For general laboratory use. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

# Transcriptor First Strand cDNA Synthesis Kit

**Version July 2006**

| | |
|---|---|
| **Cat. No. 04 379 012 001** | Kit for 50 reactions including 10 control reactions |
| **Cat. No. 04 896 866 001** | Kit for 100 reactions |
| **Cat. No. 04 897 030 001** | Kit for 200 reactions |

**Store the kit at −15 to −25°C**

⚠ Store Control RNA (vial 7 in Cat. No 04 379 012 001) at −70°C or below.

**www.roche-applied-science.com**

# Table of Contents




PROTOCOL

# 1. What this Product Does

**Number of Tests** The kit is designed for 50, 100 or 200 reactions (depending on pack size).

**Kit Contents**

| Vial/ Cap | Label | Content<br>a) Cat. No. 04 379 012 001<br>b) Cat. No. 04 896 866 001<br>c) Cat. No. 04 897 030 001 |
|---|---|---|
| **1 red** | Transcriptor Reverse Transcriptase | a) 1 vial, 25 µl (20 U/µl)<br>b) 1 vial, 50 µl (20 U/µl)<br>c) 2 vials, each 50 µl (20 U/µl)<br>• Storage buffer: 200 mM potassium phosphate, 2 mM dithiothreitol, 0.2% Triton X-100 (v/v), 50% glycerol (v/v), pH approx. 7.2 |
| **2 color-less** | Transcriptor RT Reaction Buffer (5×) | a) 1 vial, 1 ml<br>b) 1 vial, 1 ml<br>c) 2 vials, each 1 ml<br>• 5× conc.: 250 mM Tris/HCl, 150 mM KCl, 40 mM $MgCl_2$, pH approx. 8.5 (25°C) |
| **3 color-less** | Protector RNase Inhibitor | a) 1 vial, 50 µl (40 U/µl)<br>b) 1 vial, 100 µl (40 U/µl)<br>c) 2 vials, each 100 µl (40 U/µl)<br>• Storage buffer: 20 mM Hepes-KOH, 50 mM KCl, 8 mM dithiothreitol, 50% glycerol (v/v), pH approx. 7.6 (at 4°C) |
| **4 yellow** | Deoxynuc-leotide Mix | a) 1 vial, 100 µl<br>b) 1 vial, 200 µl<br>c) 2 vials, each 200 µl<br>• 10 mM each dATP, dCTP, dGTP, dTTP |
| **5 blue** | Anchored-oligo$(dT)_{18}$ Primer | a) 1 vial, 100 µl (50 µM)<br>b) 1 vial, 200 µl (50 µM)<br>c) 2 vials, each 200 µl (50 µM) |
| **6 blue** | Random Hexamer Primer | a) 1 vial, 100 µl (600 µM)<br>b) 1 vial, 200 µl (600 µM)<br>c) 2 vials, each 200 µl (600 µM) |
| **7 green** | Control RNA | a) 1 vial, 20 µl (50 ng/µl)<br>• contains a stabilized solution of a total RNA fraction purified from an immortalized cell line (K562) |
| **8 green** | Control Primer Mix PBGD | a) 1 vial, 40 µl<br>• 5 µM forward and reverse primer specific for human porphobilinogen deaminase (PBGD) |

| Vial/ Cap | Label | Content<br>a) Cat. No. 04 379 012 001<br>b) Cat. No. 04 896 866 001<br>c) Cat. No. 04 897 030 001 |
|---|---|---|
| **9 (7 for b,c) color-less** | Water, PCR-grade | a) 1 vial, 1 ml<br>b) 2 vials, each 1ml<br>c) 3 vials, each 1 ml<br>In Cat. No. 04 896 866 001 and Cat. No. 04 897 030 001 the control reagents (vial 7 and 8) are not included. Therefore, in these kits vial 7 is Water, PCR Grade. |

**Storage and Stability**

Store the kit at -15 to -25°C through the expiration date printed on the label.

The Control RNA (vial 7 in Cat. No. 04 379 012 001) should be stored at −70°C.

Avoid repeated freezing and thawing.

**Additional Equipment and Reagents Required**

- general laboratory equipment
  - nuclease-free, aerosol-resistant pipette tips
  - pipettes with disposable, positive-displacement tips
  - sterile reaction tubes for preparing master mixes and dilutions
  - standard benchtop microcentrifuge
  - thermal block cycler with a heated lid
  - sequence-specific PCR primers (optional)
- for control reactions in combination with a LightCycler® Instrument:
  - LightCycler® 1.5 Instrument*, LightCycler® 2.0 Instrument* or LightCycler® 480 Instrument*

** available from Roche Applied Science*

**Application**

The Transcriptor First Strand cDNA Synthesis Kit is designed to reverse transcribe RNA (mRNA, total RNA, viral RNA, and *in vitro* transcribed RNA) from a variety of sources for the following applications:

- Study gene expression levels, via two-step RT-PCR, using qualitative RT-PCR on conventional thermal cyclers or quantitative RT-PCR on the LightCycler® Carousel-Based System, the LightCycler® 480 System, or other real-time PCR instruments.
- Generate cDNA libraries with large and full-length inserts.
- Clone genes of interest.

The kit contains all components required for cDNA reactions for use with conventional thermal cyclers and real-time PCR instruments. In addition, the 50-reaction pack size includes 10 control reactions.

# 2. How to Use this Product

## 2.1 Before You Begin

**Precautions**

Special precautions should be taken when working with RNA:

- Always wear gloves when working with RNA. After putting on gloves, do not touch surfaces and equipment to avoid reintroduction of RNases to decontaminated material.
- Designate a special area for RNA work only.
- Treat surfaces of benches and glassware with commercially available RNase inactivating agents. Clean benches with 100% ethanol.
- Use commercially available sterile and RNase-free disposable plasticware only.
- Purchase reagents that are free of RNases. Reserve separate reagents for RNA work only. All solutions should be made with DEPC - treated $H_2O$.
- All required reagents should be kept on ice.
- Extract RNA as quickly as possible after obtaining samples. For best results, use either fresh samples or samples that have been quickly frozen in liquid nitrogen and stored at – 70°C.

**Sample Material**

Template RNA: Isolated total RNA, mRNA, viral RNA or *in vitro* transcribed RNA.

⚠ High quality intact RNA, free of residual genomic DNA, RNase, and inhibitors is essential for good results. In particular, take the following precautions to avoid contaminating RNA with RNase at any step in the isolation process (starting with cell lysis):

- Use either RNase inhibitors such as Protector RNase Inhibitor or isolation conditions that inactivate RNases.
- If necessary, analyze different steps in the process (lysis, isolation) by gel electrophoresis (ethidium bromide staining) to ensure that the sample is still RNase-free.
- Remember that RNases can also be present on contaminated glassware.

