AffinityScript QPCR cDNA Synthesis Kit

Instruction Manual

Catalog #600559

Revision E.0

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AffinityScript QPCR cDNA Synthesis Kit

Contents

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MATERIALS PROVIDED

Materials provided	Concentration	Quantity[a]
cDNA Synthesis Master Mix	2×	500 µl
AffinityScript RT/ RNase Block Enzyme Mixture	—	50 µl
Oligo(dT) primer	100 ng/µl	15 µg
Random primers	100 ng/µl	15 µg
RNase-free H_2O	—	1.2 ml

[a] The AffinityScript QPCR cDNA synthesis kit provides enough reagents for fifty 20-µl reactions total (control and experimental reactions combined).

STORAGE CONDITIONS

All Reagents: –20°C

Revision E.0

INTRODUCTION

The AffinityScript QPCR cDNA Synthesis Kit is designed for the highest efficiency conversion of RNA to cDNA and is fully optimized for two-step quantitative reverse transcription-PCR (QRT-PCR) applications. A two-step RT-PCR format is useful for amplifying multiple targets from a single cDNA source, for maintaining archival cDNA, and for providing maximum flexibility in selecting a downstream QPCR reagent system. The AffinityScript QPCR cDNA synthesis kit provides a streamlined master mix format and fast protocol, with a 15-minute cDNA synthesis step and an overall protocol time of just 25 minutes. The kit is formatted for high-yield production of cDNA up to 12 kb and each reaction accommodates a range of RNA amounts from fg to µg.

Advantages of the AffinityScript QPCR cDNA Synthesis Kit

- Robust cDNA synthesis across a broad range of temperatures from 37–55°C
- High affinity for primer-template complexes promotes optimal cDNA yield
- Produces excellent yields of amplifiable cDNA
- Can synthesize long cDNA targets, up to 12 kb, from small amounts of input RNA
- Able to reverse transcribe through RNA secondary structures
- Strict enzyme purity specifications ensure the absence of contaminating RNase and exonuclease activities

Kit Components

The AffinityScript QPCR cDNA synthesis kit provides a comprehensive set of reagents necessary to generate high-quality, QPCR-ready cDNA templates from either poly(A)$^+$ mRNA or total RNA. Key to the performance of the kit is the QPCR-grade AffinityScript Reverse Transcriptase (RT) enzyme and the corresponding QPCR-optimized RT buffer. The kit is formatted as a convenient master mix with few pipetting steps, saving you time and ensuring experiment-to-experiment reproducibility.

cDNA Synthesis Master Mix

The cDNA synthesis master mix contains a buffer that is specifically optimized for QRT-PCR performance, allowing a fast protocol and reducing variability in Ct measurements between reactions. In addition to the optimized buffer, the master mix contains $MgCl_2$ and dNTPs.

cDNA Primers

The cDNA priming strategy can affect cDNA yield, sensitivity, and detection of certain targets, such as GC-rich targets or sequences located at the 5' or 3' end of a transcript.[1] For this reason, individually packaged random nonamers and oligo(dT) primers are provided separately from the master mix, allowing you to use the best priming strategy for your specific target.[2]

AffinityScript RT/RNase Block Enzyme Mixture

The kit includes our QPCR-grade AffinityScript Multiple Temperature Reverse Transcriptase, a genetically engineered version of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) which is highly thermostable, allowing you to reverse transcribe at your preferred reaction temperature. AffinityScript RT is provided, in combination with RNase block, in a separate tube so that *no-RT control* reactions may be included in the QRT-PCR experiments. The RNase block serves as a safeguard against contaminating RNases.

QPCR-grade AffinityScript RT is stringently quality-controlled to verify the absence of nuclease contaminants that adversely affect cDNA synthesis, particularly from small input RNAs. It has been tested in QRT-PCR experiments to ensure sensitive and reproducible performance over a broad range of RNA template amounts and over a variety of RNA targets that vary in size, abundance, and GC-content.

Options for QPCR Analysis

The AffinityScript QPCR cDNA synthesis kit is designed for maximum flexibility in downstream QPCR analysis options, and is qualified for use with SYBR® Green-based and probe-based detection. The kit is tested in 2-step QRT-PCR using Agilent QPCR master mix kits. See Table 1 for a list of QPCR master mix kits and other QPCR reagents available from Agilent.

TABLE 1 Recommended Kits for QPCR Analysis

Detection Method	Product Name	Agilent Part Number
SYBR® Green dye	Brilliant II SYBR® Green QPCR Master Mix, High ROX	600829
	Brilliant II SYBR® Green QPCR Master Mix, Low ROX	600830
Probe	Brilliant II QPCR Master Mix, High ROX	600805
	Brilliant II QPCR Master Mix, Low ROX	600806
	Brilliant Multiplex Kit	600553
Any	Passive Reference Dye (ROX)	600536
	QPCR Reference Total RNA, Human, 10 cell lines	750500
	QPCR Reference Total RNA, Mouse, 11 cell lines	750600

PREPROTOCOL CONSIDERATIONS

RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA. Total RNA can be rapidly isolated and purified from cells using Agilent Absolutely RNA isolation kits, which are available for nano-, micro- and miniprep scale RNA purifications (Catalog #400753, #400805, and #400800, respectively). Total RNA may also be isolated from formalin-fixed, paraffin-embedded (FFPE) tissues using the Absolutely RNA FFPE kit (Catalog #400809 and #400811).

Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although including this step may improve the yield of specific cDNA templates. The Absolutely mRNA purification kit (Catalog #400806) is recommended for this application.

For QRT-PCR experiments with mammalian cells, we offer the SideStep Lysis and Stabilization Buffer (Catalog #400900), which allows you to skip RNA isolation steps and analyze gene expression in cell lysates directly. Since the SideStep lysates also contain genomic DNA, it is critical to design PCR primers to span adjacent exons in order to selectively amplify cDNA and prevent amplification of the intron-containing genomic DNA.

Preventing RNase Contamination

Take precautions to minimize the potential for contamination by ribonucleases (RNases). RNA isolation should be performed under RNase-free conditions. Wear gloves and use sterile tubes, pipet tips, and RNase-free water. The RNase block that is included in the cDNA synthesis reaction mixture provides additional protection against RNase contamination.

Preventing Genomic DNA Contamination

Contaminating DNA can be removed from the RNA preparation using an RNase-free DNase. All of the Agilent Absolutely RNA kits include RNase-free DNase which is used in a rapid on-column DNase treatment protocol. Additionally, PCR primers may be designed to span adjacent exons in order to prevent amplification of the intron-containing genomic DNA.

Using a Reference RNA for QRT-PCR Experiments

In order to reliably compare data across multiple experiments and instruments, it is essential to have a constant reference material to assess the performance of each QPCR run and to quantify gene expression levels. Agilent QPCR Reference Total RNA is a high-quality control for quantitative PCR gene-expression analysis. Including a standard curve with the Agilent QPCR Reference Total RNA in every experiment allows you to assess QPCR assay efficiency and precision, and to quantitate relative to an unchanging reference standard. In addition, the broad gene coverage allows you to use the reference material for nearly any human or mouse gene being investigated, thus eliminating the extra work required in generating new standards for each new gene target.

We offer QPCR Reference Total RNAs for both the human system (Catalog #750500) and the mouse system (Catalog #750600). The QPCR Human Reference Total RNA is composed of total RNA from 10 human cell lines with quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance. The QPCR Mouse Reference Total RNA is derived from RNA pooled from 11 mouse cell lines. These reference RNAs are carefully screened for contaminating genomic DNA, the presence of which can complicate interpretation of QRT-PCR assay data.

cDNA Synthesis Reaction

Primer Selection

The optimum primer type [oligo(dT) or random primer] varies for different targets and should be determined empirically with each target. Aglient QPCR Reference Total RNA (available separately) can be used for this step. (See *Using a Reference RNA for QRT-PCR Experiments* for more details.) For most targets, the best results are achieved using either oligo(dT) or random primers. For some challenging targets (long or secondary structure-rich targets), however, using a mixture of oligo(dT) and random primers may increase cDNA yield. When testing the use of mixed primers, adding a mixture of 170 ng oligo(dT) primer and 30 ng random primers to each 20-µl reaction is a good starting point.

Performing No-RT Control Reactions

Perform no-RT control reactions for each RNA sample by omitting AffinityScript RT/ RNase block from the reaction. The no-RT control is expected to generate no signal in subsequent QPCR if there is no amplification of genomic DNA. See *Preventing Genomic DNA Contamination* in *RNA Isolation*.

THE REVERSE TRANSCRIPTASE-MEDIATED POLYMERASE CHAIN REACTION PROTOCOL

Synthesis of First-Strand cDNA Using Reverse Transcriptase

Notes *Before use, mix each component and spin in a microcentrifuge.*

It is prudent to include a No-RT Control *reaction for each RNA sample by omitting the AffinityScript RT/ RNase Block enzyme mixture. This control verifies that signal detected in the subsequent QPCR is not due to genomic DNA contamination.*

1. Prepare the first-strand cDNA synthesis reaction in a microcentrifuge tube by adding the following components *in order*:

   RNase-free H_2O to a total volume of 20 µl
   10.0 µl of first strand master mix (2×)
   3.0 µl of oligo(dT) primer OR random primers (0.1 µg/µl)
   1.0 µl of AffinityScript RT/ RNase Block enzyme mixture
   X µl of RNA (0.3 pg–3 µg total RNA)

   Note *The optimum primer type [oligo(dT) or random primer] varies for different targets and should be determined empirically. See* Primer Selection *in* PreProtocol Considerations *for more information.*

2. Incubate the reaction at 25°C for 5 minutes to allow primer annealing.

3. Incubate the reaction at 42°C for 15 minutes to allow cDNA synthesis.

   Notes *This protocol has been extensively tested and is ideal for most targets up to 12 kb. Increasing the incubation time to 45 minutes at 42°C or raising the incubation temperature from 42°C to 55°C may increase cDNA yield for longer or secondary structure-rich targets, respectively.*

   When using oligo(dT) to prime cDNA synthesis at an incubation temperature of 55°C, incubate the reactions at 42°C for 5 minutes before transferring to 55°C for first-strand synthesis.

