RESEARCH

Validation of the TEMPER thermocycler in nucleic acid amplification by polymerase chain reaction

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ABSTRACT

The thermocycler is a programmable cycling incubator that automatically performs the cyclic changes in temperature required for Polymerase Chain Reaction (PCR). In this paper we present the results of the Operational Qualification (OQ) of TEMPER thermocyclers (Center for Immunoassays, CIE). OQ is carried out to ensure that they meet the previously defined functional and performance specifications. The performance of two thermocycler models produced at CIE: TEMPER 1 and TEMPER 2 were evaluated using two commercial diagnostic systems. The commercial thermocycler PTC150 (MJ Research) was used as reference equipment. Intra-assay and inter-assay precision, and the agreement among data sets obtained with different equipment, were estimated using the UMELOSA® Cualitativo HCV Test (CIE). The accuracy of the results was assessed with the quantitative AMPLICOR HBV MONITOR™ Test (Roche Diagnostics), using Hepatitis B Virus (HBV) calibrated standards. Both devices showed 100% performance efficiency; and the agreement with the PTC150 thermocycler were 95.8% and 97.9% for within and between assay precision, respectively. When positive and negative HCV samples were evaluated with UMELOSA® HCV CUALITATIVO, a 100% within assay precision among thermocyclers was documented. Moreover, the values of HBV DNA standards agreed with the previously reported ranges, which demonstrate the high accuracy of the test (tolerance limits of 0.5 $\log_{10}$ viral genome copies). The results indicate that TEMPER thermocyclers can be successfully used for diagnostic purposes or other applications involving PCR amplification of nucleic acids.

Key words: thermocycler, precision, accuracy, PCR

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RESUMEN

Validación del termociclador TEMPER en ensayos basados en la amplificación de ácidos nucleicos por reacción en cadena de la polimerasa. El termociclador es un equipo que permite realizar, de manera automática, los cambios cíclicos de temperatura que ocurren en la reacción en cadena de la polimerasa (RCP). En este artículo se exponen los resultados de la etapa de calificación del funcionamiento de la serie cero del termociclador TEMPER (Centro de Inmunoensayo, CIE), según sus especificaciones de operación. Se emplearon dos sistemas comerciales para evaluar el funcionamiento de dos termocicladores del modelo TEMPER, fabricados en el CIE: TEMPER 1 y TEMPER 2. El equipo que se usó como referencia fue el termociclador comercial PTC 150 (MJ Research). Al ensayar muestras positivas y negativas al virus de la hepatitis C (VHC), se evaluó la precisión intraensayo e interensayo del equipo y la concordancia entre los resultados, mediante el empleo del UMELOSA HCV CUALITATIVO. Por último, se evaluó la exactitud en los resultados mediante el sistema cuantitativo AMPLICOR HBV MONITORTM Test (Roche Diagnostics), con el empleo muestras estándares de concentraciones conocidas del virus de hepatitis B (VHB). Al evaluar los equipos TEMPER 1 y TEMPER 2 y el termociclador de referencia PTC 150, la eficiencia del funcionamiento fue del 100%, la concordancia en uno de los montajes fue de 95.8%, la concordancia (precisión interensayo) fue de 97.9%. Al ensayar muestras positivas y negativas al VHC, provenientes de pacientes, mediante el UMELOSA® HCV CUALITATIVO, la concordancia fue del 100%. Se evidenció exactitud en los resultados, cuyos valores estaban dentro de rangos válidos, reportados en la literatura (límites de aceptación de 0.5 log10 copias de genomas virales) en el ensayo de estándares a diferentes concentraciones de ácido dexosirribonucleico (ADN) del VHB. Los resultados de este estudio avalan el empleo del TEMPER en el diagnóstico y otras aplicaciones relacionadas con la tecnología de amplificación de ácidos nucleicos mediante la RCP.