To prepare total RNA or mRNA, we recommend using Roche Applied Science reagents. For a selection of products which produces high quality intact RNA templates suitable for RT-PCR please refer to section 6. Supplementary Information, Ordering Information or to our Special Interest Site on manual nucleic acid isolation and purification at www.roche-applied-science.com/napure. For information on automated nucleic acid isolation using the MagNA Pure LC System or the MagNA Pure Compact System, visit www.magnapure.com.

**Primer**

Depending on the type of analysis, to which the cDNA is to be subjected, use one of three different priming methods described below:

- **Random hexamer primer**:
  In general, to reversely transcribe 5 μg of total RNA with random hexamers a final primer concentration of 60 μM is sufficient. Increasing the concentration of hexamers to higher concentrations for the transcription of 5 μg RNA may increase yield of small PCR products (< 500 bp), but may decrease the yield of longer PCR products and full-length transcripts. Note that random hexamer priming is the most non-specific priming method and specificity is only obtained by PCR primers in the following PCR reaction.
- **Anchored-oligo(dT)$_{18}$ primer**:
  As anchored-oligo(dT)$_{18}$ primers are specific to the small pool of poly(A)$^+$ RNA in the whole total RNA pool (1-2%), the amount of cDNA resulting from reverse transcription reactions with anchored-oligo(dT)$_{18}$ primers is considerably lower than with random hexamers. Anchored-oligo(dT)$_{18}$ priming is recommended when performing RT-PCR for new mRNA targets. Anchored-oligo(dT)$_{18}$ produces an RT-PCR product more consistently than random hexamers or gene-specific primers.
- **Sequence-specific primer**:
  The use of gene-specific primers (recommended final concentration is 2 μM) is the most specific priming method, but sometimes fails to prime cDNA even though the same primers work in PCR on DNA templates. If gene-specific priming fails in RT-PCR, repeat first-strand synthesis using anchored-oligo(dT)$_{18}$ primers.

| Type of RT Primer | Binds | Advantages/Comments |
|---|---|---|
| Anchored-oligo(dT)$_{18}$ | Very beginning of the poly(A) tail | • Prevents priming from internal sites of the poly(A) tail.<br>• Generates full-length cDNA.<br>• Preferred priming method for most two-step RT-PCR.<br>• Available as part of the Transcriptor First Strand cDNA Synthesis Kit only. |
| Random hexamer | Many sites throughout the length of an RNA | • Provides uniform representation of all RNA sequences in mRNA.<br>• Can prime cDNA transcription from RNAs that do not carry a poly(A) tail.<br>• The ratio of random primers to RNA in the RT reaction determines the average length of cDNAs generated. Example: A high ratio will generate relatively short cDNAs, increasing the chance of copying the complete target sequence.<br>• Short cDNA transcripts may help to overcome difficulties caused by RNA secondary structures. |

| Type of RT Primer | Binds | Advantages/Comments |
|---|---|---|
| Sequence-specific | Only sequences that are exactly complementary to the primer sequence | • Selects for a particular RNA.<br>• Greatly increases the specificity of the RT-PCR. |

Whenever possible, design primers that anneal to exon sequences on both sides of an intron or on exon/exon boundaries. This will allow differentiation of the amplified cDNA from contaminating genomic DNA because amplification of DNA will result in longer amplicons due to the additional intron sequence.

⚠ Primers should not be self-complementary.

**Standard RT-PCR Procedure**

Two different procedures are provided:

- **A:** Reverse transcription using either anchored-oligo(dT)$_{18}$ priming **OR** random hexamer priming **OR** sequence-specific priming. In the majority of cases, cDNA is generated with only one sort of primers.
- **B:** Reverse transcription using a combination of anchored-oligo(dT)$_{18}$ priming **AND** random hexamer priming. This can be the method of choice to increase sensitivity, but the specificity of the reaction might be reduced compared to single anchored-oligo(dT)$_{18}$ priming .

Depending on which type of primer system you have decided to use, follow Procedure A or B described below. If you are going to use a sequence-specific primer follow Procedure A.

Preheat the thermal block cycler to the temperature of the RT reaction (see step 6 below) or set-up the experimental protocol for the LightCycler® Instrument before starting the procedure.

**Procedure A:** cDNA Synthesis with anchored-oligo(dT)$_{18}$ primer **OR** random **OR** sequence-specific primer

The following conditions describe a first-strand cDNA synthesis for a two-step RT-PCR. Fig.1 shows the standard procedure for cDNA synthesis used for single reactions and the simplified one if multiple reactions should be performed.

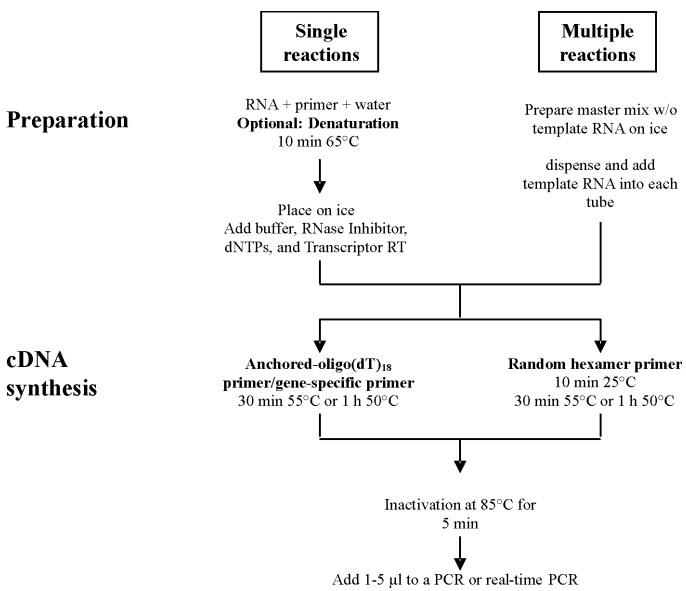



**Fig. 1:** Overview of cDNA synthesis procedures in single and multiple reactions

❶ • Thaw all frozen reagents before use.
• Briefly centrifuge them before starting the procedure.
• Keep all reagents on ice while setting up the reactions.

❷ In a sterile, nuclease-free, thin-walled PCR tube on ice, prepare the template-primer mixture for one 20 µl reaction by adding the components in the order listed below.

⚠ Always wear gloves when handling RNA.

