CE 1023

IVD For *in Vitro* Diagnostic Use

For Professional Use Only

Toxoplasma gondii Real-TM

Handbook

Real Time PCR kit for qualitative detection of *Toxoplasma gondii*

REF P1-50FRT

50

NAME

Toxoplasma gondii Real – TM

INTENDED USE

Toxoplasma gondii Real-TM PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Toxoplasma gondii* DNA in the clinical material (white blood cells of whole peripheral blood, autopsy material, cerebrospinal fluid, amniotic fluid) by means of real-time hybridization-fluorescence detection.

⚠ The results of PCR analysis are taken into account in complex diagnostics of disease.

PRINCIPLE OF ASSAY

Toxoplasma gondii detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region by using specific primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run. **Toxoplasma gondii Real-TM** PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using chemically modified polymerase (TaqF), which is activated by heating at 95 °C for 15 min.

Toxoplasma gondii DNA detection in clinical samples includes:

(a) Total DNA extraction from white blood cells of peripheral and umbilical cord blood, biopsy and autopsy material, cerebrospinal fluid, and amniotic fluid simultaneously with the exogenous Internal Control.

(b) Multiplex real-time PCR of a DNA fragment of a nonstructural repeated gene (529 bp long) encoding *Toxoplasma gondii* protein and an artificial DNA fragment cloned into phage λ, which is used as a noncompetitive exogenous Internal Control.

Toxoplasma gondii DNA amplification is detected in the **JOE/Yellow/HEX/Cy3** channel, the noncompetitive exogenous **Internal Control** amplification is detected in the **FAM/Green** channel. The exogenous Internal Control allows monitoring the main steps of PCR analysis (DNA extraction and amplification). The main advantage of a noncompetitive exogenous Internal Control is the extension of the linear detection range and, therefore, an increase in the analytical sensitivity of the test.

MATERIALS PROVIDED

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FRT *T.gondii*	colorless clear liquid	0.6	1 tube
PCR-mix-2-FRT	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	2 tubes
Pos Control DNA *T. gondii* /STI (C+)	colorless clear liquid	0.1	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-) *	colorless clear liquid	1.2	1 tube
Internal Control STI-87 (IC) **	colorless clear liquid	1.0	1 tube

Contains reagents for 110 tests.

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *add 10 µl of Internal Control STI-87 (IC) to each sample during the DNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit.
- Transport medium.
- Disposable powder-free gloves.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers.
- Disposable polypropylene 1,5/2,0 ml tubes.
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes.
- PCR Workstation.
- Real Time Thermal cycler.
- Disposable polypropylene microtubes for PCR.
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

STORAGE INSTRUCTIONS

The components of the **Toxoplasma gondii Real-TM** PCR kit must be stored at 2–8 ºC excepting **Polymerase (TaqF)** and **PCR-mix-2-FRT** that must be stored at -16°C or below. The kit can be shipped at 2-8°C no longer than 5 days but should be stored at 2-8°C and -16°C or below immediately on receipt.

STABILITY

Toxoplasma gondii Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

The shelf life of reagents before and after the first use is the same, unless otherwise stated.

WARNINGS AND PRECAUTIONS

IVD ***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose of all specimens and unused reagents in accordance with local regulations.
6. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
7. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
8. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
9. Material Safety Data Sheets (MSDS) are available on request.
10. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
11. PCR reactions are sensitive to contamination. Measures to reduce the risk of contamination in the laboratory include physically separating the activities involved in performing PCR in compliance with good laboratory practice.
12. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the PCR and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

Sampling of biological materials for PCR-analysis, transportation, and storage are described in details in the handbook of the manufacturer. It is recommended that this handbook is read before beginning of the work.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Toxoplasma gondii Real-TM PCR kit is intended to analyze DNA extracted from:

- *Whole peripheral blood.* Blood should be collected to a tube with 6% EDTA solution at a ratio 20:1 (20 portions of blood per 1 portion of EDTA) after overnight fasting. Invert the tube several times to ensure proper mixing.

  

  Whole peripheral blood can be stored at 20-25 °C for 12 hours and at 2-8 °C for 1 day. Do not freeze the whole blood samples!

- *White blood cells.* Collect 2.5-10 ml blood samples according to standard procedures in tubes containing anticoagulant (recommended anticoagulant is EDTA). Centrifuge samples at ~1500-2000 *X* g for 10-15 min. This will separate the blood into an upper plasma layer, a lower red blood cell (RBC) layer, and a thin interface containing the WBCs, also called the buffy coat. Remove the plasma with a transfer pipet, being careful not to disturb the WBCs. Samples with exceptionally high WBC counts will have a thicker buffy coat. Use transfer pipet to carefully aspirate the exposed WBC layer in a volume of about 0.5 ml or less. Aspirate slowly, using a circular motion, to pull all the visible buffy coat material into the transfer pipet. Some contamination of the WBCs with the underlying RBCs is expected.

  

  Add 300 µl of Solution for Lysis to the tube with the obtained leukocyte sample (for **DNA/RNA-Prep** protocol).

- *Autopsy material* is obtained from the expected location of the pathogen, from the damaged tissue or from the area adjoining with the damaged tissue. Collect the samples into a 2-ml tube with 0.3 ml of transport medium. The samples can be stored at room temperature for 6 hours, at 2-8 °C for 3 days, and at the temperature from -24 to -16 °C if necessary to store it for more than 3 days.

  Transfer the sample to a porcelain mortar; add an equal volume of saline or PBS. Thoroughly homogenize the specimen with a porcelain pestle. Take a 100-µl aliquot and transfer to a sterile tube for DNA extraction. The suspension can be stored at the temperature from -24 to -16 °C.

