



AffinityScript Multiple Temperature cDNA Synthesis Kit

Instruction Manual

Catalog #200436

Revision C0

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AFFINITYSCRIPT MULTIPLE TEMPERATURE cDNA SYNTHESIS KIT

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AffinityScript Multiple Temperature cDNA Synthesis Kit

MATERIALS PROVIDED

Materials Provided	Quantity ^a
AffinityScript Multiple Temperature Reverse Transcriptase	50 reactions
10× AffinityScript RT buffer	100 µl
RNase Block Ribonuclease Inhibitor (40 U/µl)	1000 U
Oligo(dT) primer (0.5 µg/µl)	25 µg
Random primers (0.1 µg/µl)	15 µg
100 mM dNTPs (25 mM each dNTP)	40 µl
RNase-free water	1.2 ml

^a The AffinityScript Multiple Temperature cDNA Synthesis Kit provides enough reagents for 50 reactions.

STORAGE CONDITIONS

All Reagents: –20°C

Revision C0

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INTRODUCTION

The AffinityScript Multiple Temperature cDNA Synthesis Kit is ideal for the production of high yields of cDNA from a wide range of total RNA or mRNA amounts. The kit includes our AffinityScript Multiple Temperature Reverse Transcriptase, a genetically engineered version of MMLV reverse transcriptase which is highly thermostable, allowing you to reverse transcribe at your preferred reaction temperature. This RT provides excellent cDNA yields, full-length cDNAs, and superior RT performance for your challenging RNA samples. Whether you are working with genes as small as 500 bases or as large as 20 kb, AffinityScript RT is the ideal choice to convert your RNA transcripts into full length cDNA.

Our kit provides a comprehensive set of reagents necessary to generate high-quality cDNA templates from either poly(A)⁺ mRNA or total RNA. Both random ninemers and oligo(dT) primers are provided, allowing the flexibility for you to use your preferred priming strategy. We also include high quality, PCR amplification-grade first strand reagents to ensure your RT-PCR success. This kit uses a streamlined protocol, which makes it easy and fast to set up multiple cDNA synthesis reactions, saving you valuable research time.

Additionally, using this kit for cDNA synthesis provides you the flexibility to select the PCR enzyme or master mix that is best suited to your downstream application. We include protocols for using our PicoMaxx DNA polymerase, for greatest sensitivity, and for our *PfuUltra* II Fusion HS DNA Polymerase for fast, high-fidelity two-step RT-PCR. In addition, a protocol is provided for using this kit in conjunction with our Herculanase II Fusion DNA polymerase, for high-yield one-step RT-PCR.

Advantages of the AffinityScript Multiple Temperature 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 20 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

FIRST-STRAND cDNA SYNTHESIS PROTOCOL

Notes *Wear gloves at all times during the first-strand cDNA synthesis and PCR amplification procedures and while handling materials and equipment to prevent contamination by ribonucleases (RNases).*

This procedure is optimized to convert 1 ng to 5 µg of total RNA or up to 250 ng of poly(A)⁺ RNA into first strand cDNA.

This protocol is optimized for the first step of a two-step RT-PCR procedure. Protocols for PCR amplification of the cDNA are provided in the following section. If performing one-step RT-PCR, see the One-Step RT-PCR Amplification Protocol section.

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

Note *Mix and spin each component in a microcentrifuge before use.*

1 ng–5 µg of total RNA or 1 ng–250 ng of poly(A)⁺ mRNA
RNase-free water to total volume 15.7 µl
1.0 µl of oligo(dT) primer (0.5 µg/µl) OR
3 µl of random primers (0.1 µg/µl) OR
1 µl of a gene-specific primer (100 ng/µl)

2. Incubate the reaction at 65°C for 5 minutes.
3. Cool the reaction at room temperature (~10 minutes) to allow the primers to anneal to the RNA.
4. Add the following components to each reaction, in order, for a final reaction volume of 20 µl:

2.0 µl of 10× AffinityScript RT Buffer
0.8 µl of dNTP mix (25 mM each dNTP)
0.5 µl of RNase Block Ribonuclease Inhibitor (40 U/µl)
1 µl of AffinityScript Multiple Temperature RT

Note *To prevent heat inactivation, AffinityScript RT and RNase Block must be added after the 65°C incubation is completed and the reaction has cooled to room temperature.*

5. If using random primers, incubate the reaction at 25°C for 10 minutes to extend the primers prior to increasing the reaction temperature to 42–55°C for cDNA synthesis in step 6.

