BIOTOOLS

BIOTOOLS B&M LABS.S.A.

Manufactured by:

BIOTOOLS B&M Labs, S.A.
Valle de Tobalina - 52 - Nave 39
28021 Madrid

Tel. 91 710 00 74
Fax 91 505 31 18
e-mail: info@biotools.eu
www.biotools.eu

SCRIPTOOLS-QUANTIMIX SYG KIT

One step quantitative RT-PCR with SYBR® Green I

Package Insert

Cat. No. 10.621/2/3

PLEASE READ THE INSTRUCTIONS FOR USE THOROUGHLY BEFORE USING THE KIT, ESPECIALLY IF YOU ARE NOT FAMILIAR WITH THE PROTOCOL.

SCRIPTOOLS-QUANTIMIX SYG KIT

Research Use Only (RUO)
Not for use in diagnosis procedures

Some of the applications which may be performed with this product may be in certain countries under an applicable patent. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application. Biotools does not encourage the unlicensed use of patented applications.

PLEASE CHECK INTEGRITY OF THE KIT AND REAGENTS BEFORE USE. DETERIORATED KITS MAY CAUSE EQUIVOCAL RESULTS.

1. INTENDED USE

SCRIPTOOLS-QUANTIMIX SYG Kit allows the quantification by real time PCR of any RNA target using SYBR® Green I. The Kit provides a convenient and fast procedure, one step only, for synthesizing cDNA and DNA amplification in real time. All the reagents necessary for both reactions are added in one tube at the same time which confers a rapid and easy-to-handle format saving manipulation time and simplifying its use, without compromising the efficiency and sensitivity of the kit.

The Kit can be used in a wide variety of Real Time amplification equipments, including air heating amplification systems or block heating amplification systems.

The employed methods for RNA purification can be either phenol-based or resin-based, provided they yield enough amounts of pure RNA and guaranteeing absence of PCR inhibitors. When using purification methods based on silica matrix, it is most important checking complete absence of silica particles in the sample since it inhibits amplification and fluorescence reading.

2. FEATURES AND PRINCIPLES OF THE PROCEDURE

SCRIPTOOLS-QUANTIMIX SYG Kit has been designed to deliver maximum efficiency, precision and sensitivity of quantitative RT-PCR with SYBR® Green I. For these purpose two high-performance enzymes, a themostable reverse transcriptase working at a temperature range of 37-77ºC, and a DNA polymerase with proofreading activity, carry out the reactions. Both cDNA synthesis and PCR are performed sequentially in a single tube due to a single buffer system formulated to ensure specific primer annealing over a wide range of temperatures.

Real Time detection of amplified products is performed by monitoring the increase of fluorescence after each amplification cycle. This fluorescent signal is generated by the use of SYBR® Green I. Thus, fluorescence is proportional to the amplified DNA in each cycle. The cycle number at which an increase in the fluorescence signal is detected is inversely proportional to the initially template DNA.

One step format lowers contamination risks and reduces manipulations and consequently variability, providing high yield, sensitivity and efficiency. It is not necessary to open the tube once the reverse transcription has finished. For its use simply add to a vial containing the prepared Master Mix (Retro-Quantisyg Buffer, Enzymes, Rox Dye and $MgCl_2$) the desired primers and RNA template.

The Real Time detection of amplified products is performed by monitoring the increase of fluorescence after each amplification cycle. This fluorescent signal is generated by the incorporation of SYBR® Green into the amplified products. Thus, the amount of fluorescence is proportional to the amplified DNA in each cycle. In the exponential phase there is a correlation between the amount of product and the initial template, which is missing during the final stage of the amplification process (plateau phase). The growth rate of the reactions is increasingly reduced at the end of the exponential phase due to the limiting amount of one or several of the reaction reagents or due to inhibition by other factors.

NOTE

The amplification product obtained with the Kit is blunt-end. The Kit is not recommended for certain experiments dealing with sequences homologous to Escherichia coli.