**Template-Primer Mix** (for 1 reaction)

| Component | Vol. | Final conc. |
|---|---|---|
| total RNA or poly(A)$^+$ mRNA | | 1 µg total RNA or 10 ng poly(A)$^+$ RNA[a)] |
| Primer – choose either: | | |
| Anchored-oligo(dT)$_{18}$ Primer, 50 pmol/µl (vial 5) | 1 µl | 2.5 µM |
| **OR** Random Hexamer Primer, 600 pmol/µl (vial 6) | 2 µl | 60 µM |
| **OR** Sequence-Specific Primer | variable | 0.5-2.5 µM |
| Water, PCR-grade (vials 7 or 9) | variable | to make total volume = 13 µl |
| **Total volume** | **13 µl** | |

[a)] These are the suggested concentrations for initial experiments. Suitable template concentrations may range from 10 ng to 5 µg total RNA and from 1 to 100 ng mRNA.

When working with low concentrated RNA samples (< 10 µg/ml), add 10 µg/ml MS2 RNA* to stabilize the template RNA.

❸ **Optional Step:**
• Denature the template-primer mixture by heating the tube for 10 min at 65°C in a thermal block cycler with a heated lid (to minimize evaporation). This step ensures denaturation of RNA secondary structures.
• Immediately cool the tube on ice.

❹ To the tube containing the template-primer mix, add the remaining components of the RT mix in the order listed below.

❹

| Component | Vol. | Final conc. |
|---|---|---|
| Transcriptor Reverse Transcriptase Reaction Buffer, 5× conc. (vial 2) | 4 µl | 1× (8 mM $MgCl_2$) |
| Protector RNase Inhibitor, 40 U/µl (vial 3) | 0.5 µl | 20 U |
| Deoxynucleotide Mix, 10 mM each (vial 4) | 2 µl | 1 mM each |
| Transcriptor Reverse Transcriptase, 20 U/µl (vial 1) | 0.5 µl | 10 U |
| **Final volume** | **20 µl** | |

❺
- Mix the reagents in the tube carefully.

  ⚠ Do not vortex!
- Centrifuge the tube briefly to collect the sample on the bottom of the tube.
- Place the tube in a thermal block cycler with a heated lid (to minimize evaporation).

❻ Depending on the primer used and the length of the target mRNA, incubate the RT reaction as described in the table below:

| If you are using… | And the target mRNA is… | Incubate the RT reaction…… |
|---|---|---|
| Anchored-oligo$(dT)_{18}$ primer, 50 pmol/µl **OR** Sequence–specific primer | Up to 4 kb | 30 min at 55°C |
| | >4 kb | 60 min at 50°C |
| Random hexamer primers, 600 pmol/µl | Up to 4 kb | 10 min at 25°C, followed by 30 min at 55°C |
| | >4 kb | 10 min at 25°C, followed by 60 min at 50°C |

❼
- Inactivate Transcriptor Reverse Transcriptase by heating to 85° for 5 min.
- Stop the reaction by placing the tube on ice.
- At this point the reaction tube may be stored at +2 to +8°C for 1-2 h or at -15 to -25°C for longer periods.

❽ For PCR:
The cDNA can be added to the PCR without purification.
- In general, use 1 – 5 µl of the reaction product (first-strand cDNA) as a template for PCR. For initial experiments, try using 2 µl cDNA template for a 50 µl PCR.
- For PCR on one of the LightCycler® instruments, use 2 – 5 µl of the cDNA reaction or dilutions in a 20 µl reaction.

🔍 The cDNA product does not need to be purified before it is used in PCR.

⚠ The final $MgCl_2$ concentration in the reverse transcription reaction is 8 mM. Therefore, each µl of the cDNA contributes 8 nmol $MgCl_2$ to the following reaction. Optimize the $MgCl_2$ concentration of the PCR if necessary.

⚠ Transcriptor RTase has RNase H activity. RNase H removes the RNA template after cDNA synthesis, allowing PCR primers to more easily bind the cDNA, which in some cases increases the sensitivity of the PCR (Polumuri *et al., 2002*).

**Procedure B:** cDNA Synthesis with anchored-oligo$(dT)_{18}$ primer **AND** random hexamer primer

The following conditions describe a first strand cDNA synthesis for a two-step RT-PCR with a mixture of anchored-oligo$(dT)_{18}$ primer **AND** random hexamer primers.

❶
- Thaw all frozen reagents before use.
- Briefly centrifuge them before starting the procedure.
- Keep all reagents on ice while setting up the reactions.

❷ In a sterile, nuclease-free, thin-walled PCR tube on ice, prepare the template-primer mixture for one 20 µl reaction by adding the components in the order listed below.

⚠ Always wear gloves when handling RNA.

**Template-Primer Mix** (for 1 reaction)

| **Component** | **Vol.** | **Final conc.** |
|---|---|---|
| total RNA or poly(A)$^+$ mRNA | | 1 µg total RNA or 10 ng poly(A)$^+$ RNA[a)] |
| Anchored-oligo(dT)$_{18}$ primer, 50 pmol/µl (vial 5) | 1 µl | 2.5 µM |
| **AND** random hexamer primer, 600 pmol/µl (vial 6) | 2 µl | 60 µM |
| Water, PCR-grade (vials 7 or 9) | variable | to make total volume = 13 µl |
| **Total volume** | **13 µl** | |

[a)] These are the suggested concentrations for initial experiments. Suitable template concentrations may range from 10 ng to 5 µg total RNA and from 1 to 100 ng mRNA.

When working with low concentrated RNA samples (< 10 µg/ml), add 10 µg/ml MS2 RNA* to stabilize the template RNA.

❸ **Optional Step:** (ensures denaturation of RNA secondary structures if present)

- Denature the template-primer mixture by heating the tube for 10 min at 65°C in a thermal block cycler with a heated lid (to minimize evaporation).
- Immediately cool the tube on ice.

❹ To the tube containing the template-primer mix, add the remaining components of the RT mix in the order listed below.

| **Component** | **Vol.** | **Final conc.** |
|---|---|---|
| Transcriptor Reverse Transcriptase Reaction Buffer, 5× conc. (vial 2) | 4 µl | 1× (8 mM $MgCl_2$) |
| Protector RNase Inhibitor, 40 U/µl (vial 3) | 0.5 µl | 20 U |
| Deoxynucleotide Mix, 10 mM each (vial 4) | 2 µl | 1 mM each |
| Transcriptor Reverse Transcriptase, 20 U/µl (vial 1) | 0.5 µl | 10 U |
| **Final volume** | **20 µl** | |

❺ • Mix the reagents in the tube carefully.

⚠ Do not vortex!

• Centrifuge the tube briefly to collect the sample on the bottom of the tube.
• Place the tube in a thermal block cycler with a heated lid (to minimize evaporation).

