Thermo Scientific Phusion RT-PCR Kit

F-546S, 20 reactions (cDNA synthesis in 20 µl, PCR in 50 µl)
F-546L, 100 reactions (cDNA synthesis in 20 µl, PCR in 50 µl)

Store at -20°C

Thermo SCIENTIFIC

1. Introduction

Thermo Scientific Phusion RT-PCR Kit is a complete kit designed for performing cDNA synthesis and PCR in two steps using RNA as a starting template. The kit includes all the necessary reagents for both cDNA synthesis and DNA amplification.

In the cDNA synthesis step, RNA is transcribed by Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV RT) to its complementary DNA (cDNA). Examples of RNA that may be used as a starting material are total RNA, mRNA, viral RNA, as well as *in vitro* transcribed RNA. The reverse transcriptase utilized in this kit has intrinsic RNase H activity. This simplifies the cDNA synthesis protocol as it renders a separate RNase treatment step unnecessary. For the subsequent amplification of the cDNA template, Phusion® Hot Start II High-Fidelity DNA Polymerase is provided. It is a proofreading DNA polymerase providing extreme processivity, accuracy and specificity in the amplification step.

2. Kit components

Component	Concentration	F-546S	F-546L
RT enzyme mix (M-MuLV RNase H+ RT + RNase inhibitor)		1 x 40 µl	1 x 200 µl
10x RT Buffer (includes 50 mM $MgCl_2$*)		1 x 1.5 ml	1 x 1.5 ml
Oligo$(dT)_{15}$ primers	100 ng/µl	1 x 20 µl	1 x 100 µl
Random primers (hexamers)	50 ng/µl	1 x 20 µl	1 x 100 µl
dNTP Mix	10 mM	1 x 40 µl	2 x 100 µl
Phusion Hot Start II DNA Polymerase	2U/µl	1 x 10 µl	1 x 50 µl
5x Phusion HF Buffer		1 x 1.5 ml	1 x 1.5 ml
Control RNA with carrier	10 ng MS2 RNA/µl	1 x 20 µl	1 x 20 µl
Control primer mix	25 µM each	1 x 20 µl	1 x 20 µl

* Provides 5 mM $MgCl_2$ in 1x reaction concentration.

Material safety data sheet (MSDS) is available at www.thermoscientific.com/fzmsds.

3. General considerations

3.1 cDNA synthesis

3.1.1 RNA template

The integrity and purity of the isolated RNA is crucial for a successful RT-PCR. Therefore, special attention should be paid when isolating the RNA template to ensure that it is free of any contaminating RNase and DNA. To avoid RNase contaminants, RNA isolation should be performed under RNase-free conditions. This can be done by wearing gloves and using sterile tubes and pipet tips. Examples of potential sources for RNase contamination could be glassware, plasticware, or reagent solutions. Water used for the reactions should be RNase-free; however, DEPC treated water is not recommended because traces of DEPC can inhibit the PCR step. Contaminating DNA in the RNA preparation may be removed by RNase-free DNase I treatment. This should be done especially if primers for the PCR step cannot be designed in exon-exon boundaries or in separate exons. The purity of RNA can be determined by measuring the ratio of A_{260}/A_{280}. The optimal ratio is 1.8–2.0.

3.1.2 M-MuLV RNase H+ Reverse Transcriptase

M-MuLV RNase H+ (plus) Reverse Transcriptase (M-MuLV RT) used for cDNA synthesis in this kit is an RNA-directed DNA polymerase. This enzyme can synthesize a complementary DNA strand initiating from a primer using either a single-stranded RNA or a single-stranded DNA as a template. The RNase H activity in the enzyme degrades RNA in the RNA-cDNA hybrid. This simplifies the RT-PCR protocol because, due to the RNase H activity, it is unnecessary to perform a separate RNase treatment step after cDNA synthesis.

3.1.3 RNase inhibitor

M-MuLV RT enzyme mix contains an RNase inhibitor. Its function is to inhibit contaminating RNases that may be present in the RNA preparation. It does not affect the intrinsic RNase H activity of the M-MuLV RT.

