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Protocol

Part A: Reverse transcription polymerase chain reaction (RT-PCR)

The use of *Tth*-DNA polymerase, which is also a reverse transcriptase in the presence of manganese and accepts both RNA and DNA as template, enables the whole reaction to be performed as a "one-step/one-tube" RT-PCR analysis (6). This experimental approach includes the amplification of fragments only to a maximum of 1 kb and a relatively high error rate for DNA polymerase as a result of the manganese ion concentration (7).

This one-step procedure minimizes the risk of cross-contamination of the sample.

Reverse transcription can be performed at 60° C minimizing problems from RNA secondary structure and high G/C content!

1. One step RT - PCR (One step reaction eliminates the risk of cross contaminations associated with two step RT-PCR.)

"One-step" RT-PCR means reverse transcription and amplification is mediated by one enzyme in one tube.

The "one enzyme/one step" *Tth* DNA polymerase assay uses bicine buffers containing Mn^{2+} ions that are compatible with both RT and subsequent PCR (6, 11). **NOTE:** For RT-PCR amplification (reverse transcription and amplification in one step), it might be necessary to determine **concentration of Mn^{2+} first** by testing Mn^{2+} concentrations from 1- 4mM for each reaction.

Setting up "one-step" RT-PCR Reaction

Prepare two mastermixes 25µL each before starting.

Table 1: Mix I for "one-step" RT-PCR

Component	Volume	Final concentration
dNTP-Mix (10mM each)	1.5µL	300µM
Forward primer	Var.	450µM
Reverse primer	Var.	450µM
Template RNA	Var.	Up to 1µg (in steps of 1ng; 10ng; 100ng; 1µg)
Sterile water	Up to 25µL	
Total	**25µL**	

Table 2: Mix II for "one-step" RT-PCR

Component	Volume	Final concentration
5X RT-PCR buffer	10µL	1-time
Mn(OAc)2	5µL	2.5mM
Tth DNA Polymerase	0.5-1.0µL	2.5 - 5 units
Sterile water	Var.	
Total	**25µL**	

NOTE: Combine Mix I and Mix II on ice and gently vortex the final mixture in a PCR-tube. Place tube in Thermal cycler and run below mentioned (page 4) cycling programme immediately.

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Table 1: Possible 1-tube RT-PCR Cycle Protocol

Step	Time	Temperature	Comments
RT-Reaction			
Initial Denaturation	1-3 minutes	95°C	
RT-Reaction*	30 minutes	60° - 70°C *	
PCR-Reaction			
Initial Denaturation	0.5 - 1 min.	95°C	
Cycling (1-10 step)			
Denaturation	30-60 sec.	94°C	
Annealing	30-60 sec.	50-70°C	Approximately 5°C to 8°C below Tm of primers
Elongation	45-90 sec.	72-74°C	
**Cycling (11->30 step) ** **			
Denaturation	30 sec.	94°C	
Annealing	30 sec.	50-70°C	Approximately 5°C to 8°C below Tm of primers
Elongation	45 sec.	72-74°C	
Final elongation	7 minutes	72-74°C	

*depends on your primers, 70 - 75° C is the optimal reaction temperature for *Tth* DNA polymerase. *Tth* DNA polymerase is resistant to prolonged incubations (20 min Half - Life time at 95° C) at high temperatures (94° C) and can therefore be used for PCR.

** Number of cycles ranges from 30 – 50 in the literature. If the template is limited, increased cycle numbers may result in nonspecific product yield.

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Part B: Two-step RT-PCR (optional – material not supplied)

In the two-step protocol the reversed transcription abilities of the *Tth* polymerase are used to perform a “standard” reverse transcription reaction for synthesis of cDNA. For this step the reverse primer is used together with the special RT-buffer system plus Mn^{2+}. The RT reaction is followed by a PCR reaction by adding PCR buffer, MgCl2 and forward primer.

As the error rate of *Tth* is increased at higher Mn^{2+} concentration (5), a two-step reaction is recommended if PCR products shall be used for subsequent cloning and/or sequencing experiments.