3. KIT REAGENTS AND STORAGE CONDITIONS

The Kit contains reagents for performance of 100 rxn of 25 µl (Cat. No. 10.621), 250 rxn of 25 µl (Cat. No. 10.622) or 500 rxn of 25 µl (Cat. No. 10.623). The reagents are in liquid format. **Store Kit at -20ºC**. Do not freeze/thaw repeatedly. For frequent use, we recommend **aliquoting vial contents**.

- **SCRIPTOOLS-QUANTISYG BUFFER**:
  A single buffer mixture has been formulated to optimise reverse transcription and real time PCR amplification. The mixture includes dNTPs and SYBR® Green I at the appropriate concentration. **Store vial at –20ºC and avoid exposure of the vial content to light.** After thawing **aliquot vial content**, once the aliquot has been defrosted for its use store at 2-8ºC.
- **$MgCl_2$ SOLUTION (50 mM)**:
  Mix well before use. Initial testing at several $MgCl_2$ concentrations is advisable. **Store at –20ºC**.
- **SCRIPTOOLS-QUANTISYG ENZYMES**:
  Contains a thermostable reverse transcriptase which exhibits high affinity for ARN and presents activity at a temperature range 37-77ºC and Biotools *Pfu* DNA polymerase with proofreading activity for second strand cDNA synthesis and DNA amplification. **Store at –20ºC**.

REAGENT	Cat. No. 10.621 (100 rxn)	Cat. No. 10.622 (250 rxn)	Cat. No. 10.623 (500 rxn)
SCRIPTOOLS-QUANTISYG BUFFER	400 µl	1000 µl	2x 1000 µl
$MgCl_2$ solution (50 mM)	1.8 ml	1.8 ml	1.8 ml
SCRIPTOOLS-QUANTISYG ENZYMES	220 µl	550 µl	2x 550 µl

4. INSTRUCTIONS FOR USE

Thaw and handle Kit components, primers and template RNA on ice. Before use mix well kit components and spin in a microcentrifuge. Protect vials with* SCRIPTOOLS-QUANTISYG BUFFER *from light at all times. Keep reaction vials refrigerated until their introduction in the thermal cycler. Use of reaction mixes and vials in non-refrigerated conditions may cause a drastic decrease in sensitivity and quality of the obtained fluorescence curves. Be careful not to wet the reactions vials.

1.- Final reaction volume is 25 µl (Master Mix + Template RNA). **Prepare the Master Mix** following the Table 1 according to the number of reactions to be performed. For each round of analysis include one negative control NTC (control without RNA) and the necessary standards for the calibration curve. To ensure sufficient volume for all desired reactions prepare the master mix for several additional reactions. Mix gently and thoroughly the prepared Master Mix.

Table 1. Reaction Mixture Preparation

COMPONENT		*Volume 1 Reaction*	*Final Concentration*
MASTER MIX	RT-PCR SYBR GREEN BUFFER	3.65 µl	1 X
	$MgCl_2$ (50 mM)*	1 µl	2 mM
	SCRIPTOOLS-QUANTISYG ENZYMES	2 µl	--
	RNase-free water+	variable	--
	Primers+	variable	0.5 - 1 µM
TEMPLATE RNA	Purified RNA	variable	
TOTAL VOLUME		25 µl	

*For each experiment the user has to determine the optimal concentration of $MgCl_2$. Good results has been obtained with 2 mM

+Not included in the Kit

NOTE

When required (e.g. ABI PRISM thermal cyclers), prepare fresh dilutions of the reference dye (ROX) prior to setting up the reactions. Keep all tubes containing the reference dye protected from light at all times.

If Real Time assay are to be carried out in the glass capillary tubes of the Roche LightCycler, Biotools recommends the addition of 0.5 mg/ml (final concentration) bovine serum albumin (BSA) to the Master Mix.

2.- Dispense the appropriate volume of **Master Mix** in each reaction vial and store vials on ice. The Master Mix contains the reagents necessary for the RT-PCR except the template RNA.

