4BB™ SunScript® One Step RT-PCR Kit

HANDBOOK

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ORDERING INFORMATION

PRODUCT	SIZE	CAT. NO.
4BB™ SunScript® One Step RT-PCR Kit	25 reactions	430025
4BB™ SunScript® One Step RT-PCR Kit	100 reactions	430100

KIT CONTENTS

DESCRIPTION	CAP COLOR	25 REACTIONS	100 REACTIONS
Enzyme Mix	Orange	1 x 25 µl	100 µl
2x Reaction Buffer	Yellow	625 µl	2 vials / 1250 µl
H_2O	Blue	500 µl	2 vials / 1000 µl

SHIPPING AND STORAGE

This product is shipped in dry ice. Upon receipt, it should be stored immediately at -20°C in a non-frost-free (constant-temperature) freezer. If stored correctly, the product can be kept for at least six months after shipping without displaying any reduction in performance. For longer periods of time, store the kit at -80°C.

HANDLING

Always wear gloves when working with RNA to avoid contaminations from human skin. Change them frequently, especially after touching skin, surfaces, etc. Use RNase free materials and reagents. Glassware should be heat-treated (250°C O/N). In doubt, rinse containers with 0.1 N NaOH/1 mM EDTA and then DEPC-treated water. Solutions should be treated by adding DEPC to 0.05%, incubating overnight and autoclaving. Design an area in the laboratory where to work exclusively with RNA and use a separate set of pipets only for RNA work.

All chemicals should be considered as potentially hazardous. This material may contain substances or activities that are harmful to human health. It should not be ingested, inhaled, or brought into contact with skin and handled with appropriate care in accordance with the principles of good laboratory practice. In case of contact with skin wash immediately with water. For more specific information please consult the Material Safety Data Sheets (MSDS) available online at www.4basebio.com.

QUALITY CONTROL

Each batch of 4BB™ SunScript® One Step RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality. Enzymes used in the kit have been tested separately to ensure adherence to specifications.

REAGENTS AND EQUIPMENT TO BE SUPPLIED BY THE USER

- Sterile nuclease-free tubes, pipettes and pipette tips.
- Microcentrifuge
- Real time PCR instrument
- Vortexer

DESCRIPTION

4BB™ SunScript® One Step RT-PCR Kit is an easy and reliable system designed for fast, specific and sensitive end point RT-PCR reactions. The kit contains all the components needed to perform both reverse transcription and PCR amplification in the same single tube by using gene specific primers, in a "one step" reaction. This minimizes contaminations and constitutes a convenient system to process multiple samples.

The system uses an Enzyme MIX containing 4BB™ SunScript® Reverse Transcriptase RNaseH-, a high quality HotStart Taq Polymerase, and RNase inhibitor for a robust and effective amplification, which enables working from starting total RNA amounts of 1µg to picograms.

4BB™ SunScript® Reverse Transcriptase RNaseH- is an engineered version of the well characterized HIV-1 RT with increased thermostability and absence of RNaseH activity. It minimizes RNA degradation for higher cDNA yields and makes it posible to perform the reverse transcription step at temperatures up to 85ºC if needed (although the recommended temperature for the RT reaction is 60ºC for most samples).

These features make this kit the best choice for amplifying difficult RNAs, with high degree of secondary structure or high GC content. The reverse transcription reaction can be effectively achieved, while Taq Polymerase remains inactive during this step due to blocking antibodies. The inactivation/activation step at 95ºC provides an automatic "hot start" for the Taq Polymerase to amplify the reverse transcribed cDNA.

The 2x Reaction Buffer contains a proprietary formulation which has been optimized for both the reverse transcription and the PCR reaction, including dNTPs, MgCl2 and stabilizers (to provide a final concentration of 250 nM for each dNTP and 3.5 nM MgCl2 in the reaction).

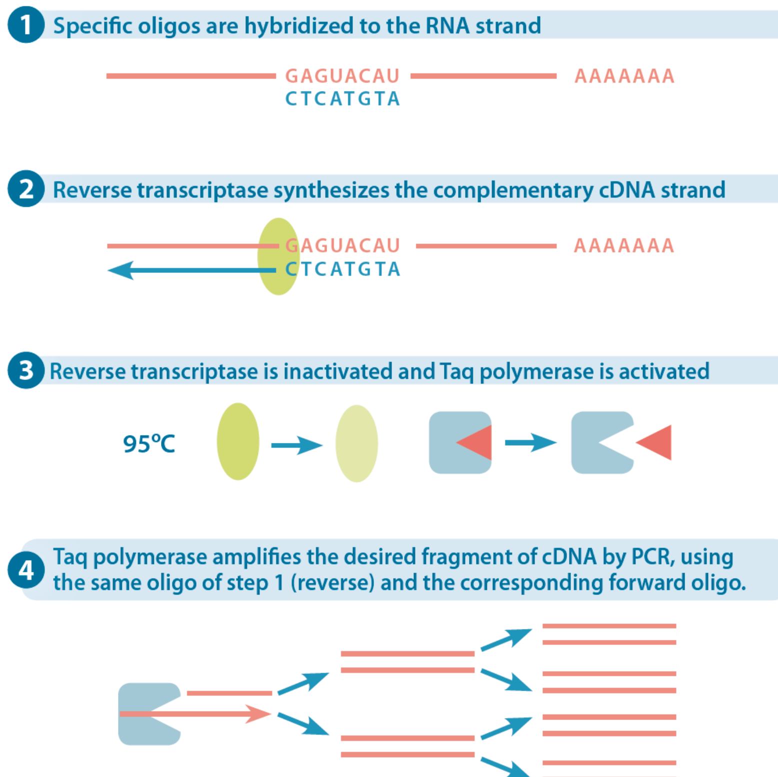

Figure 1. Overview over the One-Step RT-PCR reaction.