6. Mix the reaction components gently, and then place the tube in a temperature-controlled thermal block at 42–55°C. Incubate the reaction for 60 minutes.

Notes *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.*

AffinityScript multiple temperature reverse transcriptase performs optimally over the full range of 42–55°C. Typically, 42°C is a good starting point. For RNAs containing secondary structure and other challenging targets, a synthesis temperature of 55°C may be used without loss of performance.

7. Terminate the reaction by incubating at 70°C for 15 minutes.
8. Place the completed first-strand cDNA synthesis reactions on ice for subsequent PCR amplification (see *PCR Amplification of First-Strand cDNA*).

PCR AMPLIFICATION OF FIRST-STRAND cDNA

Notes *Reaction products should be stored at -20°C until needed.*

Mix and spin each component in a microcentrifuge before use.

Protocols are provided below for amplification using PicoMaxx DNA Polymerase (recommended for greatest sensitivity; Agilent Catalog # 600420), and for using PfuUltra II Fusion HS DNA polymerase (recommended for greatest fidelity; Catalog #600670). The protocols may be adapted for amplification using other PCR enzymes, using reaction conditions and cycling parameters recommended by the PCR enzyme manufacturer.

High-Sensitivity Amplification using PicoMaxx DNA Polymerase

1. Add the following components *in order* to a sterile thin-walled PCR tubes^{||} or a 0.5-ml microcentrifuge tube for each amplification reaction:

39.6 μl of RNase-free water (for final volume of 50 μl)

5 μl of 10 \times PicoMaxx PCR buffer

0.4 μl of dNTP mix (100 mM)

1 μl of upstream primer (0.1 $\mu\text{g}/\mu\text{l}$)

1 μl of downstream primer (0.1 $\mu\text{g}/\mu\text{l}$)

2 μl of first-strand cDNA reaction

1 μl of PicoMaxx DNA polymerase (2.5 U/ μl)

2. If the thermal cycler does not have a hot top assembly, overlay each reaction with one or two drops (20–40 μl) of nuclease-free mineral oil to prevent evaporation and condensation during thermal cycling.

^{||} Thin-walled tubes are highly recommended for use with thermal cyclers. These tubes ensure ideal contact with the multiblock design, permit efficient heat transfer, and maximize thermal-cycling performance.

3. Place the amplification reactions in a thermal cycler, and run the thermal-cycling program below.

Cycling Program for PicoMaxx DNA Polymerase

Thermal cycler	Cycles	Temperature	Duration
Single or multiple block	1	95°C	2 minute
	40	95°C	30 seconds
		$T_m - 5^\circ\text{C}^a$	30 seconds
		72°C	1 minute/kb ^b
	1	72°C	10 minutes

^a Use the annealing temperature appropriate for your gene-specific primer pair.

^b For targets <1 kb, use a 1 minute extension time.

Analyzing the PCR Products

Load 10 µl of each PCR amplification reaction taken from below the mineral oil layer into separate lanes of a 0.8% (w/v) agarose gel.

High Fidelity Amplification using *PfuUltra* II Fusion HS DNA Polymerase

1. Add the following components *in order* to a sterile thin-walled PCR tubes^{||} or a 0.5-ml microcentrifuge tube for each amplification reaction:

39.5 µl of RNase-free water (for final volume of 50 µl)

5 µl of 10× *PfuUltra* II Reaction Buffer

0.5 µl of dNTP mix (100 mM)

1 µl of upstream primer (10 µM)

1 µl of downstream primer (10 µM)

2 µl of first-strand cDNA reaction

1 µl of *PfuUltra* II Fusion HS DNA polymerase

2. If the thermal cycler does not have a hot top assembly, overlay each reaction with one or two drops (20–40 µl) of nuclease-free mineral oil to prevent evaporation and condensation during thermal cycling.
3. Place the amplification reactions in a thermal cycler, and run the thermal-cycling program below.

