



RetroTools® Two Step KIT *with DNA-Amplification Buffer MgCl₂ FREE*

including a recombinant *Tth* DNA Polymerase thermostable

STORE AT - 20°C

The RetroTools® Two Step Kit allows the amplification of a specific RNA sequence using a single enzyme, *Tth* DNA Polymerase, in a two step process in one tube. The *Tth* DNA Polymerase catalyses in the presence of Mn²⁺ and RNA template the polymerization of nucleotides into cDNA, whereas in the presence of Mg²⁺ the enzyme catalyses the polymerisation of dNTPs into duplex DNA. The thermostability of the *Tth* DNA Polymerase confers higher specificity to the kit because the transcription reaction can be performed at elevated temperatures. Furthermore, secondary ARN structures can be resolved employing higher temperatures.

RT-PCR PROTOCOL USING SPECIFIC PRIMERS

1st-STEP: Reverse transcription (RT).

Add the following reagents to a sterile tube:

5X RT Buffer	2 µl
5 mM MnCl ₂ Solution	2 µl
dNTP Mix, 10 mM each	0,25 µl
RNA Template	10 pg to 1 µg
<i>Downstream primer</i>	20 pmoles
RetroTools® Polymerase	1,25 µl
Bidistilled sterile water	To a final volume of 10 µl

Incubate the reaction mixture at 60°C-70°C for 15 to 60 minutes. The incubation time will depend on the length of the desired fragments and must be optimised accordingly. The preannealing (see Note 1) and annealing temperatures used for the reverse transcription reaction should also be optimised for each *downstream primer*.

NOTE 1: In case the downstream primer has a T_m lower than 50°C, it is recommended including a preannealing step of 3 minutes at 45°C-50°C in order to increase the efficiency of the transcription reaction, and consequently the amplification yield.

NOTE 2: Once the RT has finished, keep the vials on ice, and immediately proceed to the next step, or keep them at -20°C. If the vials are frozen add 0.3 µl fresh RetroTools® polymerase to the amplification reaction, as freezing inactivates the enzyme in the mixture.

NOTE 3: For detection of low-copy messengers, a cyclic reverse transcription protocol is highly recommended: 94°C for 1 min (initial denaturing step) + 4 cycles (48°C for 5 min, 60°C for 15 min).

2nd-STEP: Amplification Reaction.

Once the reverse transcription has been performed, the reaction volume for each tube should be made up 50 µl by adding the following reagents:

5X DNA-Amplification Buffer MgCl ₂ FREE	8 µl
“Upstream” primer	20 pmoles
50 mM MgCl ₂ Solution	Minimum 2 µl (2 mM final concentration)
Bidistilled sterile water	to 40 µl

Recommended conditions for the amplification:

94°C	for 2 minutes	Initial denaturing
94°C	for 15 seconds	
55-65°C	for 30 seconds	
72°C	1 minute per Kb	x 30 to 45 cycles
72°C	for 3 minutes	
4°C		Final extension

The annealing temperature depends on the T_m of the primer pair used. The products obtained can be analysed by electrophoresis in agarose or polyacrylamide gels.

RT-PCR PROTOCOL USING NON-SPECIFIC PRIMERS FOR THE RT

1st-STEP: Reverse transcription (RT).

Add the following reagents to a sterile tube:

5X RT Buffer	6 µl
5 mM MnCl ₂ Solution	6 µl
dNTP Mix, 10 mM each	0,25-0,5 µl
<i>Non-specific primers</i>	5-10 pmoles
RNA Template	10 pg to 1 µg
RetroTools® polymerase	1,5 µl
Bidistilled sterile water	To a final volume of 30 µl

Incubate the reaction mix at 42°C for 5 minutes. Increase the temperature slowly to 60-65°C (the use of the **Ramp** option of thermal cyclers is strongly recommended) and incubate the reaction mixture for 30-60 minutes. The incubation time will depend on the length of the desired fragments, and must thus be accordingly optimised.

Following the reverse transcription reaction, proceed immediately to the amplification step. As a general rule, use 10 µl of the former reaction as template, and store the remaining 20 µl at -20°C. When detection of rare messengers is our aim, the template volume for the amplification reaction can be increased up to 30 µl.

NOTE: For using the stored template, it is necessary to add 0.3 µl of fresh RetroTools® polymerase, as freezing inactivates the enzyme.

2nd-STEP: Amplification Reaction.

Once reverse transcription has been performed, add the following reagents to 10 µl of the RT reaction (see above), bringing the final volume up to 50 µl.

5X DNA-Amplification Buffer MgCl ₂ FREE	8 µl
50 mM MgCl ₂ Solution	Minimum 2 µl (2 mM final concentration)
<i>Downstream primer</i>	5-10 pmoles
<i>Upstream primer</i>	5-10 pmoles
Bidistilled sterile water	to 40 µl

Recommended reaction conditions for the amplification:

94°C	2 minutes		Initial denaturing	
94°C	15 seconds			x 30 to 45 cycles
55-65°C	30 seconds			
72°C	1 minute per Kb			
72°C	3 minutes		Final extension	
4°C				

The annealing temperature depends on the T_m of the pair of specific primers used. The products obtained can be analysed by electrophoresis in agarose or polyacrylamide gels.

Ref.	Product
10.059	RetroTools® Two Step KIT with DNA-Amplification Buffer MgCl ₂ FREE (100 rxns)

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