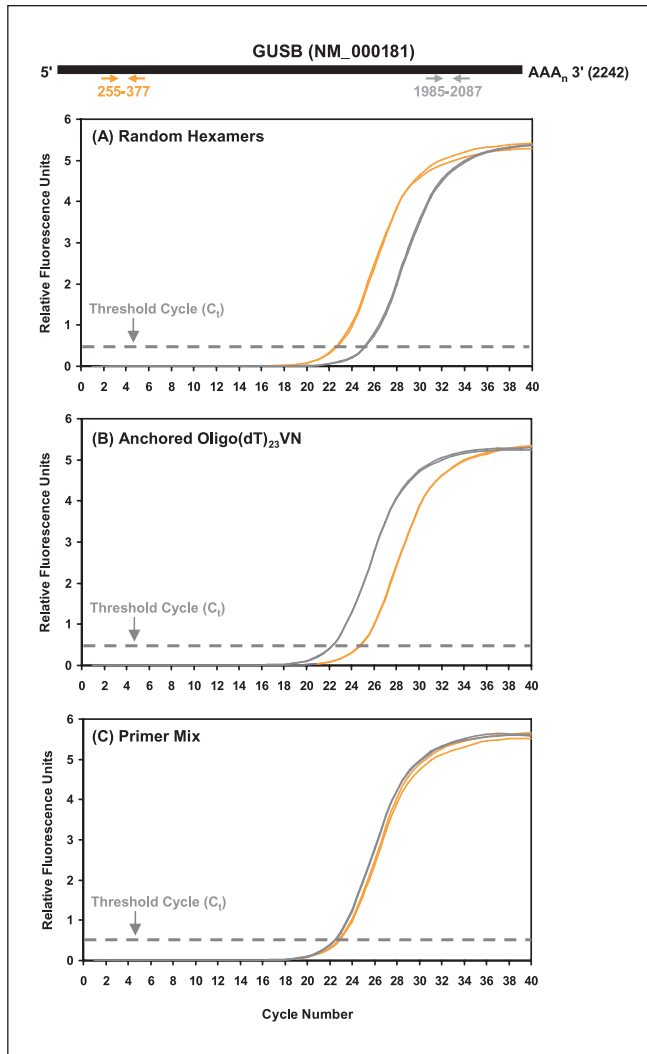


Figure 2. The Primer Mix solution greatly reduces bias for sequences near the 5' and/or 3' ends of cDNAs produced. The β -glucuronidase (GUSB) mRNA was reverse transcribed from 100 ng of HeLa cell total RNA using (A) Random Hexamers, (B) Anchored Oligo(dT)₂₃VN, or (C) the Primer Mix. Amplicons located near the 5' end (orange) or 3' end (gray) of the GUSB transcript were amplified by real-time PCR (on an ABI 7500 Fast instrument) using 1 μ l of each reverse transcription reaction in 20 μ l real-time PCR reactions (in duplicate) and HotStart-IT[®] SYBR[®] Green qPCR Master Mix (PN 75762).

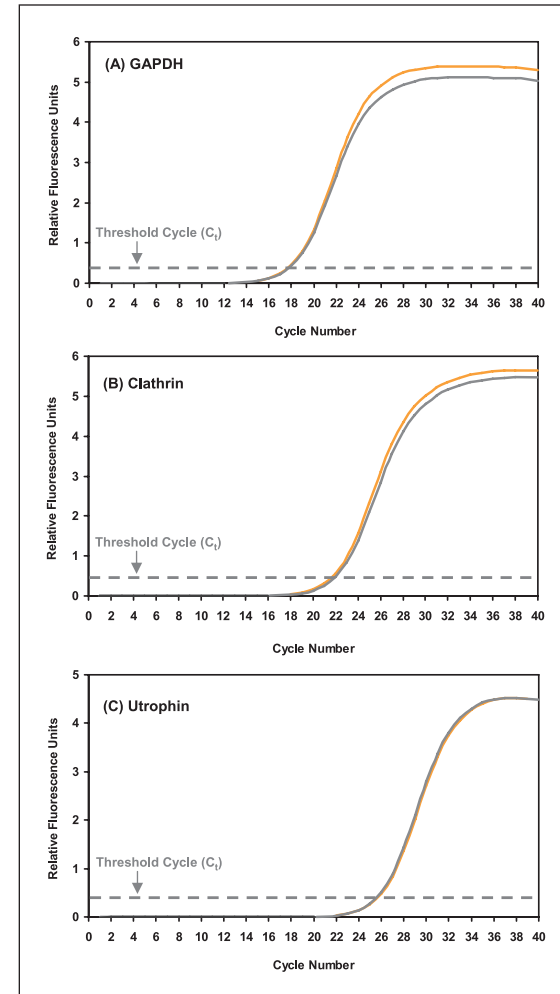


Figure 3. Assay Flexibility. The First-Strand cDNA Synthesis Kit for Real-Time PCR has been optimized for assay flexibility in carrying out reverse transcription using various RT-priming strategies and diverse RNA templates (GAPDH, ~1.5 kb transcript; Clathrin, ~6.7 kb transcript; Utrophin, ~12.5 kb transcript).