Efficiency of the reaction depends on the quality of RNA template, therefore intact RNA is essential for good results. Proceed to an area separate from that used for DNA preparations. RT-PCR must start in the next minutes after adding RNA template.

3.- Add **RNA template** (maximal concentration of RNA 1 µg) to each reaction vial containing the master mix. If necessary complete up to 25 µl final reaction volume with RNase-free water. The quantity of RNA depends on the characteristics assays (primers, RNA purity, etc). Keep reaction vials on ice.

4.- Negative Controls without RNA template (NTC) should be prepared with **RNase-free water** instead of RNA. This control will indicate the existence of DNA/RNA contamination in any reagent.

5.- Close the reaction vials and gently mix them without creating bubbles but do not vortex them. Program the thermal cycler according to the instructions in Table 2 and select the desired step for reading the fluorescence. **IMPORTANT NOTE:** ***the extension step (72ºC 12-30 s) of the amplification cycling program is absolutely necessary to achieve optimal results, therefore do not miss this step in the cycling amplification program.*** Place the reaction vials in the thermal cycler and store the remainder of the Kit reagents at the appropriate temperature.

Table 2. Guide to Thermal Cycling Program

STEP	COMMENTS
RT *1 Cycle* (need to be optimised)	***Synthesis of cDNA*** 42-65ºC^{+} 30 min According to the T_m of the RT-primer
1 Cycle	***Reverse Transcriptase is inactivated*** 95ºC 2 min
PCR *30 - 40 Cycles* *(need to be optimised)*	***Second strand cDNA synthesis and real time PCR*** 97ºC 10-20 sec Denaturation Annealing Temp 10-60 sec Annealing (approximately 5ºC bellow the calculated T_m of the primers) 72ºC 45 sec Extension depends on amplicon size allowing for 100-250 bp/45 sec ***Fluorescence reading for SYBR® Green I*** *At the extension or additional step for fluorescence adquisition* XºC 15-35 sec **If primer-dimers or inespecific amplifications occurs an additional data adquisition step can be added to the program after the extension step** T_m non-specific products (or primer-dimers) < X < T_m specific amplification products
MELTING *1 Cycle*	60-95ºC Start with a 0.5 ºC/sec ramp

$^{+}$*The reverse transcriptase included exhibits high performance over a broad range of temperature 37-77ºC. For difficult RNA template the temperature of the RT can be increased accordingly to the Tm of the RT-primer.*

6.- Perform a melting curve analysis of the obtained PCR products to verify the specificity of the reaction and to identity the obtained products. Optionally the first time the experiment is performed analyse the PCR amplification products by agarose gel electrophoresis.

5. NOTES FOR OPTIMIZATION OF SOME REACTION PARAMETERS

To check the efficiency and the sensitivity of the Kit it is highly recommended to use an endogenous control reaction. In this way the user can verify that the Kit components work well and can normalyse variations in the concentration of RNA between samples.

Follow Kit recommendations in order to obtain the best results.

REVERSE TRANSCRIPTION GUIDELINES

- *It is recommended to use gene-specific primers designed with a T_m high enough to perform the RT at 42-65ºC.*
- *The enzyme exhibits a broad activity 37-77ºC, but for most samples the reverse transcription is optimised at 42-65ºC. Since the reverse transcriptase possesses high thermal stability the temperature of the RT can be increased (up to 77ºC) for difficult RNA templates according to the Tm of the RT-Primer.*
- Experimental *samples may have different RNA concentrations. The quantity of template RNA to be added in each reaction depends on its purity and the experimental system used in every case. Therefore, we recommend determine the concentration of RNA by e.g. $A_{260/280}$ measurement or empirically. In case that the extracted RNA can not be quantified we advice to add a fixed volume of the extraction mixture to the problem samples. The purpose of this recommendation is to obtain comparable quantitative results.*