3.1.4 RT primers

Oligo(dT) primers, random primers, or gene-specific primers can be used for the RT step. Oligo(dT) primers and random primers are useful if several different amplicons need to be analyzed from a small amount of starting material. Gene-specific primers transcribe only specific sequences; in some cases their use may decrease background signal.

Use of oligo(dT) primers is recommended over random primers and gene-specific primers when using this kit. Oligo(dT) primers transcribe poly(A)+ RNAs. These include eukaryotic mRNAs and retroviruses with poly(A)+ tails. For amplicons that are located at the 5′ end of the transcript, and for transcripts that do not contain a poly(A)+ tail, it is recommended to use random primers. Random primers are used to transcribe all RNA, producing cDNA, thereby covering the whole transcript. To obtain optimal results, especially with long amplicons, the ratio of RNA and random primers may require optimization. Gene-specific primers are used to transcribe only the particular RNA of interest.

3.2 PCR

3.2.1 DNA polymerase

The DNA polymerase included in the Phusion RT-PCR Kit is Phusion Hot Start II High-Fidelity DNA Polymerase. It is a proofreading DNA polymerase that contains a unique processivity-enhancing domain. This domain makes the enzyme extremely processive, accurate and rapid. The error rate for Phusion Hot Start II DNA Polymerase (4.4×10^{-7} as determined with a modified *lacI*-based method[1]) is approximately 52-fold lower than that of *Thermus aquaticus* DNA polymerase.

Phusion Hot Start II DNA Polymerase utilizes a reversibly binding Affibody® protein.[2,3] This protein inhibits DNA polymerase activity at ambient temperatures, thus inhibiting the amplification of non-specific products. In addition, the Affibody protein blocks the 3′→5′ exonuclease activity of the polymerase preventing degradation of primers and template DNA during reaction setup. At polymerization temperatures, the Affibody protein dissociates from the polymerase, rendering the enzyme fully active.

Phusion Hot Start II DNA Polymerase possesses the following activities: 5′→3′ DNA polymerase activity and 3′→5′ exonuclease activity. When cloning fragments amplified with Phusion Hot Start II DNA Polymerase, blunt end cloning is recommended. If TA cloning is required, it can be performed by adding A overhangs to the blunt PCR product with Thermo Scientific *Taq* DNA Polymerase, for example. However, before adding the overhangs it is very important to remove all Phusion Hot Start II DNA Polymerase by purifying the PCR product carefully. Any remaining Phusion Hot Start II DNA Polymerase will degrade the A overhangs, creating blunt ends again. A detailed protocol for TA cloning of PCR fragments amplified with any of the Phusion DNA Polymerases can be found on website: www.thermoscientific.com/pcrcloning.

3.2.2 cDNA template

The cDNA synthesis reaction mixture may be used directly as a source for template in the PCR reaction. Prior purification of the cDNA is not required. The volume of the cDNA reaction mixture used should not exceed 10 % of the final PCR reaction volume. Elevated volumes of template may reduce the efficiency of the DNA amplification. Usually, 1–5 µl or less of the cDNA synthesis reaction mixture is sufficient in a 50 µl PCR reaction.

3.2.3 PCR primers

Careful primer design is important in order to minimize non-specific primer annealing and formation of primer-dimers. PCR primers should be designed to anneal to sequences in two exons on opposite sides of the intron. This design enables differentiation by size between amplified cDNA and contaminating genomic DNA. Very long introns prevent amplification of the genomic target. Alternatively, primers can be designed to anneal to the exon-exon boundary of the mRNA. With such primers, amplification of genomic DNA will be highly inefficient.

Always use the Tm calculator and instructions on website: www.thermoscientific.com/pcrwebtools to determine the Tm values of primers and optimal annealing temperature.