Reverse transcription was performed using HeLa cell total RNA (100 ng) and two different priming strategies: anchored oligo(dT)₂₃VN primers (orange) or random hexamers (gray). 1 μ l of each reverse transcription reaction was then used in 20 μ l real-time PCR reactions (on an ABI 7500 Fast instrument) using HotStart-IT[®] SYBR[®] Green qPCR Master Mix (PN 75762). (A) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (B) Clathrin, and (C) Utrophin amplicons were amplified using gene specific primers designed for real-time PCR.

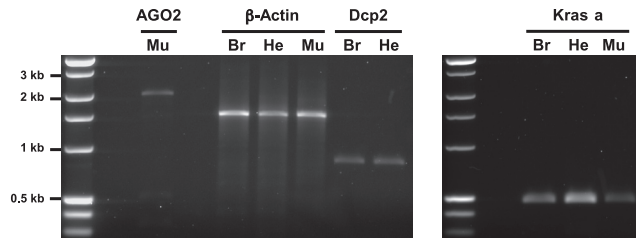


Figure 4. End-point amplification of diverse RNA targets using the First-Strand cDNA Synthesis Kit for Real-Time PCR followed by 35 PCR cycles. cDNA synthesis was performed using total cellular RNAs from human brain (Br), HeLa cells (He), or human muscle (mu) and 2 μ l of Primer Mix solution. PCR amplification was conducted using 1 μ l of RT reaction in a 25 μ l PCR reaction, 0.2 μ M of each primer and HotStart-IT[®] Taq DNA Polymerase (PN 71195). Gene specific primers for Argonaute 2 (AGO2), β -Actin, Decapping enzyme 2 (Dcp2), and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (Kras a) were designed to generate amplicon products of particular sizes, ranging from 0.5 to 2 kb, at the 5'-ends of the RNA targets. 25% of the PCR reaction was loaded on a 1% agarose gel and amplified products were visualized using ethidium bromide.

MATERIALS NOT SUPPLIED

Necessary reagents:

RNA template: Total RNA can be prepared by standard methods such as the PrepEase[®] RNA Spin Kit (PN 78766), PrepEase[®] Plant RNA Spin Kit (PN 78771), acid-guanidinium thiocyanate-phenol-chloroform extraction⁽³⁻⁴⁾, or TRIzol[®] procedures⁽⁵⁾. RNA may also be obtained from commercial suppliers. Total RNA can also be enriched for poly(A)⁺ mRNA using Oligo (dT) resin (PN 71547). RNA should be highly purified and free of RNase, polysaccharide, and proteoglycan contamination⁽⁴⁾. Ideally, RNA should also be free of DNA contamination (See Supplementary Information on RNA Quality).

Oligonucleotide primers: If desired, gene-specific oligonucleotide primers can be used in this kit and should be designed according to standard methods⁽⁶⁾. Longer oligonucleotides (> 25 bases) and those with higher melting temperatures (> 60°C) are recommended to achieve more specific amplification. Gene-specific primers which flank an intron or cross an exon-exon border are useful as a control to distinguish amplification from RNA versus contaminating DNA.

Optional reagents:

Enhancing additives: Solvents such as dimethyl sulfoxide (DMSO), glycerol, trehalose, and betaine can improve RT results on certain targets with a high degree of secondary structure⁽⁷⁻¹⁰⁾. Further details are provided in the Supplementary Information section.

Necessary equipment:

Liquid handling supplies such as PCR-grade thin-walled tubes or plates, pipettes, pipettors, and a centrifuge are required. Use plastic tubes, plates, and pipette tips that are certified RNase-free, in order to prevent RNase contamination of samples. Also, the use of barrier-tip pipettes and dedicated PCR pipettors are strongly recommended in order to avoid RNase contamination.

Latex gloves (powder-free) should be used for handling reagents and equipment in order to decrease the probability of introducing RNases into samples.

Thermal cycler for incubations between 4°C and 95°C.

Equipment such as a standard horizontal gel apparatus and a UV transilluminator or fluorescence image scanner can also be used for analysis of PCR products.

PROTOCOL

RT Priming Strategy:

Priming strategies used to convert RNA into cDNA are among the various factors that can affect the variability and sensitivity of subsequent real-time PCR reactions⁽²⁾. Oligo(dT) primer is commonly used to prime eukaryotic mRNA from their 3'-end poly(A) tails without prior enrichment for poly(A)⁺ mRNAs. Therefore, it is the primer of choice for full-length cDNA synthesis, and generally guarantees the synthesis of the longest cDNAs terminating at the 3'-end of the transcript. An anchored oligo(dT)₂₃VN primer which promotes annealing and extension at the start of the poly(A) tail is provided in this kit. This reduces "non-productive" synthesis from more distal locations along the poly(A) tail. Also provided are random hexamers which can prime throughout the entire length of the RNA. Therefore, all RNA classes present in the sample, including non-polyadenylated transcripts such as rRNA, are templates for priming and reverse transcription, resulting in more diverse cDNA products. Using random hexamers may be useful in applications such as the synthesis of cDNAs from de-adenylated transcripts (including prokaryotic and viral RNAs) and to reduce the 3'-end sequence bias that may result from the use of oligo(dT). Alternatively, when oligo(dT) and random primers lead to amplification products greatly biased for sequences near the 3'- or 5'-ends of the transcripts, we have provided a combination of anchored oligo(dT)₂₃VN and random hexamers (Primer Mix) that has been optimized to greatly reduce the production of end-biased cDNAs (Fig. 2). Alternatively, the user can also design a gene-specific primer (GSP) to use in the reverse transcription reaction to transcribe a particular target RNA. For some transcripts, the use of GSP may be appropriate for maximizing the yield of first-strand cDNA synthesis and specificity. In this scenario, adding 10 pmol of

the specific primer is recommended (optimization may be necessary to increase sensitivity). As a starting point, we recommend that the user designs and tests different GSPs to determine the one best suited for the experiment, at a concentration ranging from 0.2-5 μ M (the optimum concentration must be determined empirically).

