# AffinityScript One-Step RT-PCR Kit

## Instruction Manual

**Catalog #600188**

Revision D0

**For Research Use Only. Not for use in diagnostic procedures.**

600188-12

## LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

## ORDERING INFORMATION

Please visit www.agilent.com.

## TECHNICAL SERVICES

### United States and Canada

Email: genomics@agilent.com
Telephone: 800 227 9770 (option 3,4,3)

### All Other Locations

Please visit www.agilent.com/en/contact-us/page.

# AffinityScript One-Step RT-PCR Kit

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# AffinityScript One-Step RT-PCR Kit

## MATERIALS PROVIDED

| Materials Provided | Quantity[a] |
|---|---|
| Herculase II RT-PCR 2× Master Mix | 2.5 ml |
| AffinityScript RT/RNase Block | 100 µl |

[a] Kit provides enough reagents for 100 RT-PCR reactions.

## STORAGE CONDITIONS

**Herculase II RT-PCR 2× Master Mix:** Store at –20°C upon receipt. After thawing, the 2× master may be stored at 4°C for up to one month or returned to –20°C for long term storage.
**AffinityScript RT/RNase Block:** Store at –20°C.

## ADDITIONAL MATERIALS REQUIRED

RNase-free water
Temperature cycler
Thin-walled PCR tubes or PCR plates[II]
RT-PCR primers

[II] Agilent recommends the use of Agilent tube strips and caps (Catalog #410082 and #410086) or Agilent PCR plates (Catalog #401333 for 96-well plates and Catalog #410188 for 384-well plates). If using plates, seal the reactions with adhesive film (Catalog #410186) and a compression mat (Catalog #410187).

Revision D0

# INTRODUCTION

The AffinityScript One-Step RT-PCR Kit provides a complete system for fast, high-yield, reliable single-tube RT-PCR. The master mix format, coupled with robust performance and rapid cycling conditions, makes this system ideal for gene profiling and other high-throughput end-point RT-PCR applications.

Using this kit, cDNA is synthesized from total or poly(A)$^+$ RNA by AffinityScript reverse transcriptase (RT), a genetically engineered, highly thermostable version of MMLV RT. AffinityScript multiple temperature RT is active over a broad temperature range, from 42°C to 55°C, allowing greater flexibility in first-strand synthesis conditions. The AffinityScript RT provides excellent cDNA yields, full-length cDNAs, and superior RT performance for both routine and challenging RNA samples.

The cDNA is amplified in the same tube by Herculase II fusion DNA polymerase. In Herculase II fusion DNA polymerase, we have dramatically increased processivity of our *Pfu*-based DNA polymerase by fusing the enzyme with a high affinity double-stranded DNA binding domain. Enhanced processivity of the Herculase II fusion polymerase and the inclusion of our exclusive ArchaeMaxx PCR enhancing factor together provide superior yield and excellent reliability, with shorter PCR cycling times. The special enzyme formulation and optimized buffer system ensure robust performance when amplifying difficult and GC-rich targets. The accuracy of Herculase II fusion DNA polymerase is comparable to that of *Pfu* DNA polymerase.

## Advantages of the AffinityScript One-Step RT-PCR Kit

- Reduced PCR cycling times and one-tube, master mix format to reduce contamination, increase throughput and save valuable research time
- Optimal cDNA yield promoted by AffinityScript RT's high affinity for primer-template complexes
- Superior PCR yield and reliability promoted by the enhanced processivity of Herculase II fusion DNA polymerase and the ArchaeMaxx PCR enhancing factor
- Robust performance for difficult and GC-rich targets
- System optimized for target lengths up to 9.5 kb
- High fidelity of Herculase II fusion DNA polymerase results in low error rates for RT-PCR cloning applications

# PREPROTOCOL CONSIDERATIONS

## RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA and for optimal yields of long RT-PCR products. Total or poly(A)$^+$ RNA can be rapidly isolated and purified using Agilent's Absolutely RNA purification kits. Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although including this step may improve the yield for certain targets. RNA samples with an $OD_{260/280}$ ratio of 1.8–2.0 are optimal.

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 or DEPC-treated water.

When an RNA template is contaminated with genomic DNA, some PCR primer sets allow PCR amplification of trace amounts of genomic DNA rather than the cDNA template. Contaminating DNA can be removed from the RNA using an RNase-free DNase. A negative control reaction in which AffinityScript RT is omitted can be performed to verify that the PCR product is the result of amplification of the cDNA template. Alternatively, PCR primers can be designed to distinguish between amplification of target cDNA and possible contaminating genomic DNA (see *Primer Considerations*).

## Thermal-Cycling Program

Complementary DNA synthesis and PCR take place during an uninterrupted thermal-cycling program. cDNA synthesis at 45°C is immediately followed by an RT heat-inactivation step at 92°C and then 40 thermal cycles for PCR amplification.

### Inactivating Reverse Transcriptase

The RT enzyme must be inactivated prior to PCR to obtain high yields of amplification product. Heat treatment at 92°C for 1 minute prior to the first thermal cycle of PCR inactivates RT and also denatures the RNA–cDNA hybrid.