4. Protocol for cDNA synthesis

4.1 Guidelines for reverse transcription

- Use gloves and RNase-free plasticware to prevent RNase contamination.
- Prepare premixes to avoid pipetting very small volumes.
- Pipet all components on ice.
- Reaction volume in cDNA synthesis is 20 µl.
- Use up to 1 µg of RNA template. The minimum amount depends on both the template and the primers used.
- Recommended primer amounts in a 20 µl reaction:
  - 100 ng oligo(dT) primers (can be increased up to 1 µg) or
  - 50 ng random primers (may require optimization) or
  - 5 pmol (2–10 pmol) gene-specific primers.

Note: When determining the amount of RNA template, the expression level of the target RNA molecule should be considered, as it affects the subsequent PCR step. The volume of the cDNA reaction mixture used as a source for template in PCR should not exceed 10 % of the final PCR reaction volume. A high RNA concentration in the PCR may also inhibit the reaction.

4.2 Protocol

It is recommended that control reactions be performed in parallel with all experiments. Control RNA and primers are provided with the kit. The setup for controls is given in chapter 6.

1. Thaw template RNA, 10x RT buffer, dNTPs and primers. Mix the individual solutions to assure homogeneity and centrifuge briefly before pipetting.

2. Combine the following components in reaction tubes
   Template RNA — x µl (up to 1 µg)
   10 mM dNTP mix — 1 µl
   Oligo(dT) primer* — 1 µl
   RNase-free H_2O — Add to 10 µl

* Alternatively, random primers (1 µl of the 50 ng/µl stock, provided with the kit), or gene-specific primers (volume depends on the concentration of the primer stock) can be used. See 4.1.

3. Incubate at 65°C for 5 minutes to predenature the RNA.

4. Place the reaction tubes on ice and add to each tube
   10x RT buffer — 2 µl
   RT enzyme mix — 2 µl
   RNase-free H_2O — 6 µl

5. Program a thermal cycler as outlined in Table 1.

6. Place the tubes in the cycler and start the program.

Table 1. Cycler protocol for cDNA synthesis.

Step	Temperature	Time
Primer extension	25°C	10 min
cDNA synthesis	40°C	30 min
Reaction termination	85°C	5 min
Cooling of the sample	4°C	Hold

4.3 Notes about cDNA synthesis conditions

4.3.1 Predenaturation

A separate RNA denaturation step is recommended. The denaturation step "5 minutes at 65°C" should be performed before adding the RT buffer and reverse transcriptase to the reaction.

4.3.2 Primer extension

The incubation for 10 minutes at 25°C extends oligo(dT) and random primers before the actual cDNA synthesis. Without the incubation at 25°C, the primers may dissociate from the template when the temperature is increased. When using gene-specific primers, this extension step is not necessary.

4.3.3 cDNA synthesis

Incubation at 40°C will work for most templates, but the incubation temperature can be optimized between 37–48°C, if necessary. Increasing the temperature can be helpful if the template has strong secondary structures. A higher temperature can also improve specificity if gene-specific primers are used. Incubation above 48°C is not recommended.

In most cases, incubation for 30 minutes is sufficient. If the target is located near the 5′ end of a long transcript and oligo(dT) priming is used, or if the target is rare, cDNA synthesis time can be extended up to 60 min.

4.3.4 Reaction termination

The termination step at 85°C inactivates the M-MuLV RT. This prevents the reverse transcriptase from inhibiting the subsequent PCR reaction.
Note: A separate RNase H treatment is not required. The cDNA can be directly used as a template in the subsequent PCR or stored at -20°C, if not used immediately.

5. Amplification of the cDNA template

5.1 Guidelines for PCR

- Carefully mix and centrifuge all tubes before opening to ensure homogeneity and improve recovery.
- PCR setup can be done at room temperature.
- Prepare a master mix for the appropriate number of samples to be amplified.
- The DNA polymerase should be pipetted carefully and gently, otherwise the high glycerol content (50 %) in the storage buffer may lead to pipetting errors.

5.2 Protocol

Table 2. Pipetting instructions (add items in this order).

