YYÜ TAR BİL DERG
(YYU J AGR SCI)
2011, 21(1):35-41

Geliş Tarihi(Received): 02.11.2010
Kabul Tarihi (Accepted): 29.11.2010

Araştırma Makalesi/*Research Article (Original Paper)*

Performance Evaluation of Different Brands of Thermal Cyclers Used for Diagnostic Testing

Derya BULUT **Hikmet Murat SİPAHİOĞLU*** **Mustafa USTA**

Department of Plant Protection, Faculty of Agriculture, University of Yuzuncu Yil, 65080 Van, Turkey
*E-mail: hmsipahi@yyu.edu.tr, Tel:+90 4322251024 fax: +90 4322251104

Abstract: Reliability of PCR-based data between the thermal cyclers requires an assurance of assay reproducibility. In this work, for the validation and standardization of PCR, the precision of thermocyclers on PCR was measured in three commercial thermal cyclers. A reverse transcriptase polymerase chain reaction (RT-PCR) test was run in parallel to assess PCR results. In diagnostic testing, isolates of *Apple mosaic virus* (ApMV), *Zuchini yellow mosaic virus* (ZYMV), *Cherry leaf roll virus* (CLRV), and *Potato spindle tuber viroid* (PSTVd) were used as reference isolates. The cyclers mostly performed within the manufacturer's specification. All thermal cyclers produced the correct target for the single-product PCR assay, although slight variation in yield was observed. For two replicates of each positive sample, repeatability was demonstrated for the tested three thermal cyclers. It was concluded that, irrespective of the make and model of thermal cycler, reproducibility of PCR products by means of RT-PCR was sufficiently acceptable to have similar results from independently in separate commercial thermal cyclers.

Key words: Performance, Thermal cyclers, RT-PCR

Teşhis Çalışmalarında Kullanılan Farklı Markalara Ait PCR Cihazlarının Performans Değerlendirmesi

Özet: PCR cihazlarından elde edilen sonuçların güvenilirliği bu sonuçların tekrarlanabilirliğinin teminini gerektirir. Bu çalışmada, PCR çalışmalarının geçerliliği ve standardizasyonu için, üç ticari PCR cihazının PCR sonuçlarına olan hassas etkisi ölçülmüştür. PCR sonuçlarını değerlendirebