TRYPANOSOMA CRUZI DNA QUANTITATION (QT)

Real -Time PCR for theTrypanosoma cruzi genome Quantitation

-for “in vitro” diagnostic use only-

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REF.
TCRUZIDNAQT.CE
25/50/100/150 Tests

Trypanosoma cruzi DNA

A. INTENDED USE

The **Trypanosoma cruzi DNA Quantitation (QT)** Real-Time PCR kit coded **TCRUZIDNAQT.CE** is intended for the quantitative detection of Trypanosoma cruzi DNA in human blood samples with a simultaneous control of the extraction/amplification reaction through an **Internal Control (IC).**

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 protozoan parasite Trypanosoma cruzi, agent of Chagas disease, infected 28 million people in the Americas. The infection can be acquired mainly by triatomine faeces, blood transfusion, transplacental transmission and by an organ transplanted from an infected donor. This parasitic disease shows a variable clinical course, which ranges from asymptomatic cases, to severe chronic stages characterized by low parasitaemia and cardiac and/or gastrointestinal disorders. If untreated, infection is lifelong.

Specific treatment of infection by Trypanosoma cruzi has been performed with the only available drugs: nifurtimox and benznidazole, but the current chemotherapies are unsatisfactory and both drugs have frequently toxic side effects.

The Trypanosoma cruzi parasite are classified into two main phylogenetic lineages, named T.cruzi I (TcI) and T.cruzi II (TcII) based on different molecular markers and biological features. TcII is composed by five subdivisions designated as TcIIa to TcIIe.

Trypanosoma cruzi has about 10^5 copies of a 195bp repeats, called satellite DNA, which correspond to about 10% of the total DNA.The copy number of the satellite repeats is distinct in each one of the strains.

The current diagnosis of Chagas disease based on serology tests (indirect immunofluorescence assay, ELISA, hemagglutination and complement fixation reaction) or methods based on blood wet smear or a blood concentration technique (Strout method), hemoculture or xenodiagnoses. These methods lack sensitivity in cases of low-level parasitaemias and results inadequate for monitored parasitological reponse to treatment. The application of PCR to detect trypanosoma cruzi directly in blood samples has opened new possibilities for the diagnosis of infection and evaluation of trypanocidal treatment.

C. PRINCIPLE OF THE TEST

Importante note: The TCRUZIDNAQT.CE kit has been set up on the Trypanosoma cruzi strain Tulahuen (TcIIe).

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

Trypanosoma cruzi DNA, recovered from the biological sample under investigation through an extraction step, is amplified using Real Time amplification system. The amplified product is detected using a fluorescent reporter dye probe specific for a Trypanosoma cruzi sequence repeated for 10^4 to 10^5 copies in the parasite genome.

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

A standard curve is supplied allowing the determination of the viral load.

D. COMPONENTS

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

Component	Labelling and Contents	TCRUZIDNAQT.CE 50 Reactions
A **CODED: ALL/MM-1** **COLOR CODE: LIGHT BLUE**	Master mix	N° 2 Vials / 0.4 ml
B **CODED: TCRU/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° 4 vial /1.5 ml
NTC **CODED: ALL/NTC** **COLOR CODE: WHITE**	Negative Control	N° 1 vial /1.5 ml
STD Quantitation Standard (3400 p/ml) **CODED: TCRU/STD** **COLOR CODE: RED**	Lyophilised Quantitative High positive	N° 6 Vials (dissolve with the volume of ALL/C indicated on the vial label)
I.C. Internal Control **CODED: ALL/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	N° 1

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

1. Component A	n°1 vial/0.4 ml	n°4 vials/0.4 ml	n°6 vials/0.4 ml
2. Component B	n°1 vial	n°4 vials	n°6 vials
3. Component C	n°2 vial/1.5 ml	n°4 vial/1.5 ml	n°5 vial/1.5 ml
4. NTC	n°1 vial/1.5 ml	n°1 vial/1.5 ml	n°1 vial/1.5 ml
5. IC	n°1 vial	n°4 vials	n°6 vials
6. STD	n°3 vials	n°4 vials	n°6 vials
7.Pack. insert	n° 1	n° 1	n° 1
Number of tests	**25**	**100**	**150**
Code	**TCRUZIDNAQT.CE.25**	**TCRUZIDNAQT.CE.100**	**TCRUZIDNAQT.CE.150**

E.STORAGE AND STABILITY

The kit TCRUZIDNAQT.CE must be stored at +2…8 °C . Once dissolved Component B (coded TCRU/CB) and Component IC (coded ALL/IC) are stable for 4 months at -20°C. Once dissolved **Component STD** (coded TCRU/STD) is 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. Thermal Block
5. Microcentrifuge
6. Tube racks
7. Sterile filtered tip with aerosol barrier
8. Nuclease-Free Microtubes
9. 0,2 ml Microtubes or Pcr Microplates recommended from the Real-Time PCR instruments manufacturers
10. Disposable gloves, powder-free
11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. Vortex or similar mixing tools.
14. PBS

(*) 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.
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. Moreover 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 urethral, cervical swabs and urine 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 with Component C (Coded: 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 Area. 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 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 is drawn aseptically by venepuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis.
2. 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.