❻ Depending on the primer used and the length of the target mRNA, incubate the RT reaction as described in the table below:

| **If you are using…** | **And the target mRNA is…** | **Incubate the RT reaction……** |
|---|---|---|
| Anchored-oligo(dT)$_{18}$ primer, 50 pmol/µl **And** Random hexamer primer, 600 pmol/µl | Up to 4 kb | 10 min at 25°C, followed by 30 min at 55°C |
| | > 4 kb | 10 min at 25°C, followed by 60 min at 50°C |

❼ • Inactivate Transcriptor Reverse Transcriptase by heating to 85°C for 5 min.
• Stop the reaction by placing the tube on ice.
• At this point the reaction tube may be stored at +2 to +8°C for 1-2 h or at -15 to -25°C for longer periods.

❽ For PCR:
The cDNA can be added to the PCR without purification.
• In general, use 1 – 5 µl of the reaction product (first-strand cDNA) as a template for PCR. For initial experiments, try using 2 µl cDNA template for a 50 µl PCR.
• For PCR on one of the LightCycler® instruments, use 2 – 5 µl of the cDNA reaction or dilutions in a 20 µl reaction.

The cDNA product does not need to be purified before it is used in PCR.

⚠ The final $MgCl_2$ concentration in the reverse transcription reaction is 8 mM. Therefore, each µl of the cDNA contributes 8 nmol $MgCl_2$ to the following reaction. Optimize the $MgCl_2$ concentration of the PCR if necessary.

⚠ Transcriptor RTase has RNase H activity. RNase H removes the RNA template after cDNA synthesis, allowing PCR primers to more easily bind the cDNA, which in some cases increases the sensitivity of the PCR (Polumuri *et al., 2002*).

**cDNA Synthesis**

The control reaction which is provided in Cat. No. 04 379 012 001 includes a reverse transcription of the Control RNA followed by detection of a 151 bp fragment of PBGD in a PCR on a conventional thermal block cycler or a LightCycler® instrument.

The following conditions describe the first-strand cDNA synthesis for a two-step control RT-PCR.

⚠ Preheat the thermal block cycler to the temperature of the RT reaction (see step 4 below) before starting the procedure.

❶
- Thaw all frozen reagents before use.
- Briefly centrifuge them before starting the procedure.
- Keep all reagents on ice while setting up the reactions.

❷ In a sterile, nuclease-free, thin-walled PCR tube on ice, prepare the template-primer mixture for one 20 µl reaction by adding the components in the order listed below.

⚠ Always wear gloves when handling RNA.

**Template–Primer Mix** (for 1 reaction)

| Component | Vol. | Final conc. |
|---|---|---|
| Control RNA | 2 µl | 100 ng |
| Anchored-oligo($dT)_{18}$ primer, 50 pmol/µl (vial 5) | 1 µl | 2.5 µM |
| Water, PCR-grade | 10 µl | to make total volume = 13 µl |
| **Total volume** | **13 µl** | |

❸ Add the following components

| Component | Vol. | Final conc. |
|---|---|---|
| Transcriptor Reverse Transcriptase Reaction Buffer, 5× conc. (vial 2) | 4 µl | 1× (8 mM $MgCl_2$) |
| Protector RNase Inhibitor, 40 U/µl (vial 3) | 0.5 µl | 20 U |
| Deoxynucleotide Mix, 10 mM each (vial 4) | 2 µl | 1 mM each |
| Transcriptor Reverse Transcriptase, 20 U/µl (vial 1) | 0.5 µl | 10 U |
| **Final volume** | **20 µl** | |

- Mix well by pipetting
- Spin the tube briefly in a microfuge.

❹ Incubate 30 min at 55°C.

❺ • Inactivate Transcriptor Reverse Transcriptase by heating to 85°C for 5 min.
• Place the tube on ice.

❻ At this point the reaction tube may be stored at +2 to +8°C or at -15 to -25°C for longer periods.

**PCR for PBGD**

The resulting single-stranded cDNA can be amplified in a polymerase chain reaction utilizing the supplied PBGD-specific primers. This PCR may be done on a conventional thermal block cycler or on a LightCycler® instrument:

- Use 5 µl of the cDNA reaction for PCR on a conventional thermal block cycler with a reaction volume of 50 µl using FastStart Taq DNA Polymerase*.
- Use 2 µl of the cDNA reaction for real-time PCR on the LightCycler® 1.5 Instrument or LightCycler® 2.0 Instrument with a reaction volume of 20 µl using the LightCycler® FastStart DNA Master SYBR Green I*.
- Use 2 µl of the cDNA reaction for real-time PCR on the LightCycler® 480 Instrument with a reaction volume of 20 µl using the LightCycler® 480 SYBR Green I Master*.

For further details of the PCR or real-time PCR read the pack inserts of FastStart Taq DNA Polymerase, LightCycler® FastStart DNA Master SYBR Green I, or LightCycler® 480 SYBR Green I Master.

** available from Roche Applied Science*

**PCR in a conventional thermal block cycler**

Follow the procedure below to prepare one 50 µl standard reaction.

Perform a negative control reaction without template (using water instead of cDNA) in parallel to the RT control reaction.

❶ • Thaw all frozen reagents before use.
• Briefly centrifuge them before starting the procedure.
• Keep all reagents on ice while setting up the reaction.

❷ In a thin-walled PCR tube on ice, prepare the PCR Mix for one 50 µl reaction by adding the following components in the order mentioned below:

| Component | Vol. | Final conc. |
|---|---|---|
| FastStart Buffer with 20 mM $MgCl_2$ (10×) | 5 µl | 1× |
| PCR Nucleotide Mix* (10 mM) | 1 µl | 0.2 mM |
| Control Primer MIX PBGD (5 µM) | 2 µl | 0.2 µM |
| cDNA from Control RT reaction | 5 µl | |
| FastStart Taq DNA Polymearse* (5 U/µl) | 0.4 µl | 2 U |
| Water PCR-grade | 36.6 µl | |
| **Total volume** | **50 µl** | |

❸ Run PCR in a thermal cycler with heated lid or overlay with 30 µl mineral oil:

| | | | |
|---|---|---|---|
| 1 cycle | Initial denaturation | 94°C | 5 min |
| 35 cycles | Denaturation | 94°C | 10 s |
| | Annealing | 50°C | 20 s |
| | Elongation | 72°C | 30 s |
| 1 cycle | Final elongation | 72°C | 7 min |
| | Cooling | 4°C | |

❹ Load 15 µl on a 3% agarose gel.

See Figure 2 under Results.

**PCR on the LightCycler® Carousel-Based System**

The Control Primer Mix PBGD enclosed in Cat. No. 04 379 012 001 can be used for amplification of a 151 bp fragment detected in the SYBR Green format. Perform a negative control reaction without template (using water instead of cDNA) in parallel to the RT control reaction.

