Toxoplasma gondii Real-TM

Handbook

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

REF TP1-50FRT

Σ 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 (peripheral blood, umbilical cord blood, white cells of peripheral or umbilical cord blood, biopsy and autopsy material, cerebrospinal fluid, and 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 specidic 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

"DNA-Sorb-B": isolation of DNA from clinical specimens

- **Lysis Solution**, 15 ml;
- **Washing Solution 1**, 15 ml;
- **Washing Solution 2**, 50 ml;
- **Sorbent**, 1,25 ml;
- **DNA-eluent**, 5,0 ml.

Contains reagents for 50 extractions

"Toxoplasma gondii Real-TM": Real Time Amplification

- **PCR-mix-1-FRT T. gondii,** 0,6 ml;
- **PCR-mix-2-FRT**, 0,3 ml;
- **Polymerase (TaqF),** 2 x 0,03 ml;
- **Pos Control *DNA T.gondii/STI* (C+),** 0,1 ml;
- **DNA-buffer**, 0,5 ml;
- **Negative Control (C-)***, 1,2 ml;
- **Internal Control STI-87 (IC)**,** 1,0 ml;

Contains reagents for 55 tests

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

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g); Eppendorf 5415D or equivalent
- 60°C ± 2°C dry heat block
- Vortex mixer
- Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Biohazard waste container

Zone 2: RT and amplification:

- Real Time Thermalcycler
- Workstation
- Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

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

PRODUCT USE LIMITATIONS

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

All components of the **Toxoplasma gondii Real-TM** PCR kit (except for polymerase (TaqF), PCR-mix-2-FRT, and PCR-mix-1-FRT *Toxoplasma gondii*) are to be stored at 2–8 °C. All components of the **Toxoplasma gondii Real-TM** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

Polymerase (TaqF), PCR-mix-2-FRT, and PCR-mix-1-FRT Toxoplasma gondii are to be stored at ≤ –16 °C

PCR-mix-1-FRT Toxoplasma gondii is to be kept away from light

DNA-Sorb-B must be stored at 2-25°C.

Toxoplasma gondii Real-TM PCR kit and **DNA-Sorb-B** should be transported at 2–8 °C for no longer than 5 days but should be stored at suggested temperature immediately on receipt.

STABILITY

Toxoplasma gondii Real-TM Test 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

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

- *Whole peripheral and umbilical cord blood*
- *White cells of peripheral or umbilical cord blood*
- *Biopsy and autopsy material*
- *Cerebrospinal fluid*
- *Amniotic fluid*

- *Whole peripheral and umbilical 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. Umbilical cord blood is obtained by cordocentesis. Invert the tube several times to ensure proper mixing.

  ⚠ 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).

- *Biopsy and 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 to a 2-ml tube with 0.3 ml of transport medium. 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.
- *Cerebrospinal fluid* should be obtained by the standard procedure and collected to a sterile Eppendorf tube.
- *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. Remove the supernatant leaving 200 µl of the fluid over the pellet. Use tips with aerosol barrier. Resuspend the pellet.

DNA ISOLATION

The following kit is recommended:

- ⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B): whole blood, cerebrospinal fluid, amniotic fluid, white cells
- ⇨ **DNA-Sorb-C** (Sacace, REF K-1-6/50): biopsy and autopsy material
- ⇨ **DNA/RNA-Prep** (Sacace, REF K-2-9): whole blood, cerebrospinal fluid, amniotic fluid, white cells
- ⇨ **SaMag Bacterial DNA Extraction Kit** (Sacace, REF SM006): cerebrospinal fluid.

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

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

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60°C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes.
3. Add to each tube **300 µl** of **Lysis Solution** and **10 µl** of **IC**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control**) to labeled C*neg*.
6. Vortex the tubes, incubate 5 min at 65°C and centrifuge for 5 sec.
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically
9. Centrifuge all tubes for 1 min at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 1 min at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
11. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
12. Repeat step 11.
13. Incubate all tubes with open cap for 5 min at 65°C.
16. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.

17. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. The amplification can be performed on the same day of extraction.

PROTOCOL (Reaction volume 25 µl):

1. Prepare required quantity of reaction tubes for samples (N) and controls (N+2).
2. Prepare in the new sterile tube for each sample **10*N µl** of **PCR-mix-1**, **5,0*N** of **PCR-mix-2-FRT** and **0,5*N** of **TaqF Polymerase.** 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.

Re-centrifuge all the tubes with extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction!

4. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control (NCA);
   - add **10 µl** of ***Toxoplasma gondii* C+** to the tube labeled. *C+*;

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

AMPLIFICATION

Program the real-time instrument according to the manual provided by the manufacturer.

Amplification program for rotor-type instruments[1]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 s		

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*

Amplification program for plate-type instruments[2]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
1	95 °C	15 min	–	1
2	95 °C	5 s	–	5
	60 °C	20 s	–	
	72 °C	15 s	–	
3	95 °C	5 s	–	40
	60 °C	30 s	FAM, HEX/Cy3/JOE	
	72 °C	15 s		

[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

INSTRUMENT SETTINGS

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

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*0.03*	*10 %*	*On*
JOE/Yellow	*0.03*	*10 %*	*On*

Plate-type instruments (iQ5, Mx300P, ABI 7500/7300)

Channel	Threshold
FAM	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.

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 in channel		Interpretation
		FAM/Green	JOE/Yellow/HEX	
NCE	DNA extraction	≤ 35	Neg	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	≤ 35	≤33	OK

1. The sample is considered **positive** if Ct values detected in the FAM/Green is less than the boundary Ct value in the result grid and JOE/Yellow/HEX channel with Ct ≤ 38. 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 results of analysis are considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

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.

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 or DNA-sorb-C) 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: 529bp tandem repeat

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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 samples 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 samples 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.

KEY TO SYMBOLS USED

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

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
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