TOXOPLASMA GONDII DNA

Qualitative Real-Time PCR for detection of Toxoplasma Gondii

4BShopLab

Rev. 6

TOXOPLASMA GONDII DNA

A. INTENDED USE

The **Toxoplasma gondii DNA** Real-Time PCR kit coded **TOXODNA.CE** is intended for the qualitative detection of Toxoplasma Gondii DNA in human samples (plasma and amniotic fluid) with a simultaneous control of the extraction/amplification reaction through an **Internal Control (IC).**

TOXODNA.CE assay was standardised against the 1st WHO International Standard for Toxoplasma gondii DNA (NIBSC code 10/242) to express positive controls concentration both in tachyzoites/ml and in International Unit (IU/ml).

The kit has been adapted for the use on the Real-Time Thermacyclers and ABI 7500 Sequence Detection System® (Software SDS version 1.3.1, Applied Biosystems™*) or MX3000P (Software MxPro version 4.01, Stratagene™***).

* Applied Biosystems is a registered trademark and ABI PRISM® is a trademark of Applera Corporation or its subsisdiaries in the US and/or certain other countries.
***Stratagene is a registered trademark.

B. INTRODUCTION

The parasite Toxoplasma gondii is distributed widely in the human population, and is estimated to affect up to a 3rd of the world's population.

It is an obligate intracellular protozoan belonging to the phylum Apicomplexa, subclass Coccidia, that infects warm-blooded vertebrates, including man.

The genome is about 80Mb in size and consists of 11 chromosomes.

The most important mode of transmission of infection to humans is through the ingestion of poorly cooked meat containing encysted organism.

Farm animals in the food chain are significant reservoirs of T. gondii, which is important because of possible transmission to man, and toxoplasmosis also causes significant veterinary losses.

Toxoplasmosis has a variable outcome, depending on the interaction of many factors, including the functions of the immune system.

Most infections are asymptomatic or take the form of a mild, self-limiting illness. However, the patient is left with life-long latent infection caused by the presence of tissue cysts.

The disease is dangerous when contracted in utero, and also for patients with immunodeficiency, especially if adaptive immunity is involved (e.g., AIDS patients, leukaemia patients, transplant patients).

Acute toxoplasmosis acquired during pregnancy may result in fetal death or in serious complications such as blindness, deafness or central nervous system disorders. These complications may manifest in neonates, or later in life for children infected congenitally.

The contact with contaminated faces may constitute a potential hazard to pregnant women.

Diagnosis of toxoplasmosis usually is based on the detection of antibodies by serology by ELISA, agglutination tests or other immunometric methods such as Western blot or the Sabin-Feldman dye test.

Serological techniques may, meanwhile, be inferior in immunocompromised individuals, in AIDS patients or in prenatal cases. In those patients it is relevant the use of PCR as a diagnostic tool.

C. PRINCIPLE OF THE TEST

The TOXODNA.CE Kit is based on a Real Time chemistry which uses specific Primers and Probes.

The **Toxoplasma gondii DNA,** recovered from the biological sample under investigation through an extraction step, is amplified using the Real Time amplification system. The amplified product is detected using a fluorescent reporter dye probe specific for a TOXO highly repeated (200 to 300 times) genomic sequence.

Heterologous Internal Control (IC) serves as an Extraction/Amplification control for each individually processed specimen aiming to the identification of reaction inhibitors.

An High Positive control (CTRL-H) and Low Positive control (CTRL-L) are supplied as controls of the PCR reaction.

D. COMPONENTS

The standard format of the product code TOXODNA.CE contains reagents for 50 tests.

Component	Labelling and Contents	TOXODNA.CE 50 Reactions
A **CODED: ALL/MM4** **COLOR CODE: CLEAR**	Master mix	N°1 vials / 0.825 ml
B **CODED: TOXO/CB** **COLOR CODE: YELLOW**	Lyophilised Primers/Probes	N°2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
C **CODED: ALL/C** **COLOR CODE: RED**	MG Water	N°2 vials /1.5 ml
NTC **CODED: ALL/NTC** **COLOR CODE: WHITE**	Negative Control	N°1 vials /1.5 ml
CTRL-L Low Positive Control ($4.0x10^1$ tachyzoites/ml or $3.5x10^1$ IU/ml) **CODED:TOXO/CTRL-L** **COLOR CODE: PINK**	Lyophilised Qualitative Low Positive	N° 8 vials (Dissolve with the volume of ALL/C indicated on the vial label)
CTRL-H High Positive Control ($4.0x10^4$ tachyzoites/ml or $3.5x10^4$ IU/ml) **CODED:TOXO/CTRL-H** **COLOR CODE: VIOLET**	Lyophilised Qualitative High Positive	N° 8 vials (Dissolve with the volume of ALL/C indicated on the vial label)
IC Internal Control **CODED: TOXO/IC** **COLOR CODE: GREEN**	Lyophilised Internal Control	N°2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
Package Insert	Instruction for Use	1