❶
- Thaw all frozen reagents before use.
- Briefly centrifuge them before starting the procedure.
- Keep all reagents on ice while setting up the reaction.

❷ Prepare the reaction mixture. A no template control (water instead of cDNA) should be performed in parallel to the RT control reaction. Prepare a master mix for both reactions without template and dispense into two LightCycler(r) Capillaries*.

| **Component** | **Vol.** | **Final conc.** |
|---|---|---|
| LightCycler® FastStart DNA Master SYBR Green I reaction mix (10×) | 2 µl | 1× |
| Control Primer Mix PBGD (5 µM) | 2 µl | 0.5 µM |
| $MgCl_2$ (25 mM) | 2.4 µl | 4 mM (1 mM are contributed by the LightCycler® master mix) |
| Water PCR Grade | 11.6 µl | |
| **Total volume** | **18 µl** | |

❸
- Mix carefully by pipetting up and down. Do not vortex.
- Pipet 18 µl PCR mix into each precooled LightCycler® Capillary.
- Add 2 µl of the cDNA template from Control RNA or water (in case of the negative control).
- Seal each capillary with a stopper.

❹ • Place the adapters (containing the LightCycler® capillaries) into a standard benchtop microcentrifuge.

⚠ Place the centrifuge adapters in a balanced arrangement within the centrifuge.

• Centrifuge at 700 × *g* for 5 s (3,000 rpm in a standard benchtop microcentrifuge).

• Alternatively, use the LightCycler® Carousel Centrifuge for spinning the capillaries.

❺ Transfer the capillaries into the LightCycler® Sample Carousel.

❻ Run the following LightCycler® experimental protocol:

| | | | |
|---|---|---|---|
| **• Denaturation:** | | | |
| Temp. (°C) | | 95 | |
| Hold (s) | | 600 | |
| dT/dt (°C/s) | | 20 | |
| Acq. Mode | | none | |
| **• Amplification: 45 cycles** | | | |
| Temp. (°C) | 95 | 60 | 72 |
| Hold (s) | 10 | 10 | 10 |
| dT/dt (°C/s) | 20 | 20 | 20 |
| Acq. Mode | none | none | single |
| ❻ **• Melting curve** | | | |
| Temp. (°C) | 95 | 55 | 95 |
| Hold (s) | 0 | 30 | 0 |
| dT/dt (°C/s) | 20 | 20 | 0.05 |
| Acq. Mode | none | none | continuous |
| **• Cooling:** | | | |
| Temp. (°C) | | 40 | |
| Hold (s) | | 30 | |
| dT/dt (°C/s) | | 20 | |
| Acq. Mode | | none | |

For typical results see Figure 3 under Results

**PCR on the LightCycler® 480 Instrument**

The Control Primer Mix PBGD enclosed in Cat. No. 04 379 012 001 can be used for amplification of a 151 bp fragment detected in the SYBR Green format.

Perform a negative control reaction without template (using water instead of cDNA) in parallel to the RT control reaction.

❶ • Thaw all frozen reagents before use.
• Briefly centrifuge them before starting the procedure.
• Keep all reagents on ice while setting up the reaction.

❷ • Prepare a PCR mix for both reactions (control reaction and negative control):

| Component | Vol. | Final conc. |
|---|---|---|
| LightCycler® 480 SYBR Green I Master (2×) | 10 µl | 1× |
| Control Primer MIX PBGD (5 µM) | 2 µl | 0.5 µM |
| Water PCR-grade | 6 µl | |
| **Total volume** | **18 µl** | |

❸ • Pipet 18 µl PCR mix into each well of the LightCycler® 480 Multiwell Plate.
• Add 2 µl of the cDNA template from Control RNA or water (in case of the negative control).
• Seal the Multiwell Plate with LightCycler® 480 Multiwell Sealing Foil. Mix carefully by gently shaking the Multiwell Plate.

❹ • Place the Multiwell Plate in the centrifuge and balance it with a suitable counterweight (*e.g.* another Multiwell Plate).
• Centrifuge at 1500 × *g* for 2 min (3,000 rpm in a standard swing-bucket centrifuge containing a rotor for Multiwell Plates with suitable adaptors).

❺ • Load the Multiwell Plate into the LightCycler® 480 Instrument and run the following LightCycler® experimental protocol:

| **Setup** | | |
|---|---|---|
| **Detection format** | **Block type** | **Reaction vol.** |
| SYBR Green (483-533 nm) | 96 | 20 µl |
| **Program** | | |
| **Program name** | Cycles | Analysis Mode |
| Pre-Incubation | 1 | None |
| Amplification | 45 | Quantification |
| Melting Curve | 1 | Melting Curves |
| Cooling | 1 | None |

| Program | Target (°C) | Acquisition Mode | Hold (s) | Ramp Rate (°C/sec) | Acquisitions (per °C) |
|---|---|---|---|---|---|
| **Pre-Incubation** | 95°C | None | 600 | 4.4 | - |
| **Amplification** | | | | | |
| Segment 1 | 95°C | None | 10 | 4.4 | - |
| Segment 2 | 60°C | None | 15 | 2.2 | - |
| Segment 3 | 72°C | Single | 15 | 4.4 | - |
| **Melting Curve** | | | | | |
| Segment 1 | 95°C | None | 5 | 4.4 | |
| Segment 2 | 55°C | None | 30 | 2.2 | |
| Segment 3 | 95°C | Continuous | - | - | 2 |
| **Cooling** | 40°C | None | 10 | 1.0 | - |

❻ For typical results see Figure 4 under Results.

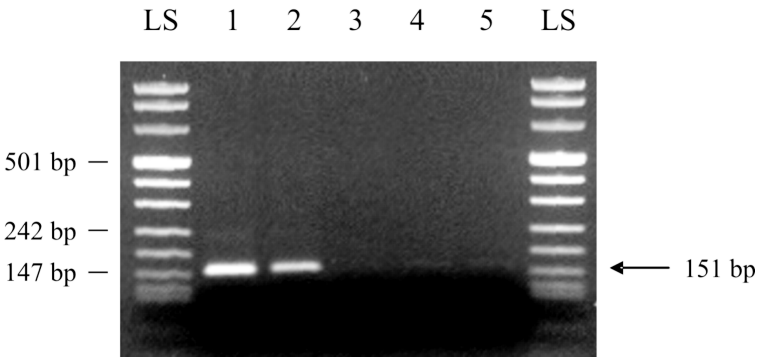



**Fig. 2:**
Control reaction with PBGD primers using FastStart Taq DNA Polymerase for the PCR. The supplied Control RNA was used in two different dilutions.
LS = Molecular weight marker VIII, 250 ng; 1 = RT step with 100 ng Control RNA; 2 = RT step with 10 ng Control RNA; 3 = negative control of PCR; 4 = RT step w/o Transcriptor RT; 5 = RT step w/o RNA; 15 µl of each reaction were loaded on a 3% agarose gel

