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Transcriptor First Strand cDNA Synthesis Kit

Version: 07
Content version: June 2017

The Transcriptor First Strand cDNA Synthesis Kit is designed for first-strand cDNA synthesis reactions up to 14 kb.

Cat. No. 04 379 012 001	1 kit 50 reactions, including 10 control reactions
Cat. No. 04 896 866 001	1 kit 100 reactions
Cat. No. 04 897 030 001	1 kit 200 reactions

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function	Catalog Number	Content
1	red	Transcriptor Reverse Transcriptase	Contains Transcriptor Reverse Transcriptase, 200 mM potassium phosphate, 2 mM dithiothreitol, 0.2% Triton X-100 (v/v), 50% glycerol (v/v), pH approximately 7.2	04379012001	1 vial, 25 µl
				04896866001	1 vial, 50 µl
				04897030001	2 vials, each 50 µl
2	colorless	Transcriptor RT Reaction Buffer 5x conc.	Contains RT Reaction Buffer, 250 mM Tris/HCl, 150 mM KCl, 40 mM $MgCl_2$, pH approximately 8.5 (+25°C)	04379012001	1 vial. 1 ml
				04896866001	1 vial, 1 ml
				04897030001	2 vials, each 1 ml
3	colorless	Protector RNase Inhibitor	Includes 20 mM Hepes-KOH, 50 mM KCl, 8mM dithiothreitol, 50% glycerol (v/v), pH approximately 7.6 (+4°C)	04379012001	1 vial , 50 µl (40 U/µl)
				04896866001	1 vial, 100 µl (40 U/µl)
				04897030001	2 vials, each 100 µl (40 U/µl)
4	yellow/ purple purple	Deoxynucleotide Mix	10 mM each dATP, dCTP, dGTP, dTTP	04379012001	1 vial, 100 µl
				04896866001	1 vial, 200 µl
				04897030001	2 vials, 200 µl
5	blue	Anchored-oligo(dT) 18 Primer		04379012001	1 vial, 100 µl (50 µM)
				04896866001	1 vial, 200 µl (50 µM)
				04897030001	2 vials, each 200 µl (50 µM)
6	blue	Random Hexamer Primer		04379012001	1 vial, 100 µl (600 µM)
				04896866001	1 vial, 200 µl (600 µM)
				04897030001	2 vials, each 200 µl (600 µM)
For Cat. No. 04896866001 and Cat. No. 04897030001					
7	colorless	Water, PCR Grade	To adjust the final volume *In Cat. No. 04896866001 and Cat. No. 04897030001 the control reagents (Vial 7 and 8) are not included. In these kits Vial 7 is Water, PCR Grade.*	04896866001	2 vials, each 1 ml
				04897030001	3 vials, each 1 ml
For Cat. No. 04379012001					

Vial / Bottle	Cap	Label	Function	Catalog Number	Content
7	green	Control RNA	Contains a stabilized solution of a total RNA fraction purified from an immortalized cell line (K562)	04379012001	1 vial , 20 µl (50ng/µl)
8	green	Control Primer Mix PBGD	Human porphobilinogen deaminase (PBGD) forward and reverse primer to amplify a 151 bp fragment	04379012001	1 vial, 40 µl, (5 µM)
9	colorless	Water, PCR Grade	To adjust the final volume *In Cat. No. 04896866 001 and Cat. No. 04897030001 the control reagents (Vial 7 and 8) are not included. In these kits Vial 7 is Water, PCR Grade.*	04379012001	1 vial, 1ml

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label.

⚠ ***Store the Control RNA (vial 7 in Cat. No. 04379012001) at −60°C or below.***

⚠ ***Avoid repeated freezing and thawing.***

Vial / Bottle	Cap	Label	Catalog Number	Storage
1	red	Transcriptor Reverse Transcriptase	all Catalog Numbers	−15 to −25°C
2	colorless	Reaction Buffer, 5x conc		
3	colorless	Protector RNase Inhibitor		
4	yellow/purple purple	Deoxynucleotide Mix		
5	blue	Anchored-oligo(dT)18 Primer		
6	blue	Random Hexamer Primer		
7	colorless	Water, PCR Grade	04896866001	
			04897030001	
7	green	Control RNA	04379012001	−60°C or below
8	green	Control Primer PBGD	04379012001	−15 to −25°C
9	colorless	Water, PCR Grade	04379012001	