3. Avoid any addition of preservatives to samples.
4. 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.
5. We recommend you, for 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.
6. When using frozen samples, thaw the samples just immediately before the extraction in order to avoid possible cases of nucleic acid degradation.
7. 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.
8. The whole peripheral blood samples for DNA extraction must be treated with one volume of Guanidine Hidrochloride 6M-EDTA 0.2 M buffer pH 8.00 solution if collected , transported and stored at room temperature. This solution was used to store whole blood specimens. The Guanidine Hidrochloride-EDTA blood samples remains undegraded for 1 month at 37°C
9. Samples have to be clearly identified with codes or names in order to avoid result misinterpretation.

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 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.

Standard Curve:

STD.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized STD with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex
- Prepare 4 Nuclease Free tubes for the preparation of the Standard Curve
- Set up a 1:10 serial dilution in Component C (ALL/C) to obtain the standard curve as table below:

	Standard curve preparation	
STD	**Calibrator 3400 p/ml**	Volume of Component C (MG water) as written on the vial label
STD 1	**340 p/ml**	10 µl (STD) + 90 µl Component C (MG water)
STD 2	**34 p/ml**	10 µl (STD 1) + 90 µl Component C (MG water)
STD 3	**3.4 p/ml**	10 µl (STD 2) + 90 µl Component C (MG water)
STD 4	**0.34 p/ml**	10 µl (STD 3) + 90 µl Component C (MG water)

Internal Control:

I.C.

- Centrifuge the vial at 11000 rpm for 1 min.
- 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 TCRUZIDNAQT.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) and NA Body Fluid Kit code:D2021 (Chemagen distributed by Dia.Pro). The end users must strictly follow the Instruction for use supplied by the manufacturers.
3. **Real-Time Thermocyclers.** The TCRUZIDNAQT.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 below.

N.1 DNA extraction

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

Manual Extraction tools

Material	Description	Kit code	Manufacturer
Blood	QIAamp DNA mini kit®	51306	Qiagen™
Blood	NucleoSpin Blood	740951	MN™

Automatic Extraction tool in combination with DIA.FASTEX Instrument

Material	Description	Kit code	Manufacturer
Blood	NA Body Fluid Kit	D-2021	Chemagen distributed by Dia.Pro

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

Important Note: The following volumes have to be strictly used in the extraction procedures:

Description	Sample volume ul	Eluition volume ul
QIAamp DNA mini kit®	200	100
NucleoSpin Blood	200	100
NA Body Fluid Kit	200	100

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

Important note: *The I.C. of the* TCRUZIDNAQT.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
- QIAamp DNA mini kit and NucleoSpin Blood: add 10 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.
- NA Body Fluid Kit: add 10ul of I.C. to sample and proceed following the instruction manual supplied in the Extraction Kit by the manufacturer.

N.2 Setting up of the reaction

TCRUZIDNAQT.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 to read the instructions 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 controls (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/well for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and the Standard Curve points** 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
I.C.	Internal Control	0,5 µl	6 µl
C	MG Water	5 µl	60 µl
Tot vol.		**20 µl**	**240 µl**

Important note: *If the Internal Control was added during the DNA isolation procedure, prepare the* ***Amplification Mix*** *for the* ***Sample,*** **NTC and Standard Curve points** *as described in the 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	5,5 µl	66 µl
Tot vol.		**20 µl**	**240 µl**

N.2.2 Amplification procedure

- Dispense 20 ul of the amplification mix in each reaction tube or microplate well
- Add 5 ul of the **Samples, NTC and standard curve** 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 Thermalcycler Thermoblock Holder.
- After the setting operations described in the Sections N3 (Instrument Programming) start the Thermalcycler run.

Important note: The Components Lyophilized after dissolution with 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, STD 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	45	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 Instruments Manual supplied by the manufacturer.