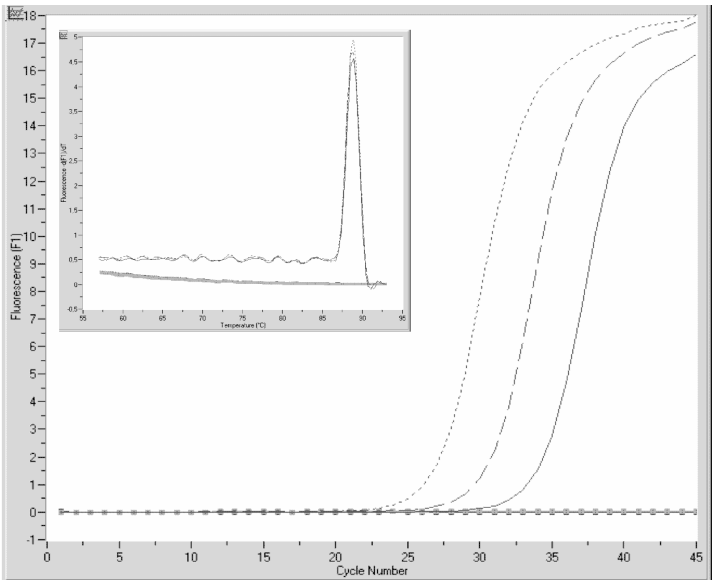
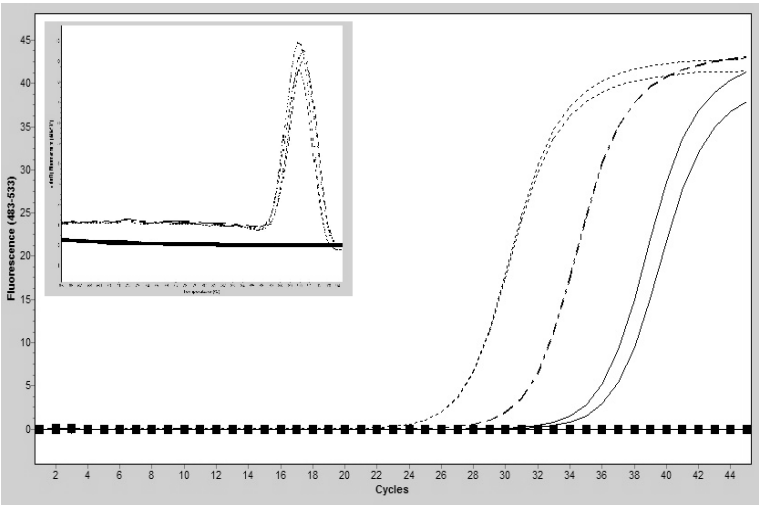



**Fig. 3:**
Control reaction with PBGD primers using LightCylcer® FastStart DNA Master SYBR Green I for the LightCycler® Carousel-Based Instrument reaction. The supplied Control RNA was used in different dilutions.




| | | |
|---|---|---|
| ------ | 100 ng Contr. RNA | 27.03 |
| ------ | 100 ng Contr. RNA | 27.01 |
| – – – | 10 ng Contr. RNA | 31.15 |
| – – – | 10 ng Contr. RNA | 31.09 |
| —— | 1 ng Contr. RNA | 35.55 |
| —— | 1 ng Contr. RNA | 36.27 |
| —▲— | w/o RNA | |
| —+— | 100 ng Contr. RNA w/o RT | |
| —■— | No template control | |

**Fig. 4:**
Control reaction with PBGD primers using LightCycler® 480 SYBR Green I Master on the LightCycler® 480 System. The Control RNA was used in different dilutions.

| | Possible Cause | Recommendation |
|---|---|---|
| **Little or no product in cDNA reaction** | Too little RNA template | • Check quality and concentration of template:<br>• Analyze an aliquot on a denaturing agarose gel to check for possible degradation or impurity. If degraded or impure, repeat purification.<br>• Amplify template with an established primer pair or in an established RT-PCR system.<br>• Determine concentration of RNA template by measuring $A_{260}$.<br>• Use purified mRNA rather than total RNA as template.<br>• Use 10 ng – 5 µg of total RNA or 1 – 100 ng of mRNA. If you must use lower amounts of RNA, try priming with a gene-specific primer.<br>Add 10 µg/ml MS2 RNA to template to stabilize low concentrations of target RNA. |
| | Too much template RNA | • Decrease amount of RNA template. Too much RNA may affect/inhibit performance of RT reaction. |
| | RNase contamination | • Protect RNA from ribonuclease degradation by adding Protector RNase Inhibitor to the cDNA reaction.<br>Inhibitor concentrations up to 60 U will not interfere with RT-PCR.<br>• Use RNase-free tubes and pipet tips.<br>• Add Control RNA to sample to determine if RNases are present in first strand reaction. |
| | Difficult template with secondary structure (GC-rich templates) | • RNA templates up to 70% GC content can be reverse transcribed if you:<br>• Increase the reaction temperature up to 65°C.<br>• Denature the template-primer mixture for 10 min at 65°C before adding reverse transcriptase.<br>• Use random hexamer primers or a gene-specific primer.<br>• Use Transcriptor Reverse Transcriptase to reverse transcribe at temperatures as high as 65°C. |
| | Enzyme concentration too high or low | • Do not use more than 10 U Transcriptor Reverse Transcriptase to transcribe 1 µg total RNA template in a 20 µl cDNA synthesis reaction.<br>• For >5 µg total RNA, increase reaction volume and amount of Transcriptor Reverse Transcriptase proportionally.<br>• For low template concentrations, use less reverse transcriptase. |