1.3. Additional Equipment and Reagents Required

Standard laboratory equipment

- Nuclease free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile reaction tubes for preparing PCR mixes and dilutions
- Standard benchtop microcentrifuge
- Standard block cycler instrument

For the RT-PCR reaction

- sequence-specific PCR primers (optional)
- Template RNA
- PCR reaction vessels (thin-walled PCR tubes or plates are recommended)
- For control reactions in combination with a LightCycler® 1.5 Instrument: LightCycler® 2.0 Instrument or LightCycler®480 Instrument

1.4. 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.

The Transcriptor First Strand cDNA Synthesis Kit provides all reagents required for first-strand cDNA synthesis reactions up to 14kb on all real-time instruments and conventional thermal cyclers. Three different priming methods can be used. The enclosed anchored-oligo(dT)$_{18}$ primer is designed to bind at the beginning of the poly(A) tail and guarantees full-length cDNA synthesis.

Transcriptor Reverse Transcriptase – the core component of the kit – has RNA-directed DNA polymerase activity, DNA-dependent DNA polymerase activity, unwinding activity, and RNase H activity that digests RNA in RNA:DNA hybrids. The latter circumvents the need to perform an additional time-consuming RNase H incubation step after reverse transcription. This shortens the reaction time and reduces costs.

Thermostable Protector RNase Inhibitor is included in the kit to protect RNA from degradation at high reaction temperatures.

1.5. Preparation Time

Time needed for cDNA synthesis: 30 min

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Template RNA such as isolated total RNA, mRNA, viral RNA or in vitro transcribed RNA are possible. Use 1 µg total RNA or 10 ng poly(A)$^+$ 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 (e.g., lysis, isolation) by gel electrophoresis (ethidium bromide staining) to ensure that the sample is RNase-free.
- Remember that RNases can also be present on contaminated glassware.

ⓘ *To prepare total RNA or mRNA, we recommend using Roche Life Science reagents. For a selection of products which produce high quality intact RNA templates suitable for RT-PCR, please refer to our Special Interest Site on Manual Nucleic Acid Isolation and Purification at www.roche-life-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.*

Control Reactions

The control reaction which is provided in Cat.No. 04379012001 includes 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.

Primers

Depending on the type of analysis, to which the cDNA is to be subjected, use one of three different priming methods described below:
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 to 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.
- Binds the very beginning of the poly(A) tail
- Prevents priming from internal sites of the poly(A) tail
- Generates full-length cDNA
- Preferred priming method for most two-step RT-PCR
- Available as part of the Transcriptor First Strand cDNA Synthesis Kit

Random hexamer primer:

- In general, reverse transcribing 4 µg total RNA using random hexamers at a final primer concentration of 60 µM is sufficient. Increasing the concentration of hexamers for the transcription of 5 µg RNA may increase yield of small PCR products (< 500 bp), but may also decrease the yield of longer PCR products and full-length transcripts. Note that random hexamer priming is the mostly used non-specific priming method; specificity is only obtained using the types of PCR primers described below.
- Binds many sites throughout the length of an RNA
- Provides uniform representation of all RNA sequences in mRNA
- Can prime cDNA transcription from RNAs that do not carry a poly(A)+ tail
- The ratio of random primers to RNA in the RT reaction determines the average length ofcDNAs generated. Example: A high ratio will generate relatively short cDNAs, increasingthe chance of transcribing the entire target sequence. Short cDNA transcripts may help to overcome difficulties caused by RNA secondary structures.

Sequence-specific primer:
- The use of gene-specific primers (recommended final concentration is 2 µM) is the most specific priming method, but this method can sometimes fail to prime cDNA even when the same primers are successful in PCR. If gene-specific priming fails in RT-PCR, repeat first-strand synthesis using anchored-oligo(dT)$_{18}$ primers.
  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 shall not be self-complementary.
- Binds only sequences that are exactly complementary to the primer sequence
- Selects for a particular RNA
- Greatly increases the specificity of the RT-PCR

Mg^{2+} Concentration

The final $MgCl_2$ concentration in the reverse transcription reaction is 8 mM. Therefore, each microliter of cDNA contributes 8 nmol $MgCl_2$ to the resulting PCR mix. Optimize the $MgCl_2$ concentration of the PCR, if necessary. Each µl of the 20 ml cDNA reaction contributes 0.4 mM $MgCl_2$ to the following PCR reaction. Optimize the $MgCl_2$ concentration of the PCR reaction if necessary.