4. Incubate the reactions at 95ºC for 5 minutes to terminate the cDNA synthesis reaction.

5. Place the completed first-strand cDNA synthesis reactions on ice for immediate use in QPCR. For long-term storage, place the reactions at –20°C.

Real-Time PCR Quantification of First-Strand cDNA

QPCR Reagent Systems

cDNA targets may be quantified using any suitable QPCR reagent system. The AffinityScript QPCR cDNA synthesis kit is specifically qualified with Agilent QPCR master mix kits. See Table 1 in the *Introduction* for a summary of the QPCR kits available to support different applications and detection methods.

Guidelines for cDNA Amounts for QPCR Analysis

The amount of the cDNA synthesis reaction that should be used for analysis depends on the target abundance, the QPCR reagent system used, and the amount of input RNA used. High amounts of reverse transcriptase or of input RNA originating from the cDNA synthesis reaction may inhibit the subsequent QPCR reaction.

For the Agilent Brilliant II QPCR master mixes or Brilliant II Fast QPCR master mixes, up to 10% of the first strand synthesis reaction, containing up to 100 ng input RNA, may be added to a 25-µl QPCR reaction. For example, if the cDNA synthesis reaction was performed with 1 µg input RNA, up to 10% of the reaction may be added to a 25-µl QPCR reaction. If the cDNA synthesis reaction was performed with 2 µg input RNA, up to 5% of the reaction may be added to a 25-µl QPCR reaction.

TROUBLESHOOTING

Observation	Suggestion
No or low yield of first-strand cDNA	Verify the integrity of the input RNA by denaturing agarose gel electrophoresis to ensure it is not degraded.
	Optimize the reaction using Agilent QPCR Reference Total RNA, which can then also be used as a calibrator for subsequent experiments.
	Prepare a new RNA sample. Use Agilent RNA isolation kits to isolate intact total RNA or mRNA.
	Isolate the RNA in the presence of a ribonuclease inhibitor, and ensure that all RT-PCR reagents and labware are free of RNases.
	Inhibitors of reverse transcription (SDS, EDTA, guanidinium chloride, formamide, Na_2PO_4, or spermidine) may be present in the RNA sample. Reduce the volume of the input RNA or remove RT inhibitors with an additional 70% (v/v) ethanol wash following ethanol precipitation.
	Increase the length of the 42°C cDNA synthesis reaction to 45 minutes for longer RNA targets.
	Increase the incubation temperature from 42°C to 55°C for secondary structure-rich targets.
	Increase the concentration of the template RNA.
	Try switching the cDNA primer composition [oligo(dT) vs. random primers]. For challenging targets, a mixture of the two primer types may also be tested.
	When using oligo(dT) to prime cDNA synthesis at an incubation temperature of 55°C, incubate the reactions at 42°C for 5 minutes before transferring to 55°C for first-strand synthesis.
No or low yield of amplification product in QPCR	See the discussion under *No or low yield of the first-strand cDNA* for suggestions related to insufficient first strand synthesis.
	Add more cDNA synthesis product to the PCR. (Typically up to 10% of the cDNA synthesis reaction may be added to a 25-µl QPCR reaction. See *Guidelines for cDNA Amounts for QPCR Analysis* for additional considerations.)
	Optimize the QPCR primer concentration, annealing temperature, and/or extension time, varying each individually and in increments. Agilent QPCR Reference Total RNAs may be used for this step to conserve experimental samples.
	Re-examine the QPCR primer design. Make sure primers are not self-complementary or complementary to each other. Verify that the primers are designed to be complementary to the appropriate strands. Try using longer primers.
	Increase the number of thermal cycles.
	To maintain adequate quality of dNTPs used in the PCR step, keep nucleotides frozen in aliquots, thaw them quickly, and keep them on ice once thawed; avoid multiple freeze–thaw cycles.
Size of the amplification product is greater than expected	The RNA preparation may be contaminated with genomic DNA. Test for the presence of contaminating DNA by performing RT-PCR in the absence of AffinityScript RT. If DNA contamination is confirmed, treat the RNA preparation with RNase-free DNase I. Alternatively, redesign the PCR primers to anneal to sequences in the exon–exon boundary of the target gene.

REFERENCES

1. Bustin, S. A. and Nolan, T. (2004) *J Biomol Tech* 15(3):155-66.
2. Stahlberg, A., Hakansson, J., Xian, X., Semb, H. and Kubista, M. (2004) *Clin Chem* 50(3):509-15. Epub 2004 Jan 15.

ENDNOTES

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.