| | Possible Cause | Recommendation |
|---|---|---|
| **Little or no product in cDNA reaction** | Reaction temperature too high or low | • Perform the RT reaction (for templates up to 4 kb) for 30 min at a temperature between 42°C and 65°C.<br>• For transcripts >4 kb, perform the reaction at a temperature between 42°C and 60°C for 1 h.<br>Prolonged incubation at lower temperatures will increase the yield of full-length product. |
| | Wrong gene-specific primer | • Try another gene-specific primer or switch to an oligo(dT) primer [*e.g.*, the anchored-oligo$(dT)_{18}$ primer included in the Transcriptor First Strand cDNA Synthesis Kit].<br>• Make sure that the gene-specific primer is able to bind to the mRNA (antisense direction). |
| | Use of Random Hexamer primer | The ratio of random primers to RNA can be adjusted to control the average length of cDNA products; high ratios as recommended in this manual will produce shorter cDNAs, but should increase the likelihood of copying the target sequence (fragments up to 6 kb were amplified by PCR using the recommended conditions). If longer cDNAs are needed, the concentration of random primers may be decreased down to 1.5 µM in the cDNA synthesis reaction. |
| | Inhibitors of RT reaction | Remove inhibitors by precipitating the mRNA, washing the precipitate with 70% ethanol, then redissolving the precipitate.<br>⚠ Remove all traces of ethanol before using RNA in RT. |
| **Little or no PCR product** | Contamination by genomic DNA | • Design primers that recognize different exons, so they can distinguish between genomic DNA contaminants and cDNA.<br>• Always include a "no RT" control that did not contain reverse transcriptase during the cDNA synthesis step.<br>Please refer to the pack inserts of the used DNA polymerase or the used kit. |
| | $MgCl_2$ concentration for PCR too low or high | • Optimize the $MgCl_2$ concentration of the PCR reaction.<br>• Optimize $MgCl_2$ concentration for each template and primer combination.<br>The final $MgCl_2$ concentration in the reverse transcription reaction is 8 mM. Therefore, each µl of the cDNA contributes 8 nmol $MgCl_2$ to the following reaction. |
| | Annealing temperature too low | Increase annealing temperature to accommodate the melting temperature of the primers. |
| | Primer design for PCR not optimal | • Design alternative primers.<br>• Both primers should have similar melting temperatures. |

| | Possible Cause | Recommendation |
|---|---|---|
| **Little or no PCR product** | Primer concentration in PCR not optimal | • Both primers should be present in the reaction at the same concentration.<br>• Try various primer concentrations (between 0.1 and 0.6 µM for each primer).<br>• Check for possible degradation of the primers (*e.g.*, on a denaturing polyacrylamide gel or by HPLC). |
| | Too much cDNA inhibits PCR | The volume of cDNA template (from the RT reaction) should not exceed 10% of the total volume of the PCR reaction. |
| | Formation of primer dimers | • Use FastStart Taq DNA Polymerase or FastStart High Fidelity PCR System.<br>• Design primers that do not contain complementary sequences.<br>• Make sure a denaturation step is included at the end of the cDNA synthesis reaction (5 min at 85°C). |
| | Difficult template with secondary structure (GC-rich templates) | • Use the GC-RICH Resolution Solution when working with FastStart Taq DNA Poly merase.<br>• Add DMSO (up to 10%) when working with FastStart High Fidelity PCR System.<br>• Use PCR primers closer to the 3' terminus of the target cDNA. |

# 5. Additional Information on this Product

**How this Product Works**

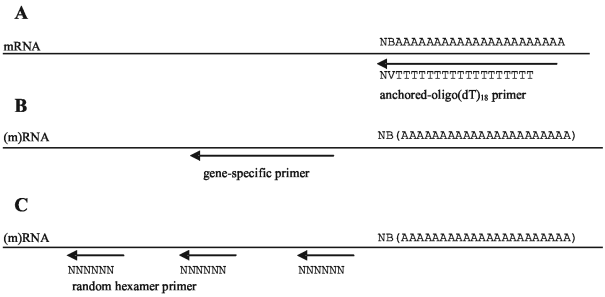
Using the Transcriptor First Strand cDNA Synthesis Kit, RNA is reverse transcribed into single-stranded cDNA, which can be used directly for subsequent PCR with gene-specific primers on conventional thermal block cyclers and real-time PCR instruments (*e.g.* the LightCycler® Carousel-Based System, the LightCycler® 480 Instrument or other real-time PCR instruments), or for other downstream applications.

Transcriptor Reverse Transcriptase is a recombinant reverse transcriptase expressed in *E. coli*. The enzyme has RNA-directed DNA polymerase activity, DNA-dependent DNA polymerase activity, unwinding activity and, very importantly, RNase H activity that degrades RNA in RNA:DNA hybrids. Thus, there is no need to perform an additional time-consuming RNase H incubation step after reverse transcription, which shortens the reaction time. Single-stranded RNA as well as ssDNA are accepted as template and are reverse transcribed in the presence of a primer.

Transcriptor Reverse Transcriptase is recommended for RT-PCR because of its high sensitivity in connection with very high thermostability: The enzyme is able to synthesize long cDNA products (up to 14 kb) and can be used at temperatures up to 65°C. Due to its thermostability, Transcriptor Reverse Transcriptase is recommended for GC-rich templates with high secondary structure without the need to include any additives in the reaction.

The kit provides all reagents required for performing first strand cDNA synthesis reactions from RNA. For priming, three different primer systems may be used as shown in Figure 5. Two cDNA synthesis primers are already provided with the kit: random hexamer primers and an anchored-oligo$(dT)_{18}$ primer. The latter is designed to bind at the very beginning of the poly(A) tail to generate full-length cDNA and to prevent priming from internal sites of the poly(A) tail. Although especially the 5´-ends of long mRNAs are often underrepresented, this priming method is preferred for most applications. The use of random hexamer primers provides priming throughout the length of RNA for uniform representation of all RNA sequences and, in addition, allows reverse transcription of RNA molecules that do not carry a poly(A) tail.

**Fig. 5**
Overview of first strand cDNA synthesis using different priming methods. A, anchored-oligo(dT)$_{18}$ primer; B, sequence-specific primer; C, random hexamer primer; V = A, C, or G; B = C, G, or T; N = A, C, G, or T.




## References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2 Sambrook, J. et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd. Edition, Cold Spring Harbour Laboratory Press.
3 Increase the Power and Sensitivity for Your cDNA Synthesis with the New Transcriptor Reverse Transcriptase (2003) *Biochemica* **3**, 17-19.
4 Protector RNase Inhibitor Enhance Protection of RNA Against Degradation (2002) *Biochemica* **4,** 29.
5 Brooke-Powell, E.T. et al (2004) Use of Transcriptor Reverse Transcriptase in Microarray Analysis. *Biochemica* **1**, 27-30.
6 Ortega, X. et al. (2005) Reconstitution of O-Specific Lipopolysaccharide Expression in Burkholderia cenocepacia Strain J2315, Which Is Associated with Transmissible Infections in Patients with Cystic Fibrosis. *J. Bacteriol.* **187**, 1324-33.
7 Tanaka, T. et al. (2004) Concerted Action of Diacetylchitobiose Deacetylase and Exo-{beta}-D-glucosaminidase in a Novel Chitinolytic Pathway in the Hyperthermophilic Archaeon Thermococcus kodakaraensis KOD1. *J. Biol. Chem.* **279,** 30021-7.
8 Wilkie et al. (2005). Embryonic Poly(A)-Binding Protein Stimulates Translation in Germ Cells. *Mol. Cell. Biol.* **25,** 2060-2071.