Component	50 µl react.	Final conc.
H_2O	add to 50 µl	
5x Phusion HF Buffer	10 µl	1x
10 mM dNTPs	1 µl	200 µM each
primer A	x µl	0.5 µM
primer B	x µl	0.5 µM
cDNA synthesis reaction mixture	x µl	Max 5 µl (see 3.2.2)
Phusion Hot Start II DNA Polymerase	0.5 µl	0.02 U/µl

5.3 Cycling conditions

Table 3. Cycling instructions.

Cycle step	Temp.	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation Annealing (see 5.4.3) Extension	98°C X°C 72°C	10 s 5–10 s 40 s /1 kb	25–40
Final extension	72°C 4°C	5 min hold	1

5.4 Notes about cycling conditions

5.4.1 Initial denaturation

Initial denaturation should be performed at 98°C, although even higher temperatures can be used. 30 seconds at 98°C is recommended for most templates. This step can be extended up to 3 minutes, if necessary.

5.4.2 Denaturation

Keep the denaturation step as short as possible. 10 seconds at 98°C is adequate for most templates.

5.4.3 Primer annealing

Note that the optimal annealing temperature for Phusion Hot Start II DNA Polymerase may differ significantly from that of *Taq*-based polymerases. Always use the Tm calculator and instructions on website: www.thermoscientific.com/pcrwebtools to determine the Tm values of primers and optimal annealing temperature. For primers >20 nt, anneal for 5 seconds at a Tm +3°C of the lower Tm primer. For primers ≤20 nt, use an annealing temperature equal to the Tm of the lower Tm primer. If necessary, use a temperature gradient to find the optimal annealing temperature for each template-primer pair combination. The annealing gradient should extend up to the extension temperature (two-step PCR). Two-step cycling without an annealing step is recommended for high Tm primer pairs.

5.4.4 Extension

Extension should be performed at 72°C. For cDNA templates, 40 seconds per 1 kb is recommended. However, many cDNA amplicons can be successfully amplified using even shorter extension times (15–30 seconds per 1 kb).

5.4.5 Number of cycles

The number of cycles required is dependent on the abundance of the original target RNA. Usually 25 cycles in PCR is adequate. If the target RNA is rare or if only a small amount of starting material is available, it may be helpful to increase the number of cycles to 35–40.

6. Control reactions

To monitor RT-PCR reactions and to facilitate optimization and possible troubleshooting, it is recommended to perform positive and negative control reactions in parallel with all experiments.

Before starting, see chapters 4.1 and 5.1 for guidelines for reaction setups. Control RNA and control primer mix are provided with the kit. Control RNA is MS2 viral RNA including a carrier RNA. A 1011 bp sequence in the viral RNA is amplified with the control primer mix.

6.1 Positive control reaction

6.1.1 cDNA synthesis

Table 4. Pipetting instructions (add items in this order).

Component	20 µl reaction
H_2O	13 µl
Control RNA	1 µl
Random primers (50 ng/µl)	1 µl
10 mM dNTPs	1 µl
10x RT Buffer	2 µl
RT enzyme mix	2 µl

Table 5. Cycler protocol for cDNA synthesis step.

Step	Temperature	Time
Primer extension	25°C	10 min
cDNA synthesis	40°C	30 min
Reaction termination	85°C	5 min
Cooling of the sample	4°C	Hold

Note: With the positive control reaction, RNA predenaturation step is not necessary due to the low complexity of the control RNA.

6.1.2 Amplification of the control cDNA template

Table 6. Pipetting instructions (add items in this order).

Component	50 µl reaction
H_2O	32,5 µl
5x Phusion HF Buffer	10 µl
10 mM dNTPs	1 µl
Control primer mix	1 µl
cDNA from 6.1.1	5 µl
Phusion Hot Start II DNA Polymerase	0.5 µl

Table 7. Cycling instructions (two-step protocol).

Cycle tep	Temp.	Time	Cycle
Initial denaturation	98°C	30 s	1
Denaturation Extension	98°C 72°C	10 s 40 s	25
Final extension	72°C 4°C	5 min hold	1

6.2 Negative control reaction (minus RT control)

Set up an identical reaction with your application, except do not add the RT enzyme mix. This negative control is performed to detect potential DNA contamination. No PCR product should be visible in a negative control reaction.