2. Two step RT - PCR:

Table 3: “two-step” RT-PCR reaction mixture – Components for RT-reaction

Component	Volume	Final concentration
10X Rev. Transcription buffer	2µL	1-time
Mn(OAc)2	2µL	0.9mM
dNTP-Mix (10mM each)	0.4µL	200µM
Reverse primer	Var.	450µM
Template RNA	Var.	up to 200ng
Tth-Polymerase	0.8µL	4 units
Sterile water	up to 20µL	
Total	**20µL**	For RT-reaction denature at 95°C for 1-3 minutes, then incubate at 60-70°C for 10-30 min.

2. Two step RT - PCR:

Table 4: “two-step” RT-PCR reaction mixture – PCR-reaction

Component	Volume	Final concentration
10X PCR reaction buffer	8µL	1-time
dNTP-Mix (10mM each)	0.4µL	200µM
Reverse primer	Var.	450µM
Forward primer	Var.	150nM
EGTA (7.5mM)*	10µL	0.75nM
Tth-Polymerase	0.8µL	4 units
Sterile water	up to 80µL	
Total	**80µL**	Gently vortex and add the 80µL PCR mastermix to the 20µL rev. transcriptase mixture after reverse transcriptase reaction. **Total PCR volume: 100µL** **Continue cycling immediately!!!**

*** For a 0.5M EGTA stock: dissolve 19.2g EGTA in 70mL deionized water, adjust pH8.0 with NaOH (10M). Add deionized water to 100mL final volume. Filter sterile with 0.22µm and store at room temperature

Table 5: Two-step PCR Protocol

Step	Time	Temperature	Comments
Initial Denaturation	1-2 minutes	95°C	
Cycling (1-10 step)			
Denaturation	30-60 sec.	95°C	
Annealing	30-60 sec.	50-70°C	Approximately 5°C to 8°C below Tm of primers
Elongation	45-90 sec.	72-74°C	
Cycling (11->30 step)**			
Denaturation	30 sec.	94°C	
Annealing	30 sec.	50-70°C	Approximately 5°C to 8°C below Tm of primers
Elongation	45 sec.	72-74°C	
Final elongation	7 minutes	72-74°C	

** Number of cycles ranges from 30 – 50 in the literature. If the template is limited, increased cycle numbers may result in nonspecific product yield.

Part C: “Standard PCR

Tth-polymerase can be used like *Taq*-polymerase for a standard PCR. As *Tth* is more unspecific for nucleotides than the other DNA-polymerases, Tth can be ideally used in the case of incorporation of modified nucleotides (digoxigenin, biotin or fluorescein). In all cases MgCl2-concentration as well as the enzyme concentration in the PCR reaction has to be optimized. Typical MgCl2 concentrations are in the range of 1 – 6mM. The supplied standard concentration is 1.5mM MgCl2. Optimal enzyme concentrations are in the range of 1.0 – 5.0 units. The standard concentration is 2.5 units per reaction.

Setting up PCR Reaction mixtures

Prepare two mastermixes 50µL each before starting.

Table 6: Mix I for standard PCR

Component	Volume	Final concentration
dNTP-Mix (10mM each)	0.4µL	200µM
Forward primer	Var.	400µM
Reverse primer	Var.	400µM
Template DNA	Var.	up to 0.5µg
Sterile water	up to 50µL	
Total	**50µL**	

Table 7: Mix I for standard PCR

Component	Volume	Final concentration
10X PCR-buffer	10µL	1-time
Tth DNA Polymerase	0.5-1.0µL	2.5 – 5 units
Sterile water	up to 50µL	
Total	**50µL**	

NOTE: Combine Mix I and Mix II on ice and gently vortex the final mixture in a PCR-tube. Place tube in Thermal cycler and run below mentioned cycling programme immediately.

Table 8: PCR Cycling Protocol

Step	Time	Temperature	Comments
PCR-Reaction			
Initial Denaturation	1 - 2 min.	95°C	
Cycling (1-10 step)			
Denaturation	30-60 sec.	94°C	
Annealing	30-60 sec.	50-70°C	Approximately 5°C to 8°C below Tm of primers
Elongation	45-90 sec.	72-74°C	
Cycling (11->30 step) *			
Denaturation	30 sec.	94°C	
Annealing	30 sec.	50-70°C	Approximately 5°C to 8°C below Tm of primers
Elongation	45 sec.	72-74°C	
Final elongation	7 minutes	72-74°C	

* Number of cycles ranges from 30 – 50 in the literature. If the template is limited, increased cycle numbers may result in nonspecific product yield.