**Quality Control**

Each lot of Transcriptor First Strand cDNA Synthesis Kit is function tested in a RT-PCR (described in the following) on a conventional thermal cycler as well as with a LightCycler® Instrument. Additionally, Transcriptor Reverse Transcriptase, Protector RNase Inhibitor, and the other components are tested independently for the absence of any contaminations.

- Function testing by two-step RT-PCR using a conventional thermal cycler:
  - Transcriptor Reverse Transcriptase is function tested using 2 µg of total human skeletal muscle RNA, 10 U Transcriptor Reverse Transcriptase and 50 pmol anchored-oligo(dT)$_{18}$ primer in a volume of 20 µl. The reaction is incubated for 1 h at 50°C. In the following PCR reaction applying 5 µl cDNA template in a total volume of 50 µl with the Expand Long Template PCR System over 30 cycles a 10 kb dystrophin fragment is visible after agarose gel electrophoresis and ethidium bromide staining.
- Function testing by two-step RT-PCR using the LightCycler® 2.0 Instrument and the LightCycler® 480 Instrument:
  - The kit is function tested using the supplied control. The control RNA (total RNA fraction from the immortalized K-562 cell line) is reverse transcribed with 10 U Transcriptor Reverse Transcriptase in a final reaction volume of 20 µl, then the reaction is incubated for 30 minutes at 55°C. Both hexamer primers and the anchored-oligo(dT)$_{18}$ primer are tested. In subsequent quantitative PCRs, using the LightCycler® 2.0 Instrument and the LightCycler® 480 Instrument, PCR reactions are performed with the PBGD control primer mix and Control RNA. The resulting curves must have defined crossing points.

# 6. Supplementary Information

## 6.1 Conventions

**Text Conventions** To make information consistent and memorable, the following text conventions are used in this Instruction Manual:

| Text Convention | Usage |
|---|---|
| Numbered stages labeled ①, ②, etc. | Stages in a process that usually occur in the order listed |
| Numbered instructions labeled ❶, ❷, etc. | Steps in a procedure that must be performed in the order listed |
| Asterisk * | Denotes a product available from Roche Applied Science. |

**Symbols** In this Instruction Manual, the following symbols are used to highlight important information:

| Symbol | Description |
|---|---|
|  | Information Note:<br>Additional information about the current topic or procedure. |
|  | Important Note:<br>Information critical to the success of the procedure or use of the product. |

## 6.2 Changes to Previous Version

- Completely revised layout.
- Two new pack sizes are introduced for this kit, now available are **Cat. No. 04 896 866 001** for 100 reactions and **Cat. No. 04 897 030 001** for 200 reactions.

## 6.3 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, www.roche-applied-science.com, and our Special Interest Sites including:

- Amplification – Innovative tools for PCR:
  http://www.roche-applied-science.com/pcr
- Nucleic Acid Isolation and Purification:
  http://www.roche-applied-science.com/napure
- Real-time PCR Systems (LightCycler® Carousel-Based System, LightCycler® 480 System, and Universal ProbeLibrary): http://www.lightcycler.com
- Automated Sample Preparation (MagNA Lyser Instrument, MagNA Pure Compact System, and MagNA Pure LC System): http://www.magnapure.com

| Product | Pack size | Cat. No. |
|---|---|---|
| Transcriptor Reverse Transcriptase | 250 U (25 react.)<br>500 U (50 react.)<br>2.000 U (200 react.) | 03 531 317 001<br>03 531 295 001<br>03 531 287 001 |
| Reverse Transcriptase M-MuLV | 500 U | 11 062 603 001 |
| Titan One Tube RT-PCR Kit<br>Titan One Tube RT-PCR System | 50 tests<br>25 react.<br>100 react. | 11 939 823 001<br>11 888 382 001<br>11 855 476 001 |
| 5'/3' RACE Kit, 2nd generation | 10 react. | 03 353 621 001 |
| FastStart Taq DNA Polymerase | 50 U<br>100 U<br>500 U<br>4 3 250 U<br>10 3 250 U<br>20 3 250 U | 12 158 264 001<br>12 032 902 001<br>12 032 929 001<br>12 032 937 001<br>12 032 945 001<br>12 032 953 001 |
| FastStart High Fidelity PCR System | 125 U<br>2 3 250 U<br>10 3 250 U | 03 553 426 001<br>03 553 400 001<br>03 553 361 001 |
| Expand Long Template PCR System | 100 U 2 3 250 U 10 3 250 U | 11 681 834 001<br>11 681 842 001<br>11 759 060 001 |
| LightCycler® FastStart DNA Master SYBR Green I | 96 react.<br>480 react. | 03 003 230 001<br>12 239 264 001 |
| LightCycler® FastStart DNA Master$^{PLUS}$ SYBR Green I | 96 reactions<br>480 reactions | 03 515 869 001<br>03 515 885 001 |
| LightCycler® FastStart DNA Master HybProbe | 96 rect.<br>480 react. | 03 003 248 001<br>12 239 272 001 |
| LightCycler® h-PBGD House-keeping Gene Set | 96 reactions | 03 146 073 001 |
| RNA, MS2 | 10 A260 units | 10 165 948 001 |
| Protector RNase Inhibitor | 2.000 U<br>10.000 U | 03 335 399 001<br>03 335 402 001 |
| PCR Nucleotide Mix | 200 µl<br>10 3 200 µl | 11 581 295 001<br>11 814 362 001 |
| PCR Nucleotide MixPLUS | 2 3100 µl | 11 888 412 001 |
| Set of Deoxy-Nucleotides, PCR Grade | 4 3 25 µmol (250 µl) | 11 969 064 001 |
| High Pure RNA Isolation Kit | 50 purifications | 11 828 665 001 |

| Product | Pack size | Cat. No. |
|---|---|---|
| High Pure RNA Tissue Kit | 50 purifications | 12 033 674 001 |
| TriPure Isolation Reagent | 50 ml<br>200 ml | 11 667 157 001<br>11 667 165 001 |
| High Pure RNA Paraffin Kit | 100 purifications | 03 270 289 001 |
| mRNA Isolation Kit | > 70 µg mRNA | 11 741 985 001 |
| mRNA Isolation Kit for Blood/Bone Marrow | 30 - 100 purifications | 11 934 333 001 |
| High Pure Viral RNA Kit | 100 purifications | 11 858 882 001 |

## 6.4 Trademarks

## Contact and Support

If you have questions or experience problems with this or any Roche Applied Science (RAS) product, please contact our Technical Support staff. Our scientists commit themselves to providing rapid and effective help.
We also want you to contact us if you have suggestions for enhancing RAS product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to RAS and the world-wide research community.

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