N.3.2 Selection of the Detectors

Following the Instruction manuals for the Real-Time thermalcyclers suggested (ABI 7500, MX3000P Stratagene) select the Detectors reported in the table here below:

Detection	Reporter	Quencher
Trypanosoma cruzi	FAM	Non Fluorescent
Internal Control (I.C.)	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 qualitative Analysis is reported here below:

Microplate or tubes

	1	2	3
A	**STD 1** 340 p/ml	Sample 4	
B	**STD 2** 34 p/ml	Sample 5	
C	**STD 3** 3.4 p/ml	Sample 6	
D	**STD 4** 0.34 p/ml	Sample 7	
E	NTC	Sample 8	
F	Sample 1	Sample 9	
G	Sample 2	Sample 10	
H	Sample 3	Sample 11	

Legend: NTC = Negative Control STD 1,2,3,4 = Trypanosoma cruzi DNA Standard Curve, 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 here 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	**0.2**
STRATAGENE™ MX3000P®	**0.2**

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

P.2 Data analysis

A check is carried out on the STD calibrators any time the kit is used in order to verify whether their Ct values are as expected and reported in the table below.

ABI™PRISM® 7500 SDS/ STRATAGENE™ MX3000P®	
Check FAM	**Requirements**
STD 1	21 ≤ Ct (Threshold Cycle) ≤ 24

Check FAM	Requirements
Slope	-3.1 < Slope <-3.9
Efficiency	R^2>0.98

Q. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

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

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

*High Initial concentration of T.cruzi DNA in the sample (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal for 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.

For each positive samples detected by kit code TCRUZIDNAQT.CE quantification ranged from 3.4E+03 to 0.34E+00 p/ml (parasite /ml)therefore TCRUZI viral load must be expressed as reported in the table below:

Sample T. CRUZI run data (p/ml)	**T . CRUZI genome load Parasite/ml or p/ml**
Quantity > 3.4E+03	T. CRUZI genome load > 3.4E+03
0.34E00 ≤ Quantity ≤ 3.4E+03	***QUANTITATION***
Quantity < 0.34E+00	T.CRUZI genome load < 0.34E+00

Troubleshooting table

	FAM	**JOE**	**Result**	**CHECK**
SAMPLE unknown	+	+/-	CORRECT RESULT *Positive*	***IMPORTANT***: High Initial concentration of TCRUZI 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 TCRUZI 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*	
STD	+	+/-	CORRECT RESULT	***IMPORTANT***: High Initial concentration of TCRUZI DNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of InteDNAl Control I.C. due to the reagents Competition.
STD	-	-	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 TCRUZI 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
STD	-	+	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 TCRUZI 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;

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.*

If the results of the test match the *CORRECT ASSAY RESULT* requirements stated above, proceed to the next section.
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.

R. QUANTITATION

The STD calibrators are treated as patient samples and the same volume,5µl, is used during the amplification step.
The STD calibrators concentration is expressed in parasite/ml.
The **Parasite Genome Concentration per mL** for each patient specimen is calculated applying the following formula:

$$\textbf{Results (parasites/ml)} \equiv \frac{\textbf{parasites/ml (run data)x Elution sample volume (ml)}}{\textbf{Sample Extraction volume (ml)}}$$

Example:

$$\textbf{Results (parasites/ml)} \equiv \frac{\textbf{1500 x 0.1}}{\textbf{0.2}}$$

Results (parasites/ml) ≡ 7.5 E+01

S. 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.

S.1 ANALYTICAL SENSITIVITY

Analytical sensitivity may be expressed as **Limit of Detection** and as **Limit of Quantitation**.
Limit of detection (LOD): it is the lowest amount of target that can be detected by a test 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 results.
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 TCRUZIDNAQT.CE the **LOD** has been determined by analysis of 24 replicates (8 replicates for three different runs) of the highest diluition of the analyte that can be detected in 100% of them.

The results are the following:

LOD Limit of Detection	
ABI™PRISM® 7500 SDS	**0.03 p/ml**
STRATAGENE™ MX3000P®	**0.03 p/ml**

This means that there is the 100% probability to detect a concentration of 0.03 p/ml with the Instrument listed above.

S.1.1 Limit of quantitation

The **Limit of Quantitation** was determined by measuring the **linearity**, the **dynamic range** and the **reproducibility.**
The **Linearity** is the measure of the degree to which a curve approximates a straight line. It is expressed with the **SLOPE** value.
The **dynamic range** is the span of analyte concentrations for which the final output value (Ct threshold cycle) of the system is directly proportional to the analyte concentration, with acceptable trueness and precision.
The boundaries of the dynamic range are the lower and upper limits of quantitation **(Limit of quantitation).**
In the kit code TCRUZIDNAQT.CE a limiting dilution curve with defined copies/ul of a plasmid carrying the specific target viral sequence were prepared. The dilution points were tested in the analytical system and their Ct (threshold cycle) determined.
The upper **limit of quantitation** is $3.53\log_{10}$ (3.4E+03 p/ml) and the lower limit of quantitation is $-0.46\log_{10}$ (0.34E+00 p/ml).