RT Reaction Set-Up:

This standard protocol applies to a single 20 μ l RT reaction. Master mixes for multiple reactions can be made by increasing the volumes of reaction components proportionally.

1. Thaw frozen reagents at room temperature (except for RNase Inhibitor and M-MLV RT) and mix thoroughly by vortexing. Briefly spin down the tube contents and then place on ice.
2. Add the following reagents in the table below to an RNase-free tube. Mix gently and briefly spin down the tube contents. Keep on ice.

Components	Volume
Primers*	2 μ l
Total RNA**	up to 1 μ g
10X RT Buffer	2 μ l
10mM dNTPs	1 μ l
RNase Inhibitor	1 μ l
M-MLV RT†	1 μ l
Water	up to 20 μ l

* If you use a gene-specific, first-strand primer, we recommend using 10 pmol as a starting amount.

** Poly(A)-enriched RNA can also be used, up to 100 ng.

† Include a negative control by replacing the enzyme with water; this RT-minus control will be used to detect the presence of genomic DNA in the subsequent PCR amplification.

RT Incubation:

The incubation step should be performed in a thermal cycler with a heated lid as follows:

1. Start the thermal cycler program shown below and transfer the reaction tube(s) from ice to the block after the block reaches the desired temperature in step one.
Step 1: 44°C, 60 min (up to 50°C)
Step 2: 92°C, 10 min
Step 3: 4°C (as needed)
2. Briefly centrifuge the samples before storing them at -20°C until ready for downstream applications (e.g. real-time PCR, etc.).

Alternative Protocol:

For RNA targets that exhibit a high degree of secondary structure, heat denaturation of the RNA/primer may be beneficial. In this case, mix RNA, primers and water together. Heat RNA/primer/water mixture at 75°C for 5 min and then cool immediately by placing on ice for at least 5 min prior to reactions. Spin briefly before adding the 10X RT Buffer, dNTPs, RNase Inhibitor, and M-MLV in the amounts indicated in the above table. In general, this step can be omitted without any adverse effect on the yield of first-strand cDNA.

PCR Suggestions:

Real-Time PCR:

This kit has been specifically tailored to real-time PCR applications in a two-step format (examples of real-time PCR analysis of RNA targets are shown in Figs. 1-3). We recommend using HotStart-IT® SYBR® Green or Probe qPCR Master Mix (PN 75762 or 75766) to detect and quantify user-defined specific transcripts. These convenient master mixes offer high specificity, sensitivity, and broad dynamic range. In general, we recommend using 1 μ l of the undiluted RT reaction per 20 μ l qPCR reaction as template, since increasing the amount of M-MLV carried over may inhibit the reactions⁽¹¹⁻¹²⁾. In general, limit the total amount of the undiluted RT reaction to 10% or less of the total volume of the qPCR reaction. For target transcripts expressed at high levels (e.g. ribosomal proteins), the RT reaction can first be diluted to 40 μ l with water, and then use 1 μ l of the diluted RT reaction as template for the PCR amplification. Alternatively, for target transcripts expressed at low levels, it may be necessary to increase the input of cDNA template to 2 μ l or increase the input of RNA used in the RT reaction. The optimum primer concentration for real-time PCR reactions lies between 0.1-1 μ M. We suggest starting with 0.2 μ M of each primer to avoid primer-dimer and non-specific products. We strongly recommend running the real-time PCR reactions in triplicate, and also including a “no RT template” to show that no DNA contamination is present in the PCR reagents. See the Supplementary Information section for design information regarding qPCR primers.

End-Point PCR:

Following first-strand cDNA synthesis, end-point PCR amplification can be performed for full-length cDNA cloning and other applications (Fig. 4). For this purpose, we recommend using HotStart-IT® FidelityTaq™ PCR Master Mix (PN 71156) for high fidelity and sensitivity. For routine end-point PCR, use HotStart-IT® Taq Master Mix (PN 71196). Amplified products can then be verified by agarose gel electrophoresis followed by PCR product purification if necessary. Typically, 1 to 5 μ l of the first-strand cDNA synthesis reaction can be used as template in a 10-50 μ l PCR reaction.

SUPPLEMENTARY INFORMATION

RNase Contamination

When working with RNA, it is critical to prevent RNase contamination that could greatly affect the outcome of your experiments. Keep your RNA samples on ice while setting up the experiment. Also, it is highly recommended to use the RNase Inhibitor provided in the kit to reduce the effect of stray RNases. These are some general guidelines that should be followed to reduce the risk of ribonuclease contamination:

- Wear gloves at all times while handling reagents, materials and equipment to prevent RNase contamination from hands. Change gloves after touching non-RNase free surfaces.
- Avoid using equipment and work areas that have been exposed to RNases. Clean the equipment and work surfaces with ethanol or commercially available RNase decontamination solutions before starting your experiments.
- Clean the interior and exterior of micropipette shafts with ethanol or commercially available RNase decontamination solutions.
- Use barrier tips.
- Use RNase-free plasticware and RNase-free buffers and reagents.