General Considerations

SPECIAL PRECAUTIONS 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. Make all solutions using DEPC - treated water.
- Keep all required reagents 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 −60°C or below.

2.2. Protocols

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 using only one type 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 may be reduced compared to single anchored-oligo(dT)$_{18}$ priming .

Depending on which type of primer system you 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.

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

Magnesium concentration
The final $MgCl_2$ concentration in the reverse transcription reaction is 8 mM. Therefore, each microliter of cDNA contributes 8 nmol $MgCl_2$ to the resulting PCR mix. Optimize the $MgCl_2$ concentration of the PCR, if necessary.

RNase H activity
Transcriptor Reverse Transcriptase has RNase H activity. RNase H digests the RNA template after cDNA synthesis, allowing PCR primers to more easily bind cDNA, which can increase PCR sensitivity.

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.

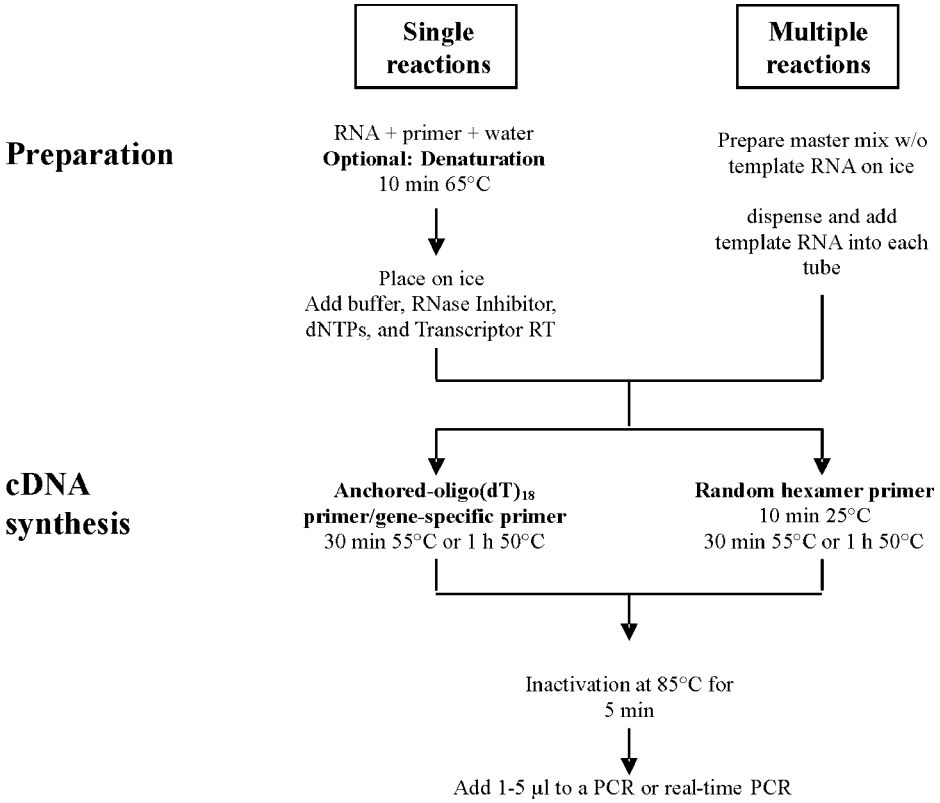

Fig. 1: Overview of cDNA synthesis procedures in single and multiple reactions.

1. Thaw the components listed below and place them on ice.

2. Briefly centrifuge all reagents before setting up the reactions.

3. Set up the reaction components in a nuclease free microcentrifuge tube placed on ice.

Reagent	Volume	Final conc.
total RNA or poly (A)+mRNA	variable	1µg total RNA *or 10 ng poly A)+mRNA
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	13 µl	

*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.*

4. Mix the reagents and centrifuge briefly to collect the sample at the bottom of the tube.

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

6. Immediately cool the tube on ice.

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

There is also the possibility to prepare master mix for multiple reactions.