### PCR Reaction

The amplification cycle consists of a denaturation step (92°C), a template–primer annealing step (typically 55–65°C), and an extension step (72°C for targets <1 kb and 68°C for targets >1 kb). PCR primer sequence is a major consideration in determining the annealing temperature of the thermal-cycling program. The annealing temperature should be 5°C below the $T_m$ of the primers.

The optimal extension temperature for Herculase II fusion DNA polymerase in RT-PCR varies with the size of the target, with 68°C optimal for most targets, and 72°C optimal for targets <1 kb. The optimal extension time for Herculase II fusion DNA polymerase in RT-PCR is 45 seconds/kb target (for targets <1 kb, 30 seconds is sufficient). A final 3–5 minute final

extension step improves the quality of the PCR products by extending truncated amplicons to full length.

### Number of Thermal Cycles

Forty cycles of amplification is sufficient to detect most RNA targets. If the target RNA is rare or if only a small amount of starting material is available, it may be necessary to increase the number of cycles.

## Primer Considerations

The gene-specific downstream primer is used to prime cDNA synthesis. The purity and quality of the primers can impact the yield of RT-PCR, particularly for low-abundance targets.

Primer pairs that exhibit similar melting temperatures and are completely complementary to the template are recommended. Primers should not be self-complementary or complementary to each other at their 3′ ends. Primer melting temperatures between 55° and 65°C generally yield the best results. The following formula is commonly used for estimating the melting temperature ($T_m$) of the primers:

$$T_m(°C) \cong 2(N_A + N_T) + 4(N_G + N_C)$$

where $N$ equals the number of primer adenine (A), thymidine (T), guanidine (G), or cytosine (C) bases.

To differentiate between amplification of target cDNA and possible contaminating genomic DNA, the primers can be designed to anneal to sequences in exons on opposite sides of an intron. With these primers, an amplification product derived from genomic DNA will be longer than the product produced by RT-PCR, making it possible to differentiate the two products by gel electrophoresis. Alternatively, PCR primers can be designed to anneal to the exon–exon boundary of the mRNA. With these primers, amplification of genomic DNA will be highly inefficient.

## General Notes

### Preventing Cross-Contamination

Take precautions to minimize the potential for carryover of nucleic acids (RNA and DNA) from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

### Mixing and Pipetting Enzymes

Enzymes (including AffinityScript RT) should be mixed by gentle vortexing without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the 50% glycerol in the buffer can lead to pipetting errors.

# PROTOCOL

## RNA Input Considerations

The optimal quantity of RNA depends on the target size, message abundance, RNA purity and primer quality. See the table below for general guidelines.

RNA should be added to the reaction mixture in a volume ≤ 5 µl for RNA samples prepared in DEPC-treated water. High concentrations of residual DEPC can inhibit RT-PCR.

| RNA | Quantity |
|---|---|
| Total RNA, target <1 kb | 1–200 ng |
| Total RNA, target >1 kb | 200–500 ng |
| mRNA (all target sizes) | 0.1–10 ng |

## RT-PCR Reaction Mixture

***Note*** *The components of this kit are specially formulated to work together for efficient, high-yield endpoint RT-PCR. Do not substitute the provided kit components with other PCR master mixes or RT formulations.*

1. Prepare the reactions by adding the following components *in order* to sterile thin-wall PCR tubes.

| Component | Volume | |
|---|---|---|
| | **<1 kb target** | **>1 kb target** |
| RNase-free water | X µl (for 50-µl final reaction volume) | X µl (for 50-µl final reaction volume) |
| Herculase II RT-PCR 2× Master Mix | 25 µl | 25 µl |
| Upstream primer (100 ng/µl) | 1 µl | 1 µl |
| Downstream primer (100 ng/µl) | 1 µl | 1 µl |
| Sample RNA | X µl (≤ 5 µl) | X µl (≤ 5 µl) |
| AffinityScript RT/RNase Block | 0.5 µl | 1 µl |

2. Vortex the reaction gently without creating bubbles.

3. Place the reaction in a thermal cycler. Run the following program:

| Cycles | <1 kb target | | >1 kb target | |
|---|---|---|---|---|
| | **Temperature** | **Duration** | **Temperature** | **Duration** |
| 1 | 45°C[a] | 5 minutes | 45°C[a] | 30 minutes |
| 1 | 92°C | 1 minute | 92°C | 1 minute |
| 40 | 92°C | 20 seconds | 92°C | 20 seconds |
| | Primer Tm – 5°C[b] | 20 seconds | Primer Tm – 5°C[b] | 20 seconds |
| | 72°C | 30 seconds | 68°C | 45 seconds/kb |
| 1 | 72°C | 3 minutes | 68°C | 5 minutes |

[a] The first strand synthesis step can be performed between 42°C and 55°C.

[b] Use the annealing temperature appropriate for the specific primer pair used in the reaction. Typical annealing temperatures will range between 55°C and 65°C.