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PROTOCOL

RT-PCR without sample denaturation: (standard samples):

IMPORTANT: The complete reaction setup must be performed ON ICE.

1. Thaw the kit components, and briefly centrifuge. Keep on ice.
2. For each sample, add the following components into a sterile, nuclease-free tube placed on ice:

COMPONENT	VOLUME / REACTION
Template RNA (10 pg - 1µg)	X µl
2x reaction buffer	25 µl
Sense primer (10 µM)	2.5 µl
Anti-sense primer (10 µM)	2.5 µl
Enzyme MIX	1 µl
H_2O (blue)	to a final volume of 50 µl

For multiple samples is convenient to pipet first the template RNA in each reaction tube, and then prepare a Master Mix by adding appropriate multiples of the rest of the components. Pipet then the corresponding amount of Master Mix to each reaction tube.

3. Gently mix and make sure that all the components are at the bottom of the tube. Centrifuge briefly if needed. Keep on ice.
4. Go to the “ Thermal Cycling parameters” section.

RT-PCR with sample denaturation: (for RNA/primer with high GC content and/or high degree of secondary structure)

IMPORTANT: The complete reaction setup must be performed ON ICE.

1. Thaw the kit components, and briefly centrifuge. Keep on ice.
2. For each sample, add the following components into a sterile, nuclease-free tube placed on ice:

COMPONENT	VOLUME / REACTION
Template RNA (10 pg - 1µg)	X µl
Sense primer (10 µM)	2.5 µl
Anti-sense primer (10 µM)	2.5 µl
H_2O (blue)	to a final volume of 24 µl

For multiple samples it is convenient to first pipet the template RNA into each reaction tube, and then prepare a Master Mix by adding appropriate multiples of the rest of the components. Pipet then the corresponding amount of Master Mix into each reaction tube.

3. Gently mix and make sure that all the components are at the bottom of the tube. Centrifuge briefly if needed. Keep on ice.
4. Denaturation and primer annealing: incubate the samples at 70ºC for 5 min and place on ice for 5 min.

5. For each sample, add the following components:

COMPONENT	VOLUME / REACTION
2x Reaction Buffer	25 µl
Enzyme MIX	1 µl

For multiple samples it is convenient to prepare a Master Mix by adding appropriate multiples of the components. Pipet then the corresponding amount of Master Mix into each reaction tube.

6. Gently mix and make sure that all the components are at the bottom of the tube. Centrifuge briefly if needed. Keep on ice.

7. Go to the “ Thermal Cycling parameters” section.

Thermal Cycling parameters

CYCLING STEP	TIME / TEMPERATURE	CYCLES
1. cDNA synthesis	15 min 60ºC*	1X
2. RT inactivation / Taq activation	10 min 95ºC	1X
3. Denaturation	30 sec 94ºC	
4. Annealing	30 sec Tann	35X
5. Extension	1 min 72ºC**	
6. Final extension	7 min 72ºC	1X

* Although 60ºC is the recommended temperature for most reactions, this step can be adjusted up to 85ºC if necessary. As a guide, the best yields are obtained in the range of 50-70ºC for most samples.
** For PCR products longer than 1 kb, increase the elongation time 1 min per kb.

Run your qPCR instrument using settings for SYBR Green I or FAM detection (intercalated SYBR Green I can be detected with an excitation wavelength ~450 nm, emission wavelength~ 520 nm).

Place the reaction tubes in the preheated thermal cycler programmed as described above, and run the programme.

Analyze the results on a 1-2% agarose gel.

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TROUBLESHOOTING GUIDE

PROBLEM	SOLUTION
Reduced yield or no amplification product	**The RNA is damaged or degraded** Check RNA integrity by agarose gel electrophoresis, or capillary gel electrophoresis (Agilent Bioanalyzer etc.). Prepare fresh RNA template taking care to prevent RNase activity. **Insuficient amount of RNA template** Increase the amount of template RNA. **Primer design is not optimal** Redesign the primers with a primer design software and test them in a control reaction, or use validated primers. Use only gene specific primers. Do not use random primers or Oligo(dT) primers. **Primer concentration is not optimal or primers are degraded** Use primer concentrations between 400 nM-1 μM. Avoid using old dilutions of primers and repeated freeze/thaw cycles. **Reverse transcription temperature is not optimal** For most reactions 60°C is suitable for the reverse transcription step, but if this does not work well for your template test temperatures in a range of 50°-70°C in small increments. **Annealing temperature too high** Decrease annealing temperature in 2°C increments. **Number of cycles too low** Increase number of cycles. **RT-PCR product is too long** The best results are obtained for RT-PCR products from 150-500 bp. For longer products, increase the PCR elongation time 1 min per kb. Note that the limit for robust amplifications is 2 kb. **Missing reagent or error in protocol setup** Check concentrations, storage conditions, volumes and dilutions of all the reagents. Perform the reaction again and include a positive control. **Template RNA has a high degree of secondary structure or GC content** Follow the protocol "RT-PCR with sample denaturation". Test temperatures for the RT step from 50ºC to 85ºC in 5ºC increments.
Non specific amplification products	**DNA contamination in the sample** Perform a control not including the reverse transcription step. Design primers that anneal in exons flanking an intron or at the exon-exon boundary of the mRNA. **Reactions set up at room temperature** Always set up RT-qPCR reactions on ice to prevent degradation of RNA and/or undefined start of the reaction. **Primer dimer formation** Avoid complementary sequences at the 3' end of the primers. **Primers are not specific enough for the target** The primer sequence is not specific to the target, or several cDNA products can be obtained from alternatively spliced genes. Check this and redesign the primers accordingly. **Annealing temperature is too low** Increase annealing temperature in 2°C increments.

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