Reagent	Volume	Final conc.
Transcriptor Reverse Transcriptase Reaction Buffer, 5x conc. (Vial 2)	4 µl	1x 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, 20U/µl (Vial 1)	0.5 µl	10 U
Total	20 µl	

8. Mix the reagents

⚠ ***Do not vortex!***
Centrifuge briefly to collect the sample at the bottom of the tube.

9. Place the tube in a thermal block cycler with a heated lid (to minimize evaporation).

⑩ 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/ml (Vial 5) **OR** sequence-specific primer	Up to 4 kb	30 min at 55°C
	>4 kb	60 min at 50°C
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

⑪ Inactivate Transcriptor Reverse Transcriptase by heating to 85° for 5 min, chill 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.

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 ml 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.

1. Thaw the components listed below and place them on ice.

2. Briefly centrifuge all reagents before setting up the reactions.

3. Set up the reaction components in a nuclease free microcentrifuge tube placed on ice.
Template-Primer Mix (for 1 reaction)

Reagent	Volume	Final conc.
total RNA or poly (A)+mRNA	variable	1µg total RNA *or 10 ng poly (A)+mRNA
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	13 µl	

*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.*

4. **Optional Step:**
Denature the template-primer mixture by heating the tube for 10 min at +65°C in a block cycler with a heated lid (to minimize evaporation).

5. Immediately cool the tube on ice.

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

Reagent	Volume	Final conc.
Transcriptor Reverse Transcriptase Reaction Buffer, 5x conc. (Vial 2)	4 µl	1x 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, 20U/µl (Vial 1)	0.5 µl	10 U
Total	20 µl	

7. Mix the reagents

⚠ ***Do not vortex!***
Centrifuge briefly to collect the sample at the bottom of the tube.

8. Place the tube in a thermal block cycler with a heated lid (to minimize evaporation).

9 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/ml (Vial 5) 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°Cfollowed by 60 min at 50°C

10 Inactivate Transcriptor Reverse Transcriptase by heating to 85° for 5 min, chill 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.

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 ml 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).

Control Reaction

cDNA Synthesis

The control reaction which is provided in Cat. No. 04379012001 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 before starting the procedure.

1 Thaw the components listed below and place them on ice.

2 Vortex briefly and centrifuge all reagents before setting up the reactions.

3 Set up the control reaction in a nuclease free microcentrifuge tube placed on ice:
Template-Primer Mix (for 1 reaction)

Reagent	Volume	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 (Vials 7 or 9)	10 µl	to make total volume = 13 µl
Total	13 µl	

4 Add the following components:

Reagent	Volume	Final conc.
Transcriptor Reverse Transcriptase Reaction Buffer, 5x conc. (Vial 2)	4 µl	1x 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, 20U/µl (Vial 1)	0.5 µl	10 U
Total	20 µl	

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

6 Incubate 30 min at 55°C.

7 Inactivate Transcriptor Reverse Transcriptase by heating to 85° for 5 min,chill 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.

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 package inserts of FastStart Taq DNA Polymerase, LightCycler® FastStart DNA Master SYBR Green I, or LightCycler® 480 SYBR Green I Master.

*available from Roche Life 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.

1 Thaw the components listed below and place them on ice.

2 Vortex briefly and centrifuge all reagents before setting up the reactions.

3 Set up the control reaction in a thin-walled nuclease free PCR tube placed on ice:

Reagent	Volume	Final conc.
FastStart buffer with 20 mM $MgCl_2$(10x)	5 µl	1x
PCR Nucleotide Mix *(10mM)	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 Polymerase* (5U/µl)	0.4 µl	2 U
Water, PCR Grade	36.6 µl	
Total	50 µl	

RUN PCR in a block cycler

Step	Action			
Sample loading	Overlay the reaction with 30 µl mineral oil, if required by the type of block cycler used.			
Initial denaturation	94°C for 5 min			
Standard PCR Profile	Setup	Temp.	Time (s)	Cycles
	Denaturation	94°C	10	35
	Annealing	50°C	20	
	Elongation	72°C	30	
	Final Elong.	72°C	7 min	1
	Cooling	4 °C		
Analyze samples	Load 15 µl on a 3% agarose gel containing Ethidium bromide. The PCR product has a size of 151 bp.			