Important note: Upon request, Dia.Pro can supply reagents for 25, 100, 150 tests, as reported below **:**

1. Component A 2. Component B 3. Component C 4. NTC 5. IC 6. CTRL-H 7. CTRL-L 8. Pack. insert	n°1 vial/0.4 ml n°1 vial n°1 vial/1.5 ml n°1 vial/1.5 ml n°1 vial n°4 vial n°4 vial n°1	n°2 vial/0.825 ml n°4 vial n°2 vial/1.5 ml n°1 vial/1.5 ml n°4 vial n°4 vial n°4 vial n°1	n°3 vial/0.825 ml n°6 vial n°3 vial/1.5 ml n°1 vial/1.5 ml n°6 vial n°6 vial n°6 vial n°1
Number of tests	**25**	**100**	**150**
Code	**TOXODNA.CE.25**	**TOXODNA.CE.100**	**TOXODNA.CE.150**

E.STORAGE AND STABILITY

The kit TOXODNA.CE must be stored at +2…8 °C . Once dissolved Component B (coded TOXO/CB) and Component IC (coded TOXO/IC) are stable for 4 months at -20°C. Once dissolved components positive controls HIGH and LOW (coded TOXO/CTRL-HIGH, TOXO/CTRL-LOW) are stable for 2 weeks at -20°C. If the components are to be used only intermittently, they should be frozen in aliquots, repeated thawing and freezing should be avoided, Only one defreezing is allowed.

F. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0.5 µl < volume <1000 µl)
2. DNA extraction kit
3. MG EtOH
4. PBS
5. Thermal Block
6. Microcentrifuge
7. Tube racks
8. Sterile filtered tips with aerosol barrier
9. Nuclease-Free Microtubes
10. 0,2 ml Microtubes or Pcr Microplates recommended from the Real-Time PCR instruments manufacturers
11. Disposable gloves, powder-free
12. Real-Time PCR Thermalcycler (*)
13. Absorbent paper tissues.
14. Vortex or similar mixing tools.

(*) Attention: A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) must be done routinely.

G. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermalcyclers, in the manipulation of Molecular Biology reagents and skilled in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, powder-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and the Components and when performing the test.
7. Components A and B are light sensitive. Protect them from strong light exposition.
8. Avoid vibration of the bench surface where the test is undertaken.
9. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
10. Do not interchange components between different lots of the kits. It is recommended that components between kits of the same lot should not be interchanged.
11. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
12. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
13. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
14. Do not use the kit after the expiration date stated on the external container label.
15. Treat all specimens as potentially infective. All human serum/plasma specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
16. Store and extract specimens separately from the other reagents and use a separate room for their handling
17. Dissolve the lyophilised reagents with the correct amount, stated in the labels, of Component C (Code: ALL/C) supplied in the kit.
18. Carry on all the working operations as quickly as possible maintaining the components on ice or in a cooling block.
19. The laboratory Workflow must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Areas. Do not return samples, equipment and reagents to the area where previous steps have been performed.
20. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring of components into automated workstations, in order to avoid cross contamination.
21. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from sample extraction procedures, has to be treated as potentially infective material and inactivated before waste. Do not put in contact the extraction waste with bleach.
22. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
23. Other waste materials generated (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

H. SPECIMEN: PREPARATION AND RECOMMENDATIONS

1.Blood has to be drawn aseptically by venepuncture and plasma has to be prepared using the standard procedures for clinical laboratory analysis.
2.Collection of amniotic fluid has to be done after 16 weeks from the beginning of gestation under continuous ultrasound control. Following the established and approved clinical guide lines.
3. No influence has been observed in the preparation of the sample with citrate, EDTA.
Attention: Heparin (≥10 IU/ml) affects the PCR reactions.
Samples, which has been collected in tubes containing heparin as an anticoagulant should not be used. Also, samples of heparinised patients must not be used.
4. Avoid any addition of preservatives to samples.
5. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.

6. Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
7. Plasma and amniotic fluid, if not used immediately, must be stored at -20°..-80°C after collection. Samples can be stored frozen at -80°C for several months. Any frozen samples should not be frozen/thawed more than once as this may affect the test result.
8.The plasma samples for DNA extraction must be collected according to the common laboratory procedures, transported and stored at +2 / +8 °C for a maximum period of 4 hours. The plasma samples can be stored frozen at – 20°C for a maximum period of 30 days or at -70°C for longer periods.
9. We recommend you, for the optimal storage of samples, to split them in several aliquots (minimum volume 300 µl) and store them frozen at -20°C for a maximum period of 30 day or -70°C for longer periods. Avoid repeated freezing / thawing cycles.
10.The amniotic fluid samples must be centrifuge before DNA extraction and dissolved in PBS according to the common laboratory procedures. The amniotic fluid samples must be transported and stored at +2 / +8 °C for a maximum period of 4 hours. The amniotic fluid samples can be stored frozen at – 20°C for a maximum period of 30 days or at -70°C for longer periods.
11.When using frozen samples, thaw the samples just before the extraction in order to avoid cases of nucleic acid degradation.
12.The whole peripheral blood samples for DNA extraction must be collected in EDTA according to laboratory advices, transported and stored at +2 / +8 °C for a maximum period of 3 days. Do not freeze the whole peripheral blood samples to avoid cell lysis and viral titre loss.

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:
Component A. Ready to use. Mix well on vortex before use and centrifuge briefly to collect the whole volume.

WARNING: Component A is light sensitive. Protect it from strong light exposition.

Primers/Probes:
Component B.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component B with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

WARNING: Component B is light sensitive. Protect it from strong light exposition.

MG Water :
Component C. Ready to use.

Negative Control :
NTC. Ready to use.

Positive Control :
Component CTRL-L.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized CTRL-L with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

Component CTRL-H.
- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized CTRL-H with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

Internal Control:
IC
- Centrifuge the vial for 1 min at 11000 rpm.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized I.C. with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-5%.
2. **Extraction Device:** The TOXODNA.CE Kit is intended to be used in combination only with QIAamp DNA Minikit Code.51306 (QIAGEN) and NucleoSpin Blood Kit Code: 740951 (Macherey-Nagel).The end users must strictly follow the Instruction for use supplied by the manufacturers.
3. **Real-Time Thermocyclers.** The TOXODNA.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI 7500, software SDS version 1.3.1 (Applied Biosystems), and MX3000P, software MxPro version 4.01 (Stratagene).
   The end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.

M. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that on the bottom of the Lyophilized components vials is present a well formed aggregate. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Dissolve the Lyophilized Components with the appropriate amount of Component C (Molecular Grade water) as described in the proper section (I).
4. Turn the Thermalcyclers on, check settings and be sure to use the right assay protocol.
5. Follow strickly the Instruments Manual supplied by the manufacturers for the correct setting of the Real-Time Thermalcyclers.
6. Check that the micropipettes are set to the required volume.
7. Check that all the other equipment is available and ready to use.
8. In case of problems, do not proceed further with the test and advise the supervisor.

N. ASSAY PROCEDURE
The assay has to be carried out according to what reported here below.

N.1 DNA extraction

The extraction step of the Toxoplasma gondii genomic DNA has to be carried out exclusively in combination with the following kits:

Material	Description	Kit code	manufacturer
Plasma/Amniotic fluid	Nucleospin Blood	740951	MN™
Plasma/Amniotic fluid	QIAamp DNA mini kit®	51306	Qiagen™

NB: *Before DNA isolation centrifuge the Amniotic fluid samples (10,000g, 5min), remove supernatant and dissolve the pellet in 200ul PBS*

The DNA isolation must be carried out only according to the Instruction Manual supplied by the Manufacturer (QIAGEN™, MN™).

WARNING*: The following volumes have to be strictly used in the extraction procedures for both the kits:*

Sample Extraction volume : 200 µl
Eluition Volume: 100 µl

The DNA collected from the samples, not used in the run , has to be stored frozen (-20°C….-80°C).