Avoiding potential sources of RNase contamination (ungloved hands, contaminated pipettors, etc.), will help to prevent sample degradation.

RNA Quality

The quality of the RNA sample is the most important factor affecting the outcome of RT reactions. Quality can be defined in terms of both purity and integrity (*i.e.* proportion of the RNA sample which is full-length). Several factors can have a negative impact on the quality and integrity of RNA that can be isolated, including tissue/organ type, sample source, and tissue storage and preservation. Although some degraded RNA samples (*e.g.* from formalin-fixed paraffin-embedded tissue) may be suitable for transcriptome analysis, the highest RNA quality should be sought for use in first-strand cDNA synthesis and subsequent downstream applications.

Many purification methods can be used to prepare RNA, provided the methods yield RNA which is essentially free of contaminating DNA, proteins, polysaccharides or proteoglycans, phenol, ethanol, and salts. These contaminants inhibit the activity of M-MLV Reverse Transcriptase, which reduces RT yields⁽⁴⁾. Total RNA can be isolated using standard methods such as acid-guanidinium thiocyanate and phenol-chloroform extraction, or TRIzol[®] procedures⁽³⁻⁵⁾. After RNA purification with protocols that use solvents such as phenol or guanidinium salts, we strongly recommend washing the RNA pellet at least twice with cold 70-75% ethanol prior to dissolving the RNA pellet in DEPC-treated solutions (to help remove any traces of these solvents). Alternatively, commercially available column-based methods (*e.g.* PrepEase[®] RNA Spin Kit, PN 78766 and PrepEase[®] Plant RNA Spin Kit, PN 78771) can also be used to purify total RNA. For some applications (*e.g.* detection of rare transcripts, cDNA library construction, etc.), poly(A)⁺ mRNA purification may be necessary. Enrichment of poly(A)⁺ mRNA can be accomplished using commercially available column-based methods (*e.g.* Oligo(dT) Resin, PN 71547).

The purity of the RNA sample should be estimated by measuring its absorbance at 260 and 280 nm in TE buffer. A_{260}/A_{280} ratios should fall between 1.7-2.1. If ratios fall outside this range, re-precipitation of the RNA or column purification may be necessary.

The integrity and quality of the RNA may be assessed by performing denaturing agarose gel electrophoresis or using a bioanalyzer. High-quality, full-length RNA will exhibit two dominant, discrete bands, composed of 28S and 18S ribosomal RNA (in mammalian systems). These rRNA bands should have minimal smearing below them, indicating absence of degradation. RNA of the highest quality has a 28S rRNA band which stains about twice as intense as the 18S rRNA band. Maintain RNA integrity by storing samples in TE (10mM Tris-HCl pH 7, 1mM EDTA), 0.1mM EDTA, or DEPC-treated Water at -80°C.

RNA samples can be treated with recombinant DNase I (PN 78311) to reduce or eliminate any suspected genomic DNA contamination. This is particularly important if experiments generate targets of the same size from RNA and contaminating genomic DNA (*i.e.* no intron present in genomic DNA target).

Amount of RNA Per Reaction

Total RNA may be used from 10 pg up to 1 µg and poly(A)⁺ mRNA may be used from 1 pg up to 100 ng per reaction. Greater representation of the specific target within a population of RNA molecules allows the use of lower amounts of RNA. Since poly(A)⁺ mRNA comprises approximately 1 to 5% of the total RNA, a specific target will be more abundant in poly(A)⁺ mRNA than in total RNA. For best results, when working with dilute stocks of RNA (less than 100 ng/µl), freshly prepare the dilute stocks from concentrated stocks rather than subjecting dilute stocks to multiple freeze-thaw cycles.

Downstream Applications

This kit is specifically tailored for real-time PCR applications. However, end-point PCR amplifications can also be performed with little to no optimization needed (Fig. 4). When performing quantitative real-time PCR or semi-quantitative end-point PCR experiments, we also recommend monitoring a “housekeeping” gene (e.g. actin, tubulin, etc.) that will be used as a reference for equal amounts of input RNA.

Primer Design

General rules for designing primers can be found in many texts⁽⁶⁾. In general, primers should range in length from 18 to 30 nucleotides, exhibit G+C content similar to each other (ideally in the range of 40 to 60%), and exhibit T_m values ranging from 55 to 65°C that are closely matched to each other. T_m values may be estimated using the following equation: $T_m(^{\circ}\text{C}) = 2(\text{A}+\text{T}) + 4(\text{G}+\text{C})$. More accurate methods for calculation of T_m values may also be applied⁽⁶⁾. When designing qPCR primers, make sure the size of the expected amplicon is between 80-200 bp, with an optimal length of 100-150 bp. Primers that do not fit these criteria may also function well, but empirical testing is required. See probe manufacturers' websites for details on the design of fluorescent probes, such as TaqMan[®], Molecular Beacons, FRET, etc.