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.

1. Thaw the components listed below and place them on ice.

2. Vortex briefly and centrifuge all reagents before setting up the reactions.

3. Set up 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® Capillaries*. :

Reagent	Volume	Final conc.
LightCycler® FastStart DNA Master SYBR Green I reaction mix (10x)	2 µl	1x
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	18 µl	

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

5. – 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.

6. Transfer the capillaries into the LightCycler® Sample Carousel.

Run the following LightCycler® experimental protocol:

LightCycler® Software Version 4.1				
Programs				
Setup	Setting			
Default Channel	Fluorescence Channel			
Seek Temperature	30°C			
Max Seek Pos.	Enter the total number of sample positions for which the instrument should look.			
Instrument Type	"6 Ch." for LightCycler® 2.0 Instrument or "3 Ch." for LightCycler® 1.5 Instrument			
Capillary Size	Select "20 µl" as the capillary size for the experiment.			
Programs				
Program Name	Cycles	Analysis Mode		
Initial Denaturation	1	None		
Amplification	45	Quantification		
Melting Curve	1	Melting Curve		
Cooling	1	None		
Temperature Targets				
	Target [°C]	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisition Mode [per °C]
Initial Denaturation	95	00:00:600	20	None
Amplification	95	00:00:10	20	None
	60	00:00:10	20	None
	72	00:00:10	20	Single
Melting Curve	95	00:00:01	20	None
	55	00:00:30	20	None
	95	00:00:00	0.05	Continuous
Cooling	40	00:00:30	20	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.

1. Thaw the components listed below and place them on ice.

2. Vortex briefly and centrifuge all reagents before setting up the reactions.

3. Set up the reaction mixture for both reactions (control reaction and negative control).

Reagent	Volume	Final conc.
LightCycler® 480 SYBR Green I Master (2x)	10 µl	1x
Control Primer Mix PBGD (5 µM)	2 µl	0.5 µM
Water, PCR Grade	6 µl	
Total	18 µl	

4 –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.

5 – Place the Multiwell Plate in the centrifuge and balance it with a suitable counterweight (e.g. another Multiwell Plate).
– Centrifuge at 1,500 x g for 2 min (3,000 rpm in a standard swing-bucket centrifuge containing a rotor for Multiwell Plates with suitable adaptors).

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

Setup					
Block Type			Reaction Volume [µl]		
96 (384)			20 (20)		
Detection Format		Excitation Filter		Emission Filter	
xxx		xxx		xxx	
xxx		xxx		xxx	
For new customized detection formats, set for all selected filters in the “Selected Filter Combination List” (under Tools), the following values:					
Melting Factor		1			
Quantification Factor		10			
Integration Time		2			
Programs					
Program Name		Cycles		Analysis Mode	
Initial Denaturation		1		None	
Amplification		45		Quantification	
Melting Curve		1		Melting Curve	
Cooling		1		None	
Temperature Targets					
	Target [°C]	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisitions [per °C]
Initial Denaturation	95	None	00:00:600	4.4	–
Amplification	95	None	00:00:10	4.4	–
	60	None	00:00:15	2.2	–
	72	Single	00:00:15	4.4	–
Melting Curve	95	None	00:00:05	4.4	–
	55	None	00:00:30	2.2	–
	95	Continuous	–	–	2 Acq. / °C
Cooling	40	None	00:00:10	1.0	–

For typical results see Figure 4 under Results

2.3. Other Parameters

Inactivation

+85°C for 5 minutes
Transcriptor Reverse Transcriptase is inactivated by incubation at +85°C for 5 minutes.

Incorporation of Modified Nucleotides

Yes
Accepts labeled nucleotides like DIG-, Biotin-, Cy3-, Cy5- or aminoallyl-dUTP.

Prevention of Carryover Contamination

DNA Contamination

Include appropriate positive and negative control reactions to exclude artifacts from DNA targets, such as residual genomic DNA contaminations from RNA preparations or contaminating DNA from previous amplifications.