REAL TIME PCR GUIDELINES

- *Design a thermal cycler program specific for the target. Optimize the annealing temperature and time by performing the amplification 5ºC bellow the calculated T_m for the primers and increase the temperature in 1ºC increments. The annealing time varies from 10-60 sec. Once all the parameters have been optimised determine empirically the number of cycles.*
- *For optimal results the size of the desired product must be between 100-250 bp, do not exceed 500 bp length. The extension time varies with the size of the amplification product for the Pfu DNA polymerase is approximately 1 min per 500 bp, for small amplicons < 250 pb allows 45 sec. Do not miss the extension step of the amplification cycling program.*
- *T_m value of the obtained products depends mainly on their composition (sequence) and employed buffer (salt concentration, existence of PCR additives e.g. betaine). The values obtained using the kit may differ from the values obtained using other commercial kits.*
- *Primer-dimers and non-specific products usually have lower melting temperature than the desired specific product.*
- *The melting step is necessary to confirm the absence of nonspecific amplifications products and primer-dimers. This step allows determining the temperature of fluorescence reading. Fluorescence data collection for a specific assay (with no primer-dimers) is at the extension step, but If primer-dimers or non-specific amplifications occurs the temperature for fluorescence reading must be > T_m non-specific products and < T_m specific amplification product. If necessary an additional data adquisition step can be added (after the extension step) to the three step cycling amplification program.*

6. TROUBLESHOOTING

PROBLEM	SUGGESTION
LITTLE OR NO PRODUCT DETECTED	Check the **starting template**. Ensure that the quality, concentration and storage of the RNA template are the optimal. If necessary make new dilutions of the template stock. The **presence of inhibitors in the template** may cause inhibition in the RT-PCR reaction. Add this starting material into an assay that is known to work. Titrate the **RNA template concentration**. If possible increase the amount of the template in the reaction. For **complex RNA template** with secondary ARN structures difficult to resolve or rich in G+C regions, increase the incubation temperature of the RT step. **Low abundance targets** or **long amplicons** may need to increase the time of the RT step up to 60 min. For optimal results the size of the amplicon should be between 100-250 bp, do not exceed 500 bp length.
	Pippeting error or missing reagent. Repeat the RT-PCR checking that all the components of the reactions are at the appropriate content.
	$MgCl_2$ concentration not optimal. Optimise the required concentration of $MgCl_2$.
	Number of amplification cycles is not sufficient. Increase the number of cycles in the PCR program.
	Extension step (72ºC) is missing. Ensure that the extension step is included in the amplification cycling. **Extension time too short**. Check the length of the amplicon and establish the time for the extension time according to the instruction in the protocol (for small amplicons < 250 pb allows 45 sec).
	Check the **annealing temperature and time** are the correct ones for the employed primers. Ensure that the annealing time and temperature is sufficient. Decrease the annealing temperature in 2ºC increments.
	Check the **primer** design and concentration. Ensure that the quality of the primers and the storage conditions are adequate. Perform a melt curve analysis or gel electrophoresis. If non-specific products are detected redesign the primers.
	Check that the **fluorescence detection** is on.
PRESENCE OF SEVERAL PEAKS IN THE DISSOCIATION CURVE	Annealing **temperature** too low. Increase the annealing temperature in 2ºC increments.
	Check the **primer design**. If necessary re-design the primers, in case this option is not possible include an additional data adquisition step (see section 5).
	Presence of **primer-dimers**. Include an additional data adquisition step (see section 5).
	Optimise **primer concentration**.
	Check the **quality of the primers**. Degraded primers may generate multiple products.
	Check the quality of the **template RNA**. The integrity and quality of the RNA template is crucial.
	Increase the incubation **temperature of the RT step**
	Contamination with **genomic DNA**. Pretreat the starting template with DNase I. Redesign your primers.
	Decrease the concentration of RNA template, enzyme and primers.
NO LINEARILTY IN RATIO Ct VALUE	The **concentration of RNA template** is too high or too low.
	Presence of **primer-dimers**. Include an additional data adquisition step (see section 5).
	Check for any **pipetting error** in the preparation of the master mix, or addition of RNA template.
FLUORESCENCE IN NO TEMPLATE CONTROL (NTC)	**Contamination of the reagents**. Perform a melt curve analysis. If the T_m of the NTC is similar to the target peak the reaction has been contaminated during reaction set-up. Repeat the assay with new reagents.
	Contamination during reaction set-up. Take appropriate safety precautions.
	Presence of **primer-dimers**. If the T_m of NTC is lower than the T_m of the target peak, primer-dimers are formed. Include an additional data adquisition step (see section 5) or redesign the primers.