Using computer programs designed to select appropriate primers and probes for qPCR in a given sequence is highly recommended. In addition, several public primer databases are available on the internet. These databases contain gene-specific primer sets that have been validated for gene expression analysis by real-time PCR. Some examples of databases include PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>) and RTPrimerDB (<http://medgen.ugent.be/rtprikerdb/>). Other resources for PCR primers, oligo databases and design tools can be found at http://www.hsls.pitt.edu/guides/genetics/obrc/dna/pcr_oligos.

When using a set of primers and probe(s) for the first time, we recommend validating this set first by running a small amount of the reaction (e.g. 5 µl) on an agarose gel to confirm the presence of a single product. This is not strictly necessary when using hybridization probes, but a “cleaner” reaction can improve the overall quantification analyses. When using SYBR[®]-based qPCR reagents for the first time on a particular amplicon, analyze the melt-curve to confirm amplification of a single product.

It is useful to design primer sets that give different amplification products from messenger RNA than from genomic DNA that may be a sample contaminant. Whenever possible, primers should flank an intron or span an exon-exon border. For primers flanking an intron, the PCR product will be smaller from RNA compared to that from contaminating genomic DNA. For primers crossing an exon-exon border, PCR product should not be generated from genomic DNA. Be aware that common housekeeping genes such as β-actin or GAPDH have intron-less pseudogenes in many organisms. In those cases, it is important to have RNA which is completely free of contaminating genomic DNA.

Suggestions for Difficult Templates

Successful amplification with the First-Strand cDNA Synthesis Kit for Real-Time PCR often requires little or no optimization. If targets have a high-degree of secondary structure and/or high G+C content (e.g. > 60%), adding certain supplements, such as DMSO, glycerol, trehalose, and/or betaine to the RT reaction may improve results⁽⁷⁻¹⁰⁾. DMSO and glycerol may be added at final concentrations ranging from 1 to 10% (v/v)⁽⁶⁾. Trehalose may be added to 0.6M final concentration⁽¹⁰⁾. Betaine (5M stock, PN 77507) may be added at 0.5M to 2.0M final concentration^(7,10). Betaine and trehalose have been reported to thermostabilize proteins in general^(7,9). Thus, the RT reaction temperature may be elevated in their presence, possibly melting secondary structure. All of these solvents tend to decrease T_m values, thus its presence in the reactions may result in a decrease in the optimum annealing temperature by several degrees. In addition, an increase in the RT reaction temperature to 50°C may provide better first-strand cDNA synthesis in those cases where the target(s) have a high degree of RNA secondary structure.

TROUBLESHOOTING

Depending on the downstream applications used following first-strand cDNA synthesis, various problems may be encountered. Here, we describe common problems and present possible causes and solutions associated with end-point and real-time PCR with specific emphasis on real-time applications.

Potential Problem	Possible Causes and Solutions
No RT-PCR product (or low yield of RT-PCR product)	<ol style="list-style-type: none"> RNA sample: <ul style="list-style-type: none"> Check the integrity of the input RNA to ensure it is not degraded. Avoid RNase contamination and always keep the RNA sample on ice when setting-up the reactions. Avoid multiple cycles of RNA freezing and thawing. Instead, aliquot the RNA sample after extraction. Use the total RNA sample and primers in the kit as a positive control. The primers provided with the kit can be used for real-time PCR amplification using as little as 1 ng of HeLa cell total RNA. Amplified control products (110 bp) can also be visualized on a 2% agarose gel. This control was functionally tested using the provided reagents and protocol. Improper storage and handling may affect the performance of the enzymes in the kit. Some reagents used for RNA extraction can inhibit the reverse transcription reaction (e.g. guanidinium salts, phenol, SDS, EDTA, etc.). We strongly recommend washing the RNA pellet at least twice with 70-75% cold ethanol to reduce the possible carrying-over of these contaminants. RNA target: <ul style="list-style-type: none"> Low expression of target RNA in the sample: test a range of RNA concentrations up to 1 µg total RNA or 100 ng poly(A)⁺ mRNA.

Potential Problem	Possible Causes and Solutions
	<ul style="list-style-type: none"> We recommend at first that the user tests all three RT primers (i.e. random hexamers, anchored oligo(dT)₂₃VN, and the Primer Mix) to determine the one best suited for the experiment. 30-35 PCR cycles are generally sufficient for most target amplifications by end-point PCR. However, it may be necessary to increase the number of cycles up to 45 if the RNA target is expressed at low levels. <ol style="list-style-type: none"> Optimization of RT/PCR conditions: <ul style="list-style-type: none"> If template and/or primers exhibit G+C contents greater than ~60%, consider supplementing reactions with reagents that improve amplification of G+C rich templates (see Supplementary Information section). The optimal amount of supplements for a given primer/template combination should be determined empirically. Perform a denaturation step prior to reverse transcription (see Alternative Protocol). Also, consider increasing the temperature of the RT step up to 50°C to help melt RNA secondary structures. For full-length cDNA synthesis of long mRNA targets, extending the RT incubation period up to 90 min may be beneficial. Increase the concentration of specific primers in both the RT and PCR steps, for example from 0.5µM to 0.8µM. RT/PCR reaction set-up: <ul style="list-style-type: none"> Master mixes and individual reactions should be well mixed (by vortexing before the enzyme is added). Be careful not to introduce air bubbles. Check that all the reaction components have been added correctly in the reaction and that the instrument (e.g. thermal cycler) was correctly programmed.