3. Results

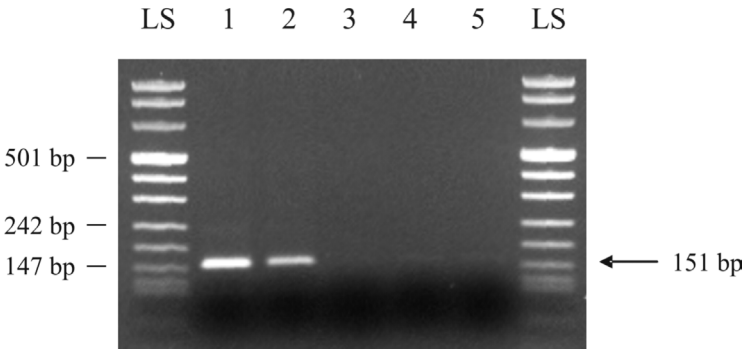

Fig. 2: Control reaction with PBGD primers using FastStart Taq DNA Polymerase for the PCR. The supplied Control RNA was used in two different dilutions.

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

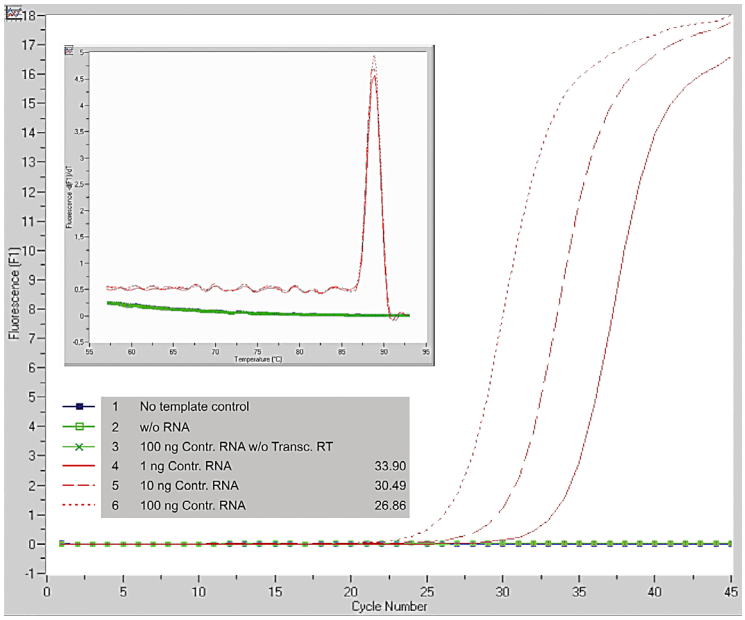

Fig. 3:
Control reaction with PBGD primers using LightCylcer® FastStart DNA Master SYBR Green I for the LightCycler® Carousel-Based Instrument reaction. The supplied Control RNA was used in different dilutions.

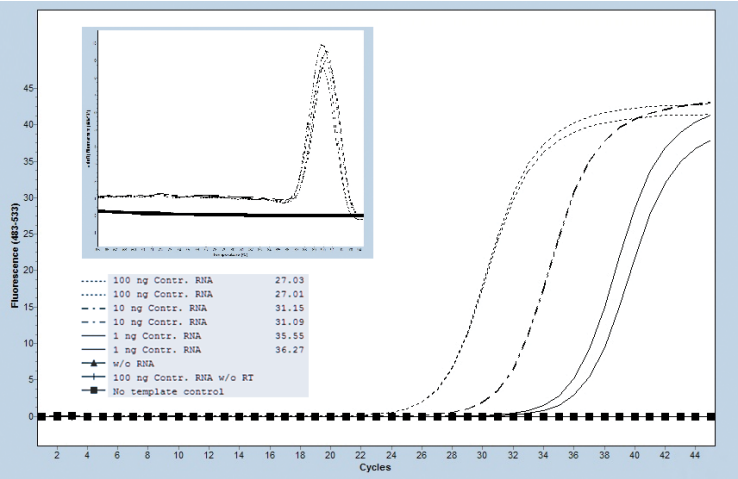

Fig. 4:
Control reaction with PBGD primers using LightCycler® 480 SYBR Green I Master on the LightCycler® 480 System. The Control RNA was used in different dilutions.