7. Troubleshooting

Possible causes	Comments and suggestions
No amplification product or low product yield	
Error in cycler set-up	• Check that instrument settings correspond with the experiment.
Missing components (e.g. primers or template) or a pipetting error	• Check the assembly of the reaction. • Check the concentrations and storage conditions of the reagents.
RNA is degraded or of poor quality	• Verify the integrity of RNA by gel electrophoresis. • Replace the RNA if necessary. • Isolate the RNA in the presence of RNase inhibitors and ensure that reagents, tips and tubes are RNase-free.
Inhibitors are present in RNA preparation	• Reduce the volume of the RNA template in cDNA synthesis reaction. • Remove inhibitors in the RNA preparation by ethanol precipitation. Note: Inhibitors of cDNA synthesis reaction include SDS, EDTA, guanidium salts, formamide, sodium phosphate, and spermidine. • Decrease the amount of cDNA added to PCR reaction.
Incorrect temperature in cDNA synthesis reaction	• The recommended temperature in cDNA synthesis step is 40°C. It can be optimized between 37–48°C.
RNase contamination	• Maintain aseptic conditions, include RNase inhibitors in RNA isolation.
Not enough starting template	• Increase the amount of template RNA (up to 1 µg).
RNA target is rare or long	• Increase the length of cDNA synthesis step to 60 minutes. • Increase the number of cycles in the PCR.
Incomplete synthesis of target cDNA because of secondary structures in the RNA template	• Make sure that the RNA predenaturation step was included in the protocol. • Optimize the temperature of cDNA synthesis step to 37–48ºC. • Try another priming method. • If random primers were used, optimize the RNA/primer ratio.
PCR reaction conditions not optimal	• Optimize annealing temperature and/or extension time varying both individually. • Increase the number of amplification cycles. • Set extension time for 40 seconds per 1 kb of target length. • Redesign PCR primers. (See 3.2.3).
Target sequence not present in RNA preparation	• Redesign experiment or try isolating target RNA from another source.
Low specificity	
Incomplete synthesis of target cDNA because of the secondary structures in the RNA template	• Make sure that the RNA predenaturation step was included in the protocol. • Optimize the temperature of cDNA synthesis step to 37–48ºC. • Try another priming method. • If random primers were used, optimize the RNA/primer ratio.
PCR reaction conditions not optimal	• Increase the annealing temperature or perform a temperature gradient PCR. • Titrate the template amount (max 10 % of the final PCR reaction volume). • Optimize extension time. • Increase magnesium chloride concentration.
Primer design not optimal	• 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 longer primers.
RNA or DNA contamination	• Use aerosol resistant tips to reduce cross-contamination during pipetting. • Use separate work areas and pipettes for pre- and post-amplification. • Always wear gloves and change them often. • Make aliquots of all reagents used and use one aliquot for one experiment only.
Non-specific products - Low molecular weight discrete bands	
RNA preparation is contaminated with genomic DNA	• Verify the presence of contaminating DNA by performing minus RT control. • Treat the RNA preparation with RNase-free DNase I. • Redesign the primers to anneal to sequences in the exon-exon boundary or separate exons in the target gene.
Multiple target sequences exist in the RNA preparation.	• Design new primers. • Very sensitive PCR amplification may also detect rare, alternatively spliced variants.

8. References

1. Frey M. & Suppmann B. (1995) *Biochemica* 2: 34–35.
2. Nord K. *et al.* (1997) *Nature Biotechnol.* 15: 772–777.
3. Wikman M. *et al.* (2004) *Protein Eng. Des. Sel.* 17: 455–462.

Shipping and storage

Phusion DNA Polymerase is shipped on gel ice. Upon arrival, store the components at -20°C.

Technical support

US: techservice.genomics@thermofisher.com
Europe, Asia, Rest of World:
techservice.emea.genomics@thermofisher.com

Web: www.thermoscientific.com/phusion
Tm-calculator: www.thermoscientific.com/pcrwebtools

v1_07.2011

Thermo SCIENTIFIC
Part of Thermo Fisher Scientific