7. MATERIALS REQUIRED BUT NOT PROVIDED

NOTE

For all equipment, regular maintenance and calibration is necessary. Follow manufacturer's instructions, and check working parameters regularly, especially thermal cyclers and pipettes. Maintenance and calibration of instruments allows their correct functioning, and helps to detect problems that may render an incorrect analysis result.

Pre-amplification Area (RNA/DNA Purification Areas and Reagent Preparation Area)

- Equipment, reagents and disposable material necessary for DNA purification (depending on the method, follow manufacturer's instructions).
- Timer.
- Automatic pipettes[1] (10, 20 and 200 µl), filter or positive displacement tips, RNase-free[2].
- Disposable examination gloves, powder-free.
- Sterile bidistilled water[3].
- Screw cap polypropylene tubes, 1.5 ml capacity, non siliconised, conical, sterile, RNase-free. It is recommended to use screw cap tubes, in order to avoid the potential contamination of samples and controls.
- Racks for reaction vials.
- Containers for disposal of potentially-infectious material.
- Disposable filter paper for working surface, cleaning paper for accidental spills.
- Termi-DNA-Tor[4] or equivalent, in order to remove DNA from working surfaces.

Amplification Area

- Real-time thermal cycler. For further information, contact our Technical Dpt. (info@biotools.eu).
- Laminar flow cabinet.
- Racks for reaction vials.
- Real Time amplification vials (as per manufacturer's requirements).
- Sterile bidistilled water or equivalent.
- Automatic pipettes (10, 20 and 200 µl), filter or positive displacement tips, RNase-free.
- Disposable examination gloves, powder-free.
- Containers for disposal of potentially-infectious material.
- Disposable filter paper for working surface, cleaning paper for accidental spills.
- Termi-DNA-Tor or equivalent, in order to remove DNA from working surfaces.

8. WARNINGS AND PRECAUTIONS

DNA amplification allows the amplification of minute quantities of template from a sample in an exponential manner. However, this means that foreign DNA present in the environment may also be amplified. Therefore, special laboratory practices are necessary in order to avoid false positive amplifications.

The list below contains several warnings and precautions that must be considered. For detailed information, we recommend to read the Material Safety Data Sheet (MSDS), available in our webpage (www.biotools.eu/msds.htm). Please contact our Technical Department for additional information. (info@biotools.eu).

A. Use of dedicated micropipettes in each area (sample preparation, amplification and pre-amplification) is highly recommended.
B. We recommend to use filter tips in order to avoid cross contamination. Pipettes must be regularly checked, in order to ensure that they are accurate within 3 % of stated volume. Use different micropipettes depending on the aliquoted volume.
C. Negative results may occur due to enzyme inhibition. RNA purification must proceed in such a way that enough amount of pure RNA is obtained. It is recommended to check suitability of RNA.

---

[1] Precision of automatic pipettes must be in the range of 3 % of the indicated volume. If necessary, callibrate and check regularly, following manufacturer's instructions. It is recommended to use RNase-free filter tips and positive displacement tips, in order to avoid cross contamination between samples and amplicons.
[2] It is recommended to use different sets of pipettes for each reaction step (pre-amplification, amplification), in order to avoid contaminations that may render false positive results.
[3] Available in Biotools catalogue (Cat. No. 20.033).
[4] Available in Biotools catalogue (Cat. No. 22.001/2).