4. Troubleshooting

Observation	Possible cause	Recommendation
No PCR product or very little amount of PCR product	Insufficient amount of template RNA	Check quality and concentration of template
		Use poly(A)+ mRNA rather than total RNA as template
		Increase amount of RNA template in cDNA reaction. Use 10 ng to 5µg of total RNA or 1 to 100 ng mRNA. Add 10 µg/ml MS2 RNA to template to stabilize low concentrations to target RNA
	Template RNA degraded	Prepare fresh RNA template, being careful to prevent RNase activity
		Check RNA preparation by gel electrophoresis
		Protect RNA from ribonuclease degradation by adding Protector RNase Inhibitor to the cDNA reaction. Inhibitor concentrations up to 60 U will not interfere with RT-PCR
	Too much template RNA	A too high amount of template RNA may affect/inhibit performance of RT reaction; decrease amount of RNA template
	RT-PCR Inhibitors are present in the RNA	Make sure that the RNA is free of RT-PCR inhibitors, such as by using Roche High Pure or MagNA Pure Kits for RNA purification and isolation
	Reaction not optimized	
		Both primers should have similar melting temperatures. Both primers should be present in the reaction at the same concentration. Try various primer concentrations (between 0.1 and 0.6 mM for each primer).
		Synthesize the cDNA for 30 min at a temperature between +42°and +60°C. Prolonged incubation at lower temperatures will increase the yield of full-length product. Use 60 min incubation time.

Observation	Possible cause	Recommendation
No PCR product or very little amount of PCR product	Enzyme concentration too high or too low	Do not use more than 10 U Transcriptor Reverse Transcrptase to transcribe 1 µg total RNA template in a 20 µl cDNA synthesis reaction
		For > 5 µg total RNA, increase reaction volume and enzyme amount proportionally.
		For low template concentrations, use less reverse transcriptase
	Template secondary structure prevented effective first strand cDNA synthesis	Raise temperature for reverse transcription reaction up to 65°C or use Transcriptor Reverse Transcriptase to reverse transcribe at temperature as high as +65 °C. Denature the template-primer mixture for 5 to 10 min at +65 °C before adding the reverse transcriptase. After the denaturation step immediately cool the tubes on ice to avoid RNA renaturation.
	Template secondary structure inhibits effective formation of full-length products	If GC content of RNA is high (>60%), increase denaturation temperature or denaturation time in PCR cycles.
	Incubation temperature too high	For higher reverse transcription reaction temperatures, primers with appropriate melting temperatures must be used. The annealing temperature in PCR depends on the melting temperature of the respective primer pair. Use an appropriate computer program to calculate the optimal temperature for the primers used. The recommended annealing temperature is the melting temperature of the primers or 2°C below.
		Perform the RT reaction (for templates up to 4 kb) for 30 min at a temperature between 42°C and 65°C. For transcripts >4 kb, perform the reaction at a temperature between 42°C and 60°C for 1 h. Prolonged incubation at lower temperatures will increase the yield of full-length product.
No PCR product or very little amount of PCR product	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 these Instructions for Use 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.
	Wrong genespecific primer	Try another gene-specific primer or switch to an oligo(dT) primer [e.g., the anchored-oligo$(dT)_{18}$ primer included in theTranscriptor First Strand cDNA Synthesis Kit]. Make sure that the gene-specific primer is able to bind to the mRNA (antisense direction).
	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.

Observation	Possible cause	Recommendation
Background smear	Secondary amplification product(s)	Check reagent concentrations and cycling conditions:
		Optimize temperature of cDNA synthesis step.
		Optimize primer concentration.
		Decrease number of cycles.
		Check and perhaps decrease concentration of template.
		Optimize $MgCl_2$ concentration of the PCR reaction and for each template and primer combination The final $MgCl_2$ concetnration in the reverse transcription is 8 mM. Therefore, each µl of the cDNA contributes 0.16 mM $MgCl_2$ to the reaction.
Nonspecific product bands	Annealing temperature too low	Increase annealing temperature during PCR to increase specificity of amplification.
	Primer-dimers formed	Design primers without complementary sequences at the 3' ends.
		Make sure a denaturation step is included at the end of the cDNA synthesis reaction (5 min at +85° C)
	Contaminating DNA in sample	Perform a control without reverse transcription step.
		Design primers that anneal to sequence in exons on both sides of an intron or at the exon/exon boundary of the mRNA to differentiate between amplified cDNA and potential contaminating DNA.