Palabras claves: termociclador, secuenciador térmico, precisión, exactitud, RCP

*I*ntroduction

According to the Food and Drug Administration (FDA) of the K.S. Equipment Qualification (EQ) is the overall process of ensuring that the equipment is appropriate for its intended use [1]. This process is often divided into four stages: design qualification (DQ), installation qualification (IQ), operational qualification (OQ) and performance qualification (PQ) [1].

The functional and operational specifications of the equipment are established during DQ. On the other hand, IQ establishes that the device is received as designed and specified, that it is properly installed in the selected environment, and that this environment is suitable for the operation and use of the device. OQ is the process of demonstrating that the equipment will function according to its operational specification

1. Equipment Validation and Qualification. 2005. [Sitio en Internet]. Disponible en: http://www.labcompliance.com. Último acceso: 10 febrero 2006.

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in the selected environment. Finally, PQ is the process that demonstrates that an equipment consistently performs according to the appropriate specification for its routine use [1].

Thermocyclers, or thermal sequencing machines, are designed to perform the polymerase chain reaction (PCR) in a rapid and efficient way. It does so by controlling the automatic and cyclic temperature changes required for the amplification of a deoxyribonucleic acid chain (DNA) with a thermostable enzyme. PCR enables for the exponential amplification of a very low initial number of DNA molecules and has a wide variety of applications in medical and biological research laboratories. The detection of gene expression or genetic diseases, the identification of genetic fingerprints, gene cloning, paternity testing, DNA information technology, and the diagnosis of infectious diseases are some of the most important ones [2].

Thermocyclers can be used in either qualitative or quantitative PCR assays. Since these techniques have a substantial impact on society, the equipment should be precise, accurate and uniform, to ensure the reliability of the results [3]. The performance of TEMPER thermocyclers was evaluated at CIE following a protocol prepared by the Central Department of Quality Control and Assurance. This protocol includes several steps to validate the new models of the existing equipment [4]. Those steps are: certification assays, safety tests, functional characterization and field tests.

In this paper we show the results of different analytical tests, carried out under controlled conditions, during the functional characterization step of the new equipment. This step is part of the OQ of the instruments, according to their operational specifications and in the selected environment [1].

TEMPER 1 and TEMPER 2, two thermocyclers manufactured at CIE were evaluated using two commercial PCR systems [5]. The commercial thermocycler PTC 150, from MJ Research [6], widely employed in PCR techniques worldwide, was used as the reference equipment because its design and technical specifications are very similar to the TEMPER series [7].

After demonstrating that TEMPER thermocyclers are precise, accurate and uniform [7], several parameters such as within and between assay precision, and the agreement of results were evaluated using the UMELOSA® HCV CUALITATIVO kit (CIE) [8, 9]. The accuracy was also assessed with the quantitative system AMPLICOR HBV MONITOR™ [10].

Material and methods

A. Thermocyclers

Thermocyclers of null series TEMPER 1 (serial N° 030001) and TEMPER 2 (serial I No 030002) (CIE) [5, 7]

Thermal range: from 0 to 105 ºC.

Accuracy: ± 0.3 ºC after 30 seconds at 90 ºC.

Control accuracy: ± 0.1 ºC.

Thermal Homogeneity: ± 0.5 ºC from well to well during 30 seconds after reaching the programmed temperature.

Ramp Velocity: for a temperature change between 50 and 95 ºC in the block, the maximal ramp for heating is 3 ºC/s and for cooling is 1 ºC/s.

Thermal range of the hot cap: from room temperature to 115 ºC.

Sample capacity: 25 tubes of 0.2 mL.

Commercial Thermocycler PTC 150 (MJ Research) [6]

Thermal range: from -9 to 105 ºC.

Accuracy: ± 0.3 ºC.

Control precision: ± 0.1 ºC.

Thermal homogeneity: ± 0.3 ºC.

Ramp velocity: for heating maximal ramp is 2.4 ºC/s and for cooling it is 1.2 ºC/s.

Sample capacity: 25 tubes of 0.2 mL.

B. Systems used

To evaluate the performance of the thermocyclers the following commercial kits were used:

1. UMELOSA® HCV CUALITATIVO (CIE) [8, 9]: Commercial assay for the detection of HCV RNA in human serum or plasma. It includes four main steps:

· Viral RNA extraction from the serum or plasma by chaotropic agents mediated virolysis; 2- propanol RNA precipitation followed by washings with 75% ethanol and acetone, and final suspension in ribonuclease (RNAse) free water.

· Reverse transcription (RT) of RNA to generate complementary deoxyribonucleic acid (cDNA), and PCR amplification of cDNA (RT-PCR) using specific primers. Amplification was carried out in a thermocycler.

· Amplification of a segment of the previously amplified fragment using inner primers (nested PCR). One of these primers is labeled with biotin.

· Hybridization of the amplified product with a specific probe and the addition of streptavidine-alkaline phosphatase tracer. Ultra-microtiter plates were used for fluorescence detection. The fluorogenic substrate is excited at 365 nm and the emitted fluorescence is detected at 450 nm.

2. AMPLICOR HBV MONITOR™ Test, Roche Diagnostics [10]: Commercial assay for the quantitative detection of HBV RNA in human serum or plasma. It is based on four main steps:

· Sample preparation by virolysis in sera and plasma and the inclusion of a neutralizing agent to enable PCR amplification.

· Simultaneous PCR amplification of target HBV DNA and internal control DNA using specific pairs of primers, one of them biotinylated. Amplification is carried out in a thermocycler.

· Hybridization of amplified products with specific oligonucleotide probes. After PCR amplification, HBV and internal control amplicons are immobilized to a streptavidine coated microtiter plate. After binding, the double stranded amplicon is chemically denaturalized and the unlabelled chain is extracted by washing, leaving the single stranded DNA bound to the plate.

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10. Roche Molecular Systems. Instructivo del ensayo AMPLICOR HBV MONITOR™ Test. Branchburg, NJ, USA; 2002.

· Detection of amplified products bound to the probe by colorimetric determination. After the previous step, the hybridization of the immobilized amplicon with dinitrophenyl (DNP) labeled oligonucleotide probes is favored. After the washing step, alkaline phosphatase conjugated anti-DNP antibody is added, followed by para-nitrophenyl phosphate. Alkaline phosphatase catalyzes the formation of a colored complex. Absorption at 405 nm is finally measured with an automatic microplate reader.

C. Samples used

Standards were prepared by diluting positive plasma up to 1.38 x 10^6 IU/mL of HCV RNA, previously determined by the AMPLICOR HCV MONITOR™ Test version 2.0 quantitative kit (Roche Diagnostics). The following dilutions were used in the experiments:

· 750 IU/mL of HCV RNA, representing approximately 7 times the detection limit (DL) of UMELOSA HCV CUALITATIVO (DL 95% =101.7 IU/mL with a confidence interval of 81 to 162.8 IU/mL of HCV RNA) [9].

· 6200 IU/mL of HCV RNA, representing approximately 61 times the detection limit (DL) of UMELOSA HCV CUALITATIVO [9].

HCV negative and positive sera from patients at the initial and final phase of antiviral treatment were also used. Those samples were previously confirmed by the AMPLICOR HCV Test version 2.0 qualitative kit (Roche Diagnostics).

HBV Viral load standards supplied in the AMPLICOR HBV MONITOR™ Test kit (Roche Diagnostics) with 10, 100, 1 000, 10 000 and 1000 000 HBV copies/reaction were used to assess accuracy.

D. Assays performed.

Within and between assay precision of UMELOSA HCV CUALITATIVO with thermocyclers TEMPER 1, TEMPER 2 and PTC 150

Within assay precision was evaluated using a secondary standard with low HCV concentration (750 IU/mL of HCV RNA). The standard was set up in 23 positions of the three equipments and a negative control was placed in the last position. This assay was repeated twice (different days and operators), to evaluate inter-assay precision. The objective was to check the reproducibility of UMELOSA HCV CUALITATIVO assay independently of the equipment used and of the small differences in temperature among the various positions in the thermocycler block.

Viral RNA was extracted from the 24 aliquots of the secondary standard and the negative control, using the UMELOSA® HCV CUALITATIVO system [8]. The 24 positive samples were mixed and homogenized in a single vial. Reverse trans-cription, PCR and nested PCR were conducted according to UMELOSA® HCV CUALITATIVO procedures [8]. Thermocyclers TEMPER 1, TEMPER 2 and the reference equipment were used for PCRs. Finally, the fluorescent signal was detected by hybridization in ultra-microtiter plates. Since this is a qualitative test, the percentage of agreement was used to evaluate the precision [11].

Agreement of the results using different PCR equipment

Samples containing 750 IU/mL and 6 200 IU/mL of HCV RNA were evaluated to assess the agreement of the results among PCR equipment. These are considered to be low viral load values for HCV RNA, since the required sensitivity for HCV RNA detection in individual samples using PCR is 5000 IU/mL, according to the European regulatory agencies such as Paul Ehrlich Institute, Germany [12].

Additionally, the agreement in of values obtained from clinical samples with TEMPER 1 and TEMPER 2 equipments, and the control equipment PTC-150 was also assessed.

Eight replicates of each positive control were tested in each one of the three equipments, together with a panel of 60 clinical samples, 37 HCV negative and 23 HCV positive samples. Extraction, amplification and detection steps were performed according to the manufacturer's recommendations [8].

Accuracy of AMPLICOR HBV MONITOR™ Test using thermocyclers TEMPER 2 and PTC 150

The accuracy was assessed using the quantitative AMPLICOR HBV MONITOR® Test, Roche Diagnostics [10]. Five HBV viral load standards provided by the kit, with 10, 100, 1 000, 10 000 and 1 000 000 HBV copies/reaction, were processed. Those samples were amplified following the procedures recommended by the manufacturer [10]. TEMPER 2 was used to amplify two replicates of each standard, while one additional replicate was run in parallel in the control PTC 150 thermocycler. All viral load values were logarithmically transformed ($\log_{10}$) and compared with the $\log_{10}$ of the expected copy numbers for each standard. The differences between those two set of values were used to evaluate the accuracy (degree of conformity of a sample to its true value) of the quantitative PCR. To detect potential manipulation errors, the same accuracy analysis was conducted with the reference equipment PTC 150.

Results and discussion

Within and between assay precision of UMELOSA HCV CUALITATIVO using TEMPER 1, TEMPER 2 and PTC 150 thermocyclers

In qualitative PCR the precision cannot be expressed as a function of the variation coefficient as in quantitative PCR. A useful indicator for precision in this type of assay could be the degree of agreement among the values obtained for several replicates of the same sample [11]. The precision of the assay can be affected by a number of factors such as time, methodology, analysts, and equipment among others [11].

UMELOSA HCV CUALITATIVO within and between assay precision was calculated as the percentage of agreement among replicates of a low viral load sample (750 IU/mL HCV RNA). Thermocyclers TEMPER 1, TEMPER 2 and PTC 150 were used.

11. Broughton PMG, Bergonzi C, Lindstedt G, Loeber JG, Malan PG. Guidelines for the evaluation of diagnostic kits. Part 2. General principles and outline procedures for the evaluation of kits for qualitative tests; 1987.

12. Gerold Z. Assessment of Validation Studies from a Fractionator's Point of View. Biologicals 1999;27:295-301.

A 100% concordance was found after amplifying the positive sample at different positions within each thermocycler block (table 1).