Important note: *The IC of the* TOXODNA.CE Kit *can be used in the isolation procedure as extraction control.*
The Internal Control Ct value for the negative samples is used to evaluate if the DNA extraction procedure has been performed correctly (see section Q).

For this application add 5 µl of I.C. to the lysis buffer and sample mixture and proceed following the instruction manual supplied by the manufacturer of the Extraction Kit.

N.2 Setting up of the reaction

TOXODNA.CE kit is intended to be used exclusively in combination with ABI 7500, software SDS version 1.3.1 (Applied Biosystems), and MX3000P, software MxPro version 4.01 (Stratagene).

N.2.1 Preparing the PCR

Important: *An example of dispensation scheme is reported in Section O. Please, refer to it before starting the operations described here below.*

- Prepare the components as described in Section I;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for the Positive control (prepared as described in section I).

Important note: *Use only optical tubes or microplates suggested by the Real-Time thermalcyclers manufacturers.*

- Consider that the samples, if possible, should be tested in duplicate;
- Include at least 1 tube for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and positive controls (CTRL-H, CTRL-L)** as table below:

Preparation of the Amplification Mix
(I.C. as Amplification Control)

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
IC	Internal Control	0,5 µl	6 µl
Tot vol.		**15 µl**	**180 µl**

Important note: *If the Internal Control was added during the DNA isolation procedure, prepare the* ***Amplification Mix*** *for* ***Samples*** **NTC and positive controls (CTRL-H, CTRL-L)** *as described in table below:*

Preparation of the Amplification Mix
(I.C. as Extraction/Amplification Control)

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
C	MG Water	0,5 µl	6 µl
Tot vol.		**15 µl**	**180 µl**

N.2.2 Amplification procedure

- Dispense 15 ul of the amplification mix in each reaction tube or microplate well
- Add 10 ul of the **Samples, NTC, CTRL-H and CTRL-L** to the reaction tubes.
- Close firmly the reaction tubes
- Centrifuge briefly the reaction tubes at 2000 rpm
- Don't leave the reaction tubes at room temperature (RT) for more than 30 minute and at light exposure (cover the tubes).
- Load the reaction tubes in the Real-Time Thermacycler Thermoblock Holder.
- After the setting operations described in the Sections N3 (Instrument Programming) start the Thermacycler run.

Important note: The Components Lyophilized after dissolution with the Component C (MG water) are stable no more than 3 hours kept in ice or at 2°…8° °C.
The not used volume of Component B, CTRL-H, CTRL-L and I.C. can be freeze at -20°C and used as described in Section E.

N.3 Instrument programming
For programming the instrument refer to the Instrumentation Instruction Manual provided by the manufacturers.

Important Note: *For Mx3000P set "Filter set gain settings" : ROX = x1, FAM = x8, JOE = x1. (see MxPro™ QPCR Software Instruction Manual, p.41)*

N.3.1 Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	2 min
1	1	95°C	10 min
2	50	95°C	15 sec
		60°C **(*)**	1 min

IMPORTANT NOTE: (*) step for the real time data collection.
WARNING: Keep attention to set up the Real-Time Thermacycler with the correct Thermal Profile following the Instrument Manual supplied by the Instrument manufacturer.

N.3.2 Selection of the Detectors

Following the Instruction manuals of the Real-Time thermalcyclers suggested (Mx3000P Stratagene and ABI 7500) select the Detectors reported in the table here below:

Detection	Reporter	Quencher
TOXO	FAM	Non Fluorescent
Internal Control (IC)	JOE	Non Fluorescent
Passive Reference	ROX	Not Present

WARNING: Keep attention to set up the Real-Time Thermacycler with the correct settings following the Instruments Manual supplied by the manufacturer.

O. ASSAY SCHEME

An example of dispensation scheme for the Analysis is reported here below:

Microplate or tubes

	1	2	3
A	**CTRL-H** 4.0x10^4 tachyzoites/ml or 3.5x10^4 IU/ml	Sample 6	
B	**CTRL-L** 4.0x10^1 tachyzoites/ml or 3.5x10^1 IU/ml	Sample 7	
C	NTC	Sample 8	
D	Sample 1	Sample 9	
E	Sample 2	Sample 10	
F	Sample 3	Sample 11	
G	Sample 4	Sample 12	
H	Sample 5	Sample 13	