5. Additional Information on this Product

5.1. Test Principle

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, significantly shortening 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 sensi-tivity 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 high thermostability, Transcriptor Reverse Transcriptase is recommended for GC-rich templates with high secondary structure without the need to include 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 5'-ends of long mRNAs can be 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 have a poly(A) tail.

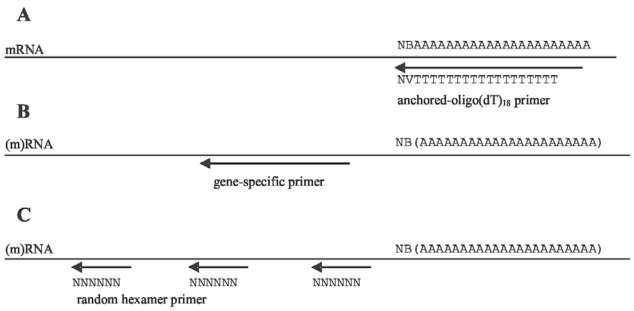

Fig. 5:
Overview of first strand cDNA synthesis using different priming methods. A, anchored-oligo(dT)18 primer; B, sequence-specific primer; C, random hexamer primer; V = A, C, or G; B = C, G, or T; N = A, C, G, or T

5.2. References

- - Protector RNase Inhibitor Enhance Protection of RNA Against Degradation (2002) *Biochemica* , 29-
- - Increase the Power and Sensitivity for Your cDNA Synthesis with the New Transcriptor Reverse Transcriptase (2003) *Biochemica* , 17-19
- Blackburn P - Ribonuclease inhibitor from human placenta: rapid purification and assay (1979) *Journal of biological chemistry* **24**, 12484-12487
- Brooke-Powell ET, Mandal TN, Ajioka JW - Use of Transcriptor Reverse Transcriptase in Microarray Analysis (2004) *Biochemica* , 27-30
- Sambrook J, Fritsch EF, Maniatis T - Molecular Cloning: A Laboratory Manual (2nd Edition) (1989) , -

5.3. Quality Control

Each lot of the kit is function tested using RT-PCR on a conventional thermal cycler, as well as the LightCycler® 2.0 Instrument. In addition, Transcriptor Reverse Transcriptase, Protector RNase Inhibitor, and the other kit components are tested independently for the absence of contamination, according to the current Quality Control procedures.

Function tested by two-step RT-PCR using a conventional thermal cycler: Transcriptor Reverse Transcriptase is function tested using 2 µg of human skeletal muscle total RNA, 10 U Transcriptor Reverse Transcriptase, and 50 pmol anchored-oligo(dT)$_{18}$ primer in a reaction volume of 20 µl. The reaction is incubated for 1 hour at +50°C. In a subsequent PCR, 5 µl cDNA template is used in a total volume of 50 µl with the Expand Long Template PCR System to amplify a 10 kb dystrophin fragment. After 30 PCR cycles, the 10 kb fragment must be clearly visible after agarose-gel electrophoresis and ethidium bromide staining.

Function tested by two-step RT-PCR using the LightCycler® 2.0 Instrument and 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; 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 LightCycler® 480 Instrument, 5 µl of the cDNA reaction is incubated with the PBGD control primer mix and the LightCycler® FastStart DNA Master SYBR Green I or the LightCycler® 480 SYBR Green I Master. Resulting curves must have specified crossing points and fluorescence intensities.

5.4. Other Parameters

RNase H Activity

The degradation of the original RNA is crucial for cDNA quality in the subsequent PCR. Using GC-rich templates for RT-PCR can lead to RNA template hybridization to newly synthesized cDNA, reducing sensitivity and specificity. It is therefore important to use reverse transcriptase enzymes with an endogenous RNase H activity. Transcriptor Reverse Transcriptase has endogenous RNase H activity that efficiently digests RNA in RNA:DNA hybrids after cDNA synthesis, allowing PCR primers to more easily bind cDNA, which can increase PCR sensitivity.

Temperature Optimum

+42 to +65°C

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Layout changes
Editorial changes

6.3. Trademarks

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6.5. Regulatory Disclaimer

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6.6. Safety Data Sheet

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6.7. Contact and Support

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