However, in a second experiment, which was set up to evaluate within (24 duplicates) and between assay precision (different days and analysts), one negative result out of the 24 replicates amplified in TEMPER 2 (table 1) was recorded, for 95.8% agreement. In the between assay precision analysis (table 2) a 97.9% agreement was achieved using the same equipment.

The false negative result found in the second precision experiment with TEMPER 2, could be related to the lack of uniform heating in the block, to the detection method or to operational errors introduced by the analyst.

Non-uniform heating of a thermocycler block could lead to false negative results. This is especially important for diagnostic PCR where multiple samples are simultaneously tested [3]. It has been determined that temperature uniformity (maximum difference in temperature between two positions) of TEMPER thermocyclers is ± 0.5 ºC [7], a value that is acceptable in diagnostic PCR and comparable to the reference equipment (PTC 150) and other commercial thermocyclers [6]. Therefore we did not consider that the within and between assay precision values obtained (95.8% and 97.9%, respectively) were due to failures of these devices.

Furthermore, it was demonstrated that the failure to amplify the samples did not occur at the detection of the amplified product step, because another negative result was obtained when the same amplified sample was retested in another ultra-microtiter plate using a different set of reagents [8].

Thus the analytic technique (UMELOSA HCV CUALITATIVO) has been validated by the satisfactory results obtained when a low viral load sample was repeatedly assayed, regardless of the analyst and the batch of reagents used [9].

Every analytical technique, if not fully automatic, is susceptible to operational errors introduced by the analyst. The lack of HCV nucleic acid amplification observed in one of the 24 duplicates analyzed in TEMPER 2 could be the consequence of this type of error. Additionally, it could be caused by other factors that can induce RNA degradation, such as ribonuclease contamination in the reaction tube, or in the tips used to transfer the RNA to the PCR tube.

Handling errors can affect the precision of the assay, especially those related to automatic pipette handling [14]. Preparation of PCR samples is a process involving many micropipetting steps, leading to either false negative results or contamination due to carryover effects [2].

The within and between assay precision values obtained with TEMPER 2 (95.8% and 97.9%, respectively) are thus considered satisfactory, and could be even better if more replicates of the positive control are tested [11].

Agreement of the results obtained with different PCR equipment

To evaluate the agreement of the results among different PCR equipment, HCV negative and positive plasma samples, and different concentrations of HCV RNA obtained by dilution of a high viral load positive control (1.38×10^6 UI/mL HCV RNA), were evaluated. Two working concentrations, differing in more than 0.5 $\log_{10}$ UI/mL of HCV RNA, were amplified in the three thermocyclers (TEMPER 1, TEMPER 2 and PTC 150). A 100% agreement was obtained for all HCV RNA samples assayed with the three thermocyclers (table 3). A similar result was found when 60 clinical samples, 23 of which were positive to HCV, were assayed (table 4).

Accuracy of AMPLICOR HBV MONITOR™ Test using thermocyclers TEMPER2 and PTC 150

The accuracy of analytical tests is defined as the distance between the average of the experimental values and the true value [14]. The accuracy of the quantitative assay AMPLICOR HBV MONITOR™ Test was assessed by testing viral load standards

13. PCR Cycler Validation-Extended Information. CYCLERtest-Products. 2004. [Sitio en Internet]. Disponible en: http://www.cyclertest.com. Último acceso: 4 agosto 2005.

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Table 3. Agreement of the results of 8 replicates of two low HCV RNA concentrations (750 and 6 200 IU/mL) placed at random positions in the block of TEMPER 1, TEMPER 2 thermocyclers and reference equipment PTC 150

Equipment used	Positive sample with 750 IU/mL of HCV RNA (n = 8)		Positive sample with 6200 IU/mL of HCV RNA (n = 8)	
	Result (+)	Percentage of agreement (%)	Result (+)	Percentage of agreement (%)
PTC 150	8		8	
TEMPER 1	8	100	8	100
TEMPER 2	8		8	