Legend: NTC = Negative Control CTRL-H, CTRL-L = Toxoplasma gondii DNA Positive Control, Sample 1,2,3 = Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre - Analysis Settings

Before starting the analysis:

- Set the "Baseline" (the background fluorescence level) as reported below:

"Baseline"	
ABI™PRISM® 7500 SDS	**Auto Baseline**
STRATAGENE™ Mx3000P®	**Adaptive Baseline (not use Mx4000 v1.00 to v3.00 algorithm)**

- Set manually the FAM/JOE fluorescence "Threshold"

FAM fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	**0.1**
STRATAGENE™ MX3000P®	**0.1**

JOE fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	**0.1**
STRATAGENE™ MX3000P®	**0.02**

P.2 Data analysis

A check is carried out on the High/Low Positive Controls any time the kit is used in order to verify whether their Ct values are as expected and reported in the table below.

Check	Requirements
CTRL-H	20 ≤ Ct (Threshold Cycle) ≤ 24
CTRL-L	30.5 ≤ Ct (Threshold Cycle) ≤ 34.5

Q. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

For each samples the FAM fluorescence (positive/negative Ct values) and the Internal Control JOE fluorescence are assumed to validate Toxoplasma DNA detection as described in the table below:

T.gondii FAM	Internal Control JOE	Assay Result
SAMPLE POSITIVE	+	CORRECT
	-	CORRECT*
SAMPLE NEGATIVE	Ct < 40	CORRECT
	Ct > 40 or undetermined	INVALID**

*High Initial concentration of TOXO DNA in the sample (Positive FAM Signal) can lead to a REDUCED or an ABSENT Fluorescent Signal for the Internal Control I.C. due to the reagents Competition.

** Problems may be occurred during the amplification step (inefficient or absent amplification) or during the extraction step (presence of inhibitors or initial sample containing an insufficient number of cells) leading to an incorrect result. The test procedure must be repeated starting from the Extraction step using a fresh sample coming from the patient.

The results obtained with this product must be interpreted taking consideration of the clinical symptoms and the other laboratory parameters related to the patient conditions.

The following results are possible:

Troubleshooting table

	FAM	JOE	Result	CHECK
SAMPLE unknown	+	+/-	CORRECT RESULT *Positive*	***IMPORTANT***: High Initial concentration of TOXO DNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition.
SAMPLE unknown	-	-	ATTENTION ! POSSIBILITY OF: Inhibition, error in the procedure or misfunctioning of the Instruments	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. that the selected detection dyes are corrected FAM for the TOXO detection and JOE for the I.C. detection;

				4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube 7. that the Extraction procedure have been executed correctly;
SAMPLE unknown	-	+	CORRECT RESULT *Negative*	
CTRL-H/CTRL-L	+	+/-	CORRECT RESULT	***IMPORTANT***: High Initial concentration of TOXO DNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition.
CTRL-H/CTRL-L	-	-	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctely 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the TOXO detection and JOE for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube
CTRL-H/CTRL-L	-	+	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the TOXO detection and JOE for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly;
NTC	-	+	CORRECT RESULT	
NTC	+	+/-	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3.that the work space and Instruments are decontaminated at regular intervals; 4. that the kit has been stored correctly;

If one of more of the problems described in the table above happen, after checking, report any residual problem to the supervisor for further actions.

Important notes:

1. *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
2. *When test results are transmitted from the laboratory to an informatics centre, attention has to be paid to avoid erroneous data transfer.*

R. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Internal Technical Specifications or ITS.

The performance evaluation was carried out in DiaPro's laboratories on materials supplied by the reference clinical labs.

R.1 ANALYTICAL SENSITIVITY

Analytical sensitivity may be expressed as **Limit of Detection**.
Limit of detection (LOD): it is the lowest amount of target that can be detected by the system with a stated probability.
For the NAT tests it is expressed as the smallest concentration of the **analyte** that tested in multiple repetitions gives a positive result.
The **limit of detection (LOD)** is determined by testing serial dilutions containing known concentrations of the analyte.
The **LOD** is the lowest concentration of analyte that can be consistently detected (e.g. in ≥ 95% of samples under routine laboratory conditions).
For the kit TOXODNA.CE the **LOD** has been determined by testing 1:2 serial dilutions (8 replicates for three different runs) of the 1st WHO International Standard for Toxoplasma gondii (NIBSC code 10/242).
The results were analized by a **Probit** analysis, to determine the detection limit at 95%.