PCR Cycling Program for *PfuUltra* II DNA Polymerase

Thermal cycler	Cycles	Temperature	Duration
Single or multiple block	1	95°C	1 minute
	40	95°C	20 seconds
		$T_m - 5^\circ\text{C}^a$	20 seconds
		72°C	30 seconds/kb ^b
	1	72°C	3 minutes

^a Use the annealing temperature appropriate for your gene-specific primer pair.

^b For targets < 1 kb, use a 30-second extension time.

Analyzing the PCR Products

Load 10 µl of each PCR amplification reaction, taken from below the mineral oil layer, into separate lanes of a 0.8% (w/v) agarose gel.

- ^{||} Thin-walled tubes are highly recommended for use with thermal cyclers. These tubes ensure ideal contact with the multiblock design, permit efficient heat transfer, and maximize thermal-cycling performance.

ONE-STEP RT-PCR AMPLIFICATION PROTOCOL

Note *A protocol is provided below for high-yield, one-step RT-PCR using reagents from this kit in conjunction with Herculase II Fusion DNA polymerase (Agilent Catalog #600675).*

1. Prepare a 1:20 dilution of AffinityScript Multiple Temperature RT by combining the following components:

17 μ l of nuclease-free water
2 μ l of 10 \times AffinityScript RT Buffer
1 μ l of AffinityScript Multiple Temperature RT

2. Add the following components *in order* to sterile thin-walled PCR tubes^{||} or 0.5-ml microcentrifuge tubes for each RT-PCR reaction:

35.6 μ l of RNase-free water (for final volume of 50 μ l)
10 μ l of 5 \times Herculase II Reaction Buffer
0.4 μ l of dNTP mix (100 mM)
0.5 μ l of forward primer (200 ng/ μ l)
0.5 μ l of reverse primer (200 ng/ μ l)
1 μ l of **diluted** (1:20) AffinityScript Multiple Temperature RT
1 μ l of RNA template (5–500 ng total RNA or 2–20 ng mRNA)
1 μ l of Herculase II Fusion DNA polymerase (2.5 U/ μ l)

Note *Be sure to add the 5 \times Herculase II Reaction Buffer to the one-step RT-PCR reactions. The 10 \times AffinityScript RT Buffer is **not** added to this reaction mixture.*

3. If the thermal cycler does not have a hot top assembly, overlay each reaction with one or two drops (20–40 μ l) of nuclease-free mineral oil to prevent evaporation and condensation during thermal cycling.

^{||} Thin-walled tubes are highly recommended for use with thermal cyclers. These tubes ensure ideal contact with the multiblock design, permit efficient heat transfer, and maximize thermal-cycling performance.

4. Place reactions in a thermal cycler, and run the program outlined below.

RT-PCR Cycling Program

Cycles	Temperature	Duration
1	42°C ^a	30 minutes
1	95°C	1 minute
40	95°C	30 seconds
	T _m -5°C ^b	30 seconds
	68°C	45 seconds/kb
1	68°C	7 minutes

^a AffinityScript multiple temperature reverse transcriptase performs optimally over the full range of 42–55°C. Typically, 42°C is a good starting point. For RNAs containing secondary structure and other challenging targets, a synthesis temperature of up to 55°C may be used without loss of performance.

^b Use the annealing temperature appropriate for your gene-specific primer pair.

5. Load 10 µl of each RT-PCR amplification reaction, taken from below the mineral oil layer, into separate lanes of a 0.8% (w/v) agarose gel.

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 RNAs, which can then 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 ₂ PO 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 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 PCR	See the discussion under <i>No or low yield of the first-strand cDNA</i> 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 the PCR reaction.)
	Optimize the PCR primer concentration, annealing temperature, and/or extension time, varying each individually and in increments. Agilent QPCR Reference Total RNA may be used for this step to conserve experimental samples.
	Re-examine the PCR 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.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at <http://www.chem.agilent.com/en-US/search/library/Pages/MSDSSearch.aspx>. MSDS documents are not included with product shipments.