Potential Problem	Possible Causes and Solutions
Non-specific, smeared RT-PCR product	<ol style="list-style-type: none"> 1. RNA sample: <ul style="list-style-type: none"> • Non-specific bands and/or smearing may occur with use of either too little or too much RNA. Test a range of RNA concentrations. 2. RNA target: <ul style="list-style-type: none"> • Non-specific amplification of the cDNAs. Design a new set of primers for the PCR/qPCR. Check the primer databases mentioned in the Supplementary Information section. • If multiple bands are observed, this may indicate the presence of several forms of alternatively spliced transcripts. Purification and sequencing of these fragments should be performed to validate this possibility. 3. Optimization of RT/PCR conditions: <ul style="list-style-type: none"> • An extremely high number of PCR cycles may lead to the amplification of non-specific products. Start using 30-35 PCR cycles first, then increase or decrease as necessary. • Decrease the concentration of specific primers in the RT and/or PCR reactions, for example from 0.5µM to 0.2µM or 0.1µM. • Supplement reactions with reagents that improve amplification of G+C rich templates (see Supplementary Information section). • Test a range of PCR annealing temperatures above and below the first one(s) tested. 4. Amplified product in the “RT-minus” control: <ul style="list-style-type: none"> • This may indicate the presence of genomic DNA contamination. • At the same time, set-up a “no RT template” control PCR reaction in which the RT template is replaced with water. If amplified products are indeed observed, this may indicate that one of the reagents may be contaminated with DNA. • Replace all the reagents with new ones. • Always clean-up all the equipment and the working area appropriately before starting an experiment.

Potential Problem	Possible Causes and Solutions
Specifics regarding real-time PCR amplification	<ol style="list-style-type: none"> 1. Refer to the Troubleshooting section of the user’s manual provided with the qPCR protocol. 2. Amplified product: <ul style="list-style-type: none"> • When designing primers, make sure the size of the expected amplicon is between 80-200 bp, with an optimal length of 100-150 bp. • Always include the “RT-minus” control in the RT reaction and the “no RNA template” control in the real-time PCR reaction. 3. Analyze the melting curve: <ul style="list-style-type: none"> • A single peak should be observed indicating the presence of a specific and single product amplified. • The presence of multiple peaks and/or shoulder(s) may indicate the presence of non-specific products and/or primer-dimer formation. The PCR cycle needs to be optimized and/or the primers must be re-designed (see Supplementary Information section).

If problems persist please contact USB Technical Support for assistance at (800) 321-9322 or techsupport@usbweb.com. Additional information such as new product listings, updated protocols and TechTips, may be found at our website, www.usbweb.com. For technical support outside the U.S., please visit our website for up-to-date contact information on the USB product distributor within your area.

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RELATED PRODUCTS

Reverse Transcription Enzymes and RT-PCR Products

Product	Application	Pack size	Product number
HotStart-IT [®] SYBR [®] Green One-Step qRT-PCR Master Mix Kit	RT-qPCR analysis in a single reaction format	100 reactions 500 reactions	75770
HotStart-IT [®] Probe One-Step qRT-PCR Master Mix Kit	RT-qPCR analysis in a single reaction format	100 reactions 500 reactions	75772
M-MLV Reverse Transcriptase	cDNA synthesis, low RNase H activity	25,000 units 100,000 units	78306
AMV Reverse Transcriptase	cDNA synthesis	200 units 1,000 units	70041Y 70041Z
Ribonuclease Inhibitor, Recombinant (40 units/μl)	Protect RNA	5,000 units	71571
Oligo dT ₍₁₂₋₁₈₎ Primer	Priming synthesis of cDNA from mRNA	100 μl (2.5 nmol)	77405
One-Step RT-PCR Kit	RT-PCR, analysis of multiple templates	50 reactions	78350
Two-Step RT-PCR Kit	RT-PCR, analysis of multiple genes	50 RT/100 PCR reactions	78355
RT Script Kit	RT synthesis of cDNA for cloning, arrays, and RT-PCR	50 reactions	78360
DNase I, Recombinant, RNase-Free	Removal of DNA prior to RT-PCR	1,000 units 2,500 units	78311

PCR Enzymes and Related Products

Product	Application	Pack size	Product number
Taq DNA Polymerase	PCR	50 units 250 units 1,000 units 5 × 250 units 5,000 units	71160
Taq PCR Kit	PCR, including all necessary reagents	100 reactions	71161
Taq PCR Master Mix (2X)	PCR reaction mix (2X), ready-to-use	100 reactions (125 units)	71162
FideliTaq [™] DNA Polymerase	PCR	50 units 250 units 1,000 units 5 × 250 units 5,000 units	71180
FideliTaq PCR Master Mix (2X)	PCR reaction mix (2X), ready-to-use	100 reactions	71182
FideliTaq PCR Master Mix Plus	PCR reaction mix, ready-to-use	100 reactions	71183
ExoSAP-IT [®]	Clean-up of PCR products	20 reactions 100 reactions 500 reactions 2,000 reactions 5,000 reactions	78250 78200 78201 78202 78205