S.2 ANALITYCAL SPECIFICITY
The Analytical specificity is the ability of the method to detect only the target DNA sequence.
The analytical specificity of TCRUZIDNA 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 Chlamydia trachomatis, and by the "ClustalX" software, in order to compare the genome target sequences of the different genotypes of T.cruzi.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Genomic DNA isolated from bacteria potential interfering organisms with Trypanosoma cruzi were obtained from was tested

The results are reported in the following table:

Organism	Results
Leishmania chagasi	Negative **(*)**
Leishmania infantum	negative

(*) for parasite load less than $6.2x10^6$ p/ml

S.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 T. cruzi DNA negative blood 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%.

S.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 TCRUZIDNAQT.CE this parameter was studied by examining 10 T. cruzi DNA positive blood samples. The samples have been studied in duplicate in the same run and then it was been calculated the percentage (%) of positive samples.

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

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

Diagnostic Sensitivity	100 %
Diagnostic Specificity	> 99.5 %

S.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 code TCRUZIDNAQT.CE, **precision** was expressed as intra-assay variability and inter-assay variability. 4 dilution points in 8 replicates 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 an established parameters in the European IVD Directive CTS we have identified the following value of acceptability for the TCRUZI DNA:
Intra-Assay Coefficient Variation (CV%) ≤ 10%.
Inter-Assay Coefficient Variation (CV%) ≤ 10%.

T. 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 thermocycling steps are essential for accurate and reproducible T. cruzi DNA detection and quantitation.
It is recommended that confidentiality, appropriate counselling and medical evaluation be considered as an essential aspect of the testing sequence.

U. BIBLIOGRAPHY

1. Comparison of Trypanosoma cruzi lineages and levels of parasitic DNA in infected mothers and their newborns. Virreira M, truyens C, Alonso-Vega C, Brutus L, Jijena J, Torrico F, Carlier Y, and Svoboda M. Am J Trop Med Hyg 2007; 77 (1): p102-106.
2. International study to evaluate PCR methods for detection of Trypanosoma cruzi DNA in blood samples from Chagas disease patients. Schijman AG, Bisio M, Ladzins et al. PLoS Negl Trop Dis 2011; 5 (1): e931, p1-13.
3. Accurate real-time PCR strategy for monitoring bloodstream parasitic loads in Chagas disease patients. Duffy T, Bisio M, Altcheh J, Burgos JM, Diez M, Levin MJ, Favaloro RR, Freilij H, and Schijman AG. PLoS Negl Trop Dis 2009; 3 (4): e419, p1-10.
4. Organization of satellite DNA in the genome of Trypanosoma cruzi. Elias MC, Vargas NS, Zingales B, Schenkman. Mol Biochem Parassitol 2003; 129 (1): p1-9.
5. Development of a real-time PCR assay for Trypanosoma cruzi detection in blood samples. Piron M, Fisa R, Casamitjana N, Lopez-Chejade P, Puig L, Verges M, Gascon J, Gomez I Prat J, Portus M, Sauleda S. Acta Trop 2007; 103(3): p195-200.
6. Genetic variability of Trypanosoma cruzi: implications for the pathogenesis of Chagas disease. Macedo AM, Pena SD. Parasitol Today 1998; 14: p 119-124.
7. Specific treatment for trypanosome cruzi: lack of efficacy of allopurinol in the human chronic phase of chagas disease. Rassi A, Luquetti AO, Da Silva IG, et al. Am J Trop Med Hyg 2007; 76(1): p 58-61.
8. Nucleotide sequenze provide evidence of genetic Exchange among distantly related lineages of Trypanosoma cruzi. Machado CA, and Ayala FJ. Proc Natl Acad Sci USA 2001; 98(13): p 7396-401.
9. Polymerase chain reaction amplification of Trypanosoma cruzi kinetoplast minicircle DNA isolated from whole blood lysates: diagnosis of chronic Chagas'disease. Avila HA, Sigman DS, Cohen LM, Millikan RC, Simpson L. Mol Biochem Parasitol 1991; 48: 211-221.

v. 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 in compliance with ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Manufacturer:
Dia.Pro Diagnostic Bioprobes Srl
via G. Carducci n° 27 – Sesto San Giovanni - Milano - Italy

CE