Table 1. UMELOSA® HCV CUALITATIVO within assay precision using TEMPER 1, TEMPER 2 thermocyclers and the reference equipment PTC 150

Equipment used	Number of replicates of the positive sample assayed (750 IU/mL HCV RNA)	Result + (Percentage of agreement)	
		Experiment 1*	Experiment 2*
PTC 150	24	24(100%)	24 (100%)
TEMPER 1	24	24 (100%)	24 (100%)
TEMPER 2	24	24 (100%)	23 (95.8%)

*24 duplicates of a low HCV RNA concentration (750 IU/mL, 7 times the 95% detection of UMELOSA® HCV CUALITATIVO) were assayed in experiments 1 and 2. Samples were placed in the 24 positions of each thermocycler. Each experiment was carried out on different days and was conducted by a different analyst.

Table 2. UMELOSA® HCV CUALITATIVO between assay precision, using TEMPER 1, TEMPER 2 thermocyclers and the reference equipment PTC 150

Equipment used	Number of replicates of the positive sample assayed (750 IU/mL HCV RNA)	Result (+)	Percentage of agreement
PTC 150	48	48	100
TEMPER 1	48	48	100
TEMPER 2	48	47	97.9

Table 4. Agreement of the results for negative and positive HCV RNA samples using thermocyclers TEMPER 1, TEMPER 2 and the reference equipment PTC 150 in the amplification step

Equipment used	HCV negative samples (n = 37)		HCV positive samples (n = 23)	
	Result (-)	Percentage of agreement (%)	Result (+)	Percentage of agreement (%)
PTC 150	37	100	23	100
TEMPER 1	37		23	
TEMPER 2	37		23	

distributed at random positions in the block of TEMPER 2 and PTC 150 thermocyclers. The experimental values obtained for each standard, expressed as DNA copy numbers, were logarithmically transformed (log_{10}), and the average of the replicates was calculated and compared with the expected values. The same procedure was conducted independently with each apparatus. Results are shown in table 5.

A limit of approximately 0.5 log_{10} between expected and experimental values has been reported as acceptable for HBV, HIV-1 and HCV viral load determination assays [15-18]. Any difference above this limit is considered to be a significant change. In our conditions, the maximum difference found was 0.2 log_{10} for the 10 copies standard measured with TEMPER 2 (table 5). It is therefore possible to conclude that accurate measurements of HBV DNA were achieved when both thermocyclers TEMPER 2 and PTC 150 were used. The acceptance limits for every concentration assayed were below 0,5 log_{10} copies/reaction (table 5).

Excellent intra-assay and inter-assay precision were found in the studies conducted to evaluate the performance of TEMPER in qualitative detection assays. Appropriate concordance among thermocyclers was also observed, either with controls containing known concentrations of HCV RNA, or with other negative and positive samples. It was thus demonstrated that TEMPER thermocyclers not only lead to the satisfactory development of qualitative PCR assays, but that they can also be used in quantitative PCR tests, where the accurate measurement of virus concentration in a clinical sample is of utmost importance.

The performance of the two null series of thermocyclers evaluated here was always comparable to the commercial thermocycler used as reference, an equipment that has been extensively validated for diagnostic and research applications. These results allow us to conclude that TEMPER thermocyclers, developed and produced at CIE, can be reliably used in techniques where nucleic acid is amplified by the PCR method.

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Table 5. Accuracy of the quantitative AMPLICOR® HBV MONITOR Test using TEMPER 2 and the reference thermocycler PTC 150 to amplify HBV DNA standards (from 10 to 10^6 copies/reaction)

Expected Copies/reaction	Log_{10} Expected Copies/reaction	Average of log_{10} experimental copies/reaction TEMPER 2	Average of log_{10} experimental copies/reaction PTC 150
10	1.00	0.80	1.10
10^2	2.00	1.85	2.0
10^3	3.00	3.10	3.0
10^4	4.00	4.00	3.9
10^6	6.00	6.00	6.1