Ultrapure Nucleotides

Product	Application	Pack size	Product number
PCR Nucleotide Mix, 10mM each of dATP, dCTP, dGTP, and dTTP	RT and/or PCR, nucleotides	500 µl	77212
PCR Nucleotide Mix, 25mM each of dATP, dCTP, dGTP, and dTTP	RT and/or PCR, nucleotides	500 µl	77119
dATP, dCTP, dGTP, dTTP (Set of Four), 2'-Deoxy-Nucleoside-5'-Triphosphates, 100mM Solution	RT and/or PCR, nucleotides 4 dNTPs per pack	4 x 25 µmol (250 µl) 1 pack	77100

Additives for PCR

Product	Application	Pack size	Product number
Magnesium Chloride, 25mM Solution	Supplement for RT and/or PCR	1 ml 5 x 1 ml	71167
Glycerol, Nuclease-Free, Ultrapure	Supplement for RT and/or PCR	500 ml 1 L	16374
Betaine, 5M Solution, Ultrapure	Supplement for RT and/or PCR	1.5 ml 5 x 1.5 ml 10 ml	77507
α,α-Trehalose, Dihydrate	Supplement for RT and/or PCR	10 gm 100 gm	22515
BSA, 50 mg/ml Solution, Non-Acetylated, Ultrapure	Supplement for RT and/or PCR	50 mg 5 x 50 mg	10921

Ultrapure RNA Reagents

Product	Application	Pack size	Product number
Diethyl Pyrocarbonate (DEPC)	RNase inactivation	25 ml 100 ml	14710
RNA Solutions Kit Contains 100 ml of: 5M Ammonium Acetate; 0.5M EDTA; 1M Magnesium Chloride; 2M Potassium Chloride; 5M Sodium Chloride; 1M Tris, pH 7.0; 1M Tris, pH 8.0; and RNase-Free Water, DEPC-Treated	Storage and handling of RNA	8 x 100 ml per pk, 1 kit	75903
TE Buffer (1X)	Storage of RNA/DNA	10 x 1 ml 100 ml 500 ml	75893
Water, RNase-Free		100 ml 500 ml 1 L 5 L	71783

Product	Application	Pack size	Product number
Water, RNase-Free, DEPC-Treated		25 ml 10 x 1 ml 100 ml 500 ml 1 L	70783

Ultrapure Electrophoresis Reagents

Product	Application	Pack size	Product number
Agarose - Separation ≥ 500 bp, Genetic Performance Certified™	Gel electrophoresis	25 gm 100 gm 250 gm 500 gm	75817
Ethidium Bromide Drops	Staining RNA and DNA	5 ml	75816
TAE Buffer (50X)	Gel electrophoresis	100 ml	74015
TBE Buffer (5X)	Gel electrophoresis	1 L 5 L	75891

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Material Safety Data Sheet

Revision: 02/21/2008



Hazard information is provided for compliance with both the UK Chemicals (Hazard Information and Packaging) (CHIP) Regulations and the US Hazard Communication Standard (HCS)

IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND COMPANY

PRODUCT NAME:
First-Strand cDNA Synthesis Kit for Real-Time PCR

PRODUCT CODE:
75780

EEC NUMBER:
None

SUPPLIER:

USB Corporation
26111 Miles Road, Cleveland, Ohio 44128 Phone: (216) 765-5000
Please visit our website at www.usbweb.com for contact information on USB product distributors within your area.

EMERGENCY CONTACT:

Chemtrec: (800) 424-9300
Outside USA & Canada: 703-527-3887

COMPOSITION/HAZARDOUS COMPONENTS

HAZARD	CAS NO.	%WT	TLV	CHIP R & S PHRASES
For Component 75781: Tris-HCl Potassium Chloride	1185-53-1 7447-40-7	~7.9% ~5.6%	— —	R:36/37/38 Irritating to eyes, respiratory system and skin. S:23 Do not breathe vapour. S:24/25 Avoid contact with skin and eyes. S:36/37 Wear suitable protective clothing and gloves.
For Components 75782 & 75784: Glycerol	56-81-5	~50%	See "Regulatory Information" Section	R:36/37/38 Irritating to eyes, respiratory system and skin. S:26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S:36/37 Wear suitable protective clothing and gloves.

For Component 75788:
HeLa Total RNA

N/A ~100%

HAZARDS IDENTIFICATION

CHIP:
Biohazard; Irritant
HCS:
Biohazard; Irritant

FIRST-AID MEASURES

EYES: Flush with water for 15 min. Seek medical advice if irritation persists.

SKIN: Flush with water, then wash thoroughly with soap and water. Remove contaminated clothing and wash before reuse. Seek medical attention if irritation persists.

INHALATION: Remove the victim from exposure and move to fresh air. If breathing is difficult, give oxygen. If not breathing, give artificial respiration. Keep victim quiet and warm. Seek immediate medical attention.

INGESTION: Drink water and seek immediate medical attention. Avoid alcoholic beverages. Never give anything by mouth to an unconscious person.

FIRE-FIGHTING INFORMATION

Use media suitable to extinguish the supporting or surrounding fire. Wear NIOSH (or equivalent) approved self contained breathing apparatus. For small fires only, use carbon dioxide, dry powder or foam. Emits toxic fumes under fire conditions. For Glycerol: Contact with strong oxidizing agents may produce an explosion. Explosion Limits for Glycerol = Lower - 1.1; Upper - Not available. Flash point for Glycerol = 193°C (379.4°F); Autoignition temperature for Glycerol = 400°C (752°F).