D. Follow general instructions for laboratory safety (e.g. do not eat, drink or smoke in laboratory work areas, wear disposable gloves, wear clean lab coats and eye protection, wash hands thoroughly after handling specimens and test reagents, etc.).
E. Open and close reagent vials carefully. Follow temperature and light exposure instructions. After use, close vials and store at indicated temperatures.
F. Do not use a kit after its expiration date.
G. Extreme care must be taken when aliquoting the different volumes in each reaction step. Mix well after addition of each reagent, unless otherwise noted. Read instructions for use of automatic pipettes .
H. Do not pipette by mouth.
I. Packaging material included within the kit is resistant to the indicated storage conditions. Storage at different conditions can cause breakage of the material, and possible contamination of the kit reagents.
J. Plastic material included within the kit is resistant under normal conditions of use. Use of plastic material in extreme conditions may cause its breakage, and therefore, the impossibility to use the kit.
K. Laboratory workflow must be unidirectional, from pre-amplification to amplification area. Specific equipment for each working area must be used, in order to avoid cross contaminations. Equipment used for amplification must remain in this area at all times.
L. Gloves must be worn in each area and must be changed before leaving that area.
M. As with any test procedure, good laboratory technique is essential for the proper performance of this assay. Due to the high analytical sensitivity of this test, extreme care should be taken to preserve the purity of all reagents. Discard any reagents that may be suspect for their purity.
N. Do not touch or wet the vials in the detection areas. Use non talcum powder gloves.

9. STORAGE AND HANDLING INSTRUCTIONS

Upon receipt, store the different reagents under the recommended conditions (-20ºC). Use non frost-free freezers. Also, for frequent use (more than once a week), aliquot the contents of the vials in different tubes, in order to avoid multiple freeze-thaw cycles.

Do not use the kit after its expiration date. If stored under the recommended conditions, the product will maintain performance through the indicated date on the label. Do not mix reagents from other kits and/or other lots. Discard any residual amount of reagents after using the kit.

10. WARRANTY

The products are warranted to the original purchaser only to conform to the quantity and contents stated on the vial and outer labels for the duration of the stated shelf life. Biotools' obligation and the purchaser's exclusive remedy under this warranty is limited either to replacement, at Biotools' expense, of any products which shall be defective in manufacture, and which shall be returned to Biotools, transportation prepaid, or at Biotools option, refund of the purchase price. Claims for merchandise damaged in transit must be submitted to the carrier.

The product has been designed for research use only, and to be used by qualified professionals only. It is the user's responsibility to ensure that a given product is fit for a given application. Any product that does not meet the performance standards stated in the product specification sheet will be replaced at no charge. This warranty limits our liability to the replacement of the product. No other warranties of any kind, express or implied, including, without limitation, implied warranties for merchantibility or fitness for a particular purpose, are provided by Biotools. Biotools shall have no liability for any direct, indirect, consequential or incidental damages arising out of the use, the results of use or the inability to use any product.

Manufactured by:

BIOTOOLS, Biotechnological & Medical Laboratories, S.A. has been evaluated and certified to accomplish ISO 9001:2000 requirements for the following activities: Research and development of biotechnology products and manufacture of biotechnology and in vitro products.
Valle de Tobalina – 52 – Nave 39, 28021 Madrid – Spain

BIOTOOLS® is a registered mark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A.
SCRIPTOOLS-QUANTIMIX SYG KIT is a trademark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A.
SCRIPTOOLS is a registered mark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A
Termi-DNA-Tor™ is a trademark of BIOTOOLS, Biotechnological & Medical Laboratories, S.A.
ROX™ s a trademark of Applera Corporation
ABI PRISM® is a registered trademark of PE Corporation

© 2009 BIOTOOLS, Biotechnological & Medical Laboratories, S.A. All rights reserved.