- *Amniotic fluid* should be obtained during amniocentesis by the standard procedure and collected to a sterile Eppendorf tube. Thoroughly resuspend the obtained sample and transfer 1 ml of it to a new sterile tube. Centrifuge the tube at 8,000–9,000 g for 10 min. Carefully remove the supernatant using a filter tip and leaving 200 µl of the liquid over the pellet. Then, resuspend the pellet on vortex.

- *Cerebrospinal fluid* should be obtained by the standard procedure and collected to a sterile Eppendorf tube. The cerebrospinal fluid can be stored at room temperature for 6 hours, at 2-8 °C for 1 day, at the temperature from -24 to -16 °C for a month, and at the temperature ≤68 °C for a long time.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.

Sacace Biotechnologies recommends to use the following kit:

⇨ **DNA/RNA Prep** (Sacace, REF K-2-9);

Please carry out DNA extraction according to the manufacturer's instruction.

Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

PROTOCOL (Reaction volume 25 µl):

The total reaction volume is **25 µl**, volume of DNA sample - **10 µl**.

Unfreeze PCR-mix-2-FRT before mixing.

1. Prepare the required number of the tubes for amplification of DNA from clinical and control samples.
2. Prepare in a new sterile tube the **Reaction Mix.** For each sample mix **10*N µl** of **PCR-mix-1-FRT *T.gondii***, **5,0*N** of **PCR-mix-2-FRT** and **0,5*N** of **Polymerase (TaqF).** Vortex and centrifuge for 2-3 sec.
3. Add **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
4. Carry out the control amplification reactions:

NCA -Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+ -Add **10 µl** of **Positive Control complex** to the tube labeled C+ (Positive Control of Amplification).

5. Insert the tubes in the thermalcycler.

Amplification

1. Create a temperature profile on your instrument as follows:

Step	Rotor type instruments[1]			Plate or modular type instruments[2]		
	Temperature, °C	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
Hold	95	15 min	1	95	15 min	1
Cycling	95	5 sec	5	95	5 sec	5
	60	20 sec		60	20 sec	
	72	15 sec		72	15 sec	
Cycling 2	95	5 sec	40	95	5 sec	40
	60	**20 sec fluorescence detection**		60	**30 sec fluorescence detection**	
	72	15 sec		72	15 sec	

[1] For example Rotor-Gene™ 3000/6000(Qiagen)

[2] For example,iCycler iQ™ / iCycler iQ5™ (BioRad); Mx3000P™ (Agilent),

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green and JOE/Yellow/Hex/Cy3 fluorescence channels.

Toxoplasma gondii on the JOE (Yellow)/HEX/Cy3 channel, IC is detected on the FAM (Green) channel.

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 3000/6000, RotorGene Q)

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 3 Fl to 8 Fl*	*0.03*	*10 %*	*On*
JOE/Yellow	*from 3 Fl to 8 Fl*	*0.03*	*10 %*	*On*

Plate- or modular type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

DATA ANALYSIS

- ***Toxoplasma gondii*** **DNA** amplification product is detected in the **JOE/Yellow/HEX** channel,
- **Internal Control** amplification product is detected in the **FAM/Green** channel.

RESULTS INTERPRETATION

The results are interpreted by the software of the PCR instrument by the crossing (or not crossing) of the fluorescence curve with the threshold line.

The analysis results are considered valid, only if the control samples results comply with the following:

Results for controls

Control	Stage for control	Ct channel Fam	Ct channel Joe	Interpretation
NCE	DNA isolation	POS	NEG	OK
NCA	Amplification	NEG	NEG	OK
C+	Amplification	POS	POS	OK

1. The sample is considered **positive** if Ct values detected in the FAM/Green and JOE/Yellow/HEX channel are less than the boundary Ct values (see below) for these channels. The fluorescence curve should have a typical sigmoid shape and cross the threshold line in the region of significant fluorescence increase only once.
2. The sample is considered **negative** if its fluorescence curve does not cross the threshold line (Ct value is absent) and does not have the typical shape.

The result of Ct values are considered reliable only if the results obtained for Positive control, Negative Control and Clinical samples are within the limits as indicated in the table:

Control	Ct in channel	
	FAM/Green	**JOE/Yellow/HEX**
NCE	≤ 30	Neg
NCA	Neg	Neg
C+	≤ 30	≤30
Clinical samples	≤ 30	≤40

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity of **Toxoplasma gondii Real-TM** PCR kit is 400 *Toxoplasma gondii* DNA copies/ml.

*The claimed analytical features of **Toxoplasma gondii Real-TM** PCR kit are guaranteed only when additional reagent kit (DNA/RNA-prep) is used.*

Specificity

The analytical specificity of **Toxoplasma gondii Real-TM** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The clinical specificity of **Toxoplasma gondii Real-TM** PCR kit was confirmed in laboratory clinical tests.

Target region

Channel for fluorophore	FAM	JOE
DNA-target	Internal Control STI-87-rec (IC)	Toxoplasma gondii
Target gene	genetically engineered construction	rep529

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam/Green): retesting of the sample is required.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
    - Improper DNA extraction.
        - ⇒ Repeat analysis starting from the DNA extraction stage
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak (Ct > 38) signal on the Joe (Yellow)/Cy3/HEX channel: the result is considered equivocal. It is necessary to repeat the analysis twice. If a positive Ct value is detected twice, the sample is considered as positive.
3. Joe (Yellow)/Cy3/HEX signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All sample results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips among tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - Contamination during PCR procedure. All sample results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive controls at the end.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

REFERENCES

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KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**C–**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

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* Rotor-Gene™ is a registered trademark of Qiagen
* MX3000P™ is a registered trademark of Agilent Technologies

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