The results are the following:

Detection Limit (p=0.05)	
ABI™PRISM® 7500 SDS	**12.44 IU/ml or 14.18 tachyzoites/ml**

***Important Note**: The target sequence is a 529 fragment repeated 200-300 fold in the genome of Toxoplasma gondii.*

R.2 ANALITYCAL SPECIFICITY

The Analytical specificity is the ability of the method to detect only the target DNA sequence.
The analytical specificity of TOXO DNA assay has been studied as follow:

1. The primer/probe Set has been choose analysing the genome target sequence with an appropriate software (Lionsoft v.1.0 supplied by Biotools and Primer Express v.3.0" supplied by Applied Biosystem Inc.).
2. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with T.gondii, and by the "ClustalX" software, in order to compare the genome target sequences of the different genotypes of Toxoplasma.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Genomic DNA isolated from bacteria potential interfering with T. gondii were obtained from American Type Culture Collection (ATCC) and Vircell and tested.

The results are reported in the following table:

Organism	Result
Human T cell Leukemia virus	negative
Candida albicans	negative
Candida glabrata	negative
Chlamydia trachomatis	negative
Pseudomonas aeruginosa	negative

R.3. DIAGNOSTIC SPECIFICITY AND SENSITIVITY

R.3.1 Diagnostic Specificity:
Diagnostic specificity is the probability that the device gives a positive result in the absence of the target marker. So the **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device

This parameter was studied by examining 20 TOXO DNA negative plasma samples:

TRUE NEGATIVES	20
FALSE POSITIVES	0
TOTAL SAMPLES	20
SPECIFICITY %	**100**

On the basis of the results obtained Diagnostic Specificity of the system has been calculated ≥99%.

R.3.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So the **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.

For the kit TOXODNA.CE the parameter was studied by examining 10 TOXO DNA positive plasma and amniotic fluid samples. The samples have been studied in duplicates in the same run and then it has been calculated the percentage (%) of positive samples.

TOXO DNA Positive samples

TRUE POSITIVES	10
FALSE NEGATIVES	0
TOTAL SAMPLES	10
SENSITIVITY %	**100**

In addition, the QCMD 2008 Toxoplasma gondii (TGDNA08) EQA Programme was tested.

The Panel contains 8 positive samples (6 amniotic fluid and 2 plasmas) and 2 negative samples (1 plasma and 1 amniotic fluid).

On the basis of the results obtained the Diagnostic Sensitivity of the system has been calculated in the 100%.

Diagnostic Sensitivity	100 %
Diagnostic Specificity	> 99.5 %

R.4 PRECISION

Precision shows the degree of the system's reliability. Every measurement procedure has an inherent random variation called "random error". Random error does not have a number value but it is determined by dispersion of measurement as standard deviation (DevST) and coefficient variation (CV%). Usually precision of an assay refers to the agreement between replicate measurements of the same material.

In the kit TOXODNA.CE, **precision** was expressed as intra-assay variability and inter-assay variability. 8 replicates of the CTRL-H and CTRL-L were tested in the same run (intra-assay) and in three different runs (inter-assay).

On the basis of the results obtained Intra and inter-assay variability were then calculated.

In absence of established International parameters we have identified the following value of acceptability for the TOXODNA.CE Kit:

Intra-Assay Coefficient Variation (CV%) ≤ 10%.

Inter-Assay Coefficient Variation (CV%) ≤ 10%.

S. LIMITATIONS

The user of this kit is advised to carefully read and understand this package insert. Strict adherence to the protocol is necessary in order to obtain reliable test results. In particular, accurate sample and reagent pipetting, application of a correct workflow along with careful programming of thermalcycling steps are essential for an accurate and a reproducible TOXO DNA detection.

The TOXO DNA determination in a patient sample has extensive medical, social, Psychological and economical implications.

It is recommended that confidentiality, appropriate counselling and medical evaluation be considered as an essential aspect of the testing sequence.

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5. Symbols

LEGENDA			
REF	Product code		Storage temperature
IVD	In Vitro Diagnostic Device		See use instructions
LOT	Lot number		Manufacturer
	Expiry date		Number of tests
CE	CE conformity mark		Date of manufacturing

All the IVD Products manufactured by the company are under the control of a certified Quality Management System approved by an EC Notified Body. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

CE
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DISTRIBUTOR

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