ACCIDENTAL RELEASE MEASURES

Caution: Catalog# 75788 is isolated from human sources. Handle all products prepared from human sources as if they were capable of transmitting infectious agents. Avoid accidental inoculation, intravenous injection or contact with open wounds. Wash thoroughly after handling. Observe universal precautions when working with this product. Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, gloves and NIOSH-approved (or equivalent) respirator appropriate for the hazard. Contain the spill with an inert absorbent and place in a suitable waste container. Avoid contact of material with skin or eyes. Use adequate ventilation.

HANDLING AND STORAGE

Caution: Catalog# 75788 is isolated from human sources. Handle all products prepared from human sources as if they were capable of transmitting infectious agents. Avoid accidental inoculation, intravenous injection or contact with open wounds. Wash thoroughly after handling. Observe universal precautions when working with this product. Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, gloves and NIOSH-approved (or equivalent) respirator. A qualified industrial hygienist should evaluate the need for respiratory protection. Use adequate ventilation. Avoid contact of material with skin or eyes. Store kit at -20°C away from incompatible materials.

PERSONAL PROTECTION

Caution: Catalog# 75788 is isolated from human sources. Handle all products prepared from human sources as if they were capable of transmitting infectious agents. Avoid accidental inoculation, intravenous injection or contact with open wounds. Wash thoroughly after handling. Observe universal precautions when working with this product. Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, gloves and NIOSH-approved respirator. A qualified industrial hygienist should evaluate the need for respiratory protection. Use respiratory protection approved by NIOSH (or equivalent) and appropriate to the hazard. Avoid contact of material with skin or eyes. Mechanical ventilation or local exhaust as needed to control exposure to dust, vapors or mists. Access to a safety shower and eye-wash.

PHYSICAL AND CHEMICAL PROPERTIES

Appearance: Kit containing vials of solutions
Boiling Point: No data available
Vapor Pressure: No data available
Vapor Density: No data available
Solubility (Water): All components are soluble
Specific Gravity: No data available
Percent Volatile: No data available
Evaporation Rate: No data available
Chemical Formula: Not applicable

STABILITY AND REACTIVITY

Product is stable under normal conditions. Avoid prolonged excessive heat which may cause decomposition. Store away from strong bases, strong acids, and strong oxidizing agents. Hazardous decomposition products may include carbon oxides. Hazardous polymerization will not occur. For Glycerol: Avoid strong oxidizing agents including mixtures with hydrogen peroxide, potassium permanganate, trifluorobromide, calcium hypochlorite, nitric acid, sulfuric acid, perchloric acid and lead oxide. Contact with Sodium Hypochlorite and Hypochlorous acid may cause an explosion.

TOXICOLOGICAL INFORMATION**EFFECTS OF OVEREXPOSURE:**

YES: Contact may cause irritation.

SKIN: Contact may cause redness, swelling and pain at any site, especially mucous membranes.

INHALATION: Excessive inhalation of vapor may cause irritation, cough and shortness of breath.

INGESTION: Ingestion or excessive exposure may lead to nausea, vomiting and diarrhea. Large amounts may cause weakness, collapse and coma.

TARGET ORGANS: Eyes and Skin.

ADDITIONAL INFORMATION:

Tris-HCl - RTECS: No data available.

Potassium Chloride: Irritation, mutation and toxicity data listed in RTECS under TS8050000.

Irritation data: Eye Rabbit 500 mg/24H = Mild (1972).

Toxicity data: Oral Rat LD50 = 2600 mg/kg (1972).

Laboratory experiments have resulted in mutagenic effects.

Glycerol: Irritation, mutation, reproductive effects and toxicity data listed in RTECS under MA8050000.

Irritation data: Eye Rabbit 500 mg/24H = Mild (1986). Skin Rabbit 500 mg/24H = Mild (1986).

Toxicity data: Oral Rat LD50 = 12600 mg/kg (1945). Inhalation Rat LC50 = >570 mg/m³/1H (1970).

CAUTION: Catalog# 75788 is isolated from human sources. Handle all products prepared from human sources as if they were capable of transmitting infectious agents. Avoid accidental inoculation, intravenous injection or contact with open wounds. Wear appropriate personal protective equipment. Wash thoroughly after handling. Observe universal precautions when working with this product.

Definition(s): RTECS = Registry of Toxic Effects of Chemical Substances.

ACGIH = American Conference of Governmental Industrial Hygienists.

OSHA = Occupational Safety and Health Administration.

No information available.

Dispose of material in accordance with applicable local, state, and federal regulations.

US DOT / IATA: No applicable information.

RCRA - No applicable information.

SARA 302 - No applicable information.

SARA 313 - No applicable information.

EPA TSCA Section 8(b) - For Glycerol, Tris-HCl, and Potassium Chloride: Chemical Inventory.

Exposure Limits - For Glycerol: ACGIH TLV-TWA 10 mg/m³ (total particulate).

OSHA PEL TWA: 15 mg/m³ (total dust).

California Proposition 65 - No applicable information.

This data sheet is based upon information believed to be reliable. The Company makes no statement or warranty as to the accuracy or completeness of the information contained herein which is offered for your consideration, investigation and verification. Any use of the information contained in this data sheet must be determined by the user to be in accordance with appropriate applicable regulations.