Store the reaction products at –20°C until needed. The RT-PCR products may be analyzed by agarose gel electrophoresis and may be purified using the StrataPrep PCR purification kit (Agilent Catalog #400771).

# TROUBLESHOOTING

| Observation | Suggestion |
|---|---|
| No or low product yield | Verify the integrity of the RNA by automated electrophoresis using the Agilent 2100 Bioanalyzer, TapeStation, or Fragment Analyzer and the respective RNA assay. Alternatively, RNA integrity may be assessed by denaturing agarose gel electrophoresis. |
| | Replace the RNA. Use Agilent Absolutely RNA isolation kits to isolate intact 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. |
| | For targets ≥1 kb, the optimal amount of AffinityScript RT/RNase Block is 1 µl per 50-µl RT-PCR reaction. When using low amounts of input RNA (1–10 pg total RNA), yield may be improved for some targets by decreasing the amount of AffinityScript RT/RNase Block to 0.5 µl per 50-µl reaction. |
| | Increase the concentration of the template RNA in the RT-PCR reaction. Optimizing the amount of input RNA is especially recommended for low abundance targets. |
| | Increase the number of thermal cycles. Optimizing cycle number is especially recommended for low abundance targets. |
| | It is not beneficial to adjust the $Mg^{++}$ concentration in the RT-PCR reaction. $Mg^{++}$ is present in the master mix at the optimal concentration for Herculase II fusion DNA polymerase, independent of the specific target and primer pair used. In contrast to *Taq* DNA polymerase, this *Pfu*-based DNA polymerase displays a relatively constant optimum $Mg^{++}$ concentration in amplifying DNA from a variety of template-primer combinations. |
| | The RNA preparation may contain a RT-PCR inhibitor (e.g. SDS, EDTA, guanidinium chloride, formamide, $Na_2PO_4$, or spermidine). Reduce the volume of the target RNA or remove potential inhibitors with an additional 70% (v/v) ethanol wash following ethanol precipitation. |
| | Make sure primers are not self-complementary and are not complementary to each other. |
| | Verify that the primers are designed to be complementary to the appropriate strands. |
| | Optimize the primer concentration, PCR annealing temperature, and PCR extension time, varying each individually and in increments. |
| | RNase Block is included with the AffinityScript RT enzyme supplied with this kit. Adding additional RNase Block to reactions may reduce amplicon yield for some targets. |
| | Verify that the times and temperatures are correct and that the programs for cDNA synthesis and PCR amplification are correctly linked. |
| Molecular weight of the amplification product is higher than expected | In some cases, the RNA preparation may be contaminated with genomic DNA. Verify the presence of contaminating DNA by performing RT-PCR in the absence of reverse transcriptase. The RNA may be treated with RNase-free DNase prior to RT-PCR to remove contaminating genomic DNA. |
| | Redesign the PCR primers to anneal to sequences in the exon–exon boundary of the target gene (see *PCR Primer Considerations*). |
| Multiple nonspecific amplification products | Increase the PCR annealing temperature. |
| | Make sure primers are not self-complementary and are not complementary to each other. |
| | Try a longer primer. |
| | Use positive displacement pipets or aerosol-resistant pipet tips to reduce cross-contamination during pipetting; use separate work areas and pipettors for pre- and post-amplification steps; wear gloves and change them often. |
| | It is possible that multiple target sequences exist in the RNA template. In this case, design new primers. |
| 2× Master Mix solution appears cloudy | The Herculase II RT-PCR 2× Master Mix solution may appear cloudy under certain conditions. It is not necessary to clarify the solution before use; the cloudy appearance does not affect the performance of the master mix. |

## MSDS INFORMATION

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

# AffinityScript One-Step RT-PCR Kit

Catalog #600188

## QUICK-REFERENCE PROTOCOL

- Combine the following components *in order*:

| Component | Volume | |
|---|---|---|
| | **<1 kb target** | **>1 kb target** |
| RNase-free water | X µl (for 50-µl final reaction volume) | X µl (for 50-µl final reaction volume) |
| Herculase II RT-PCR 2× Master Mix | 25 µl | 25 µl |
| Upstream primer (100 ng/µl) | 1 µl | 1 µl |
| Downstream primer (100 ng/µl) | 1 µl | 1 µl |
| Sample RNA | X µl (≤ 5 µl) | X µl (≤ 5 µl) |
| AffinityScript RT/RNase Block | 0.5 µl | 1 µl |

- Vortex gently without creating bubbles.

- Run the appropriate thermal-cycling program below:

| Cycles | <1 kb target | | >1 kb target | |
|---|---|---|---|---|
| | **Temperature** | **Duration** | **Temperature** | **Duration** |
| 1 | 45°C[a] | 5 minutes | 45°C[a] | 30 minutes |
| 1 | 92°C | 1 minute | 92°C | 1 minute |
| 40 | 92°C | 20 seconds | 92°C | 20 seconds |
| | Primer Tm – 5°C[a] | 20 seconds | Primer Tm – 5°C[a] | 20 seconds |
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[a] The first strand synthesis step can be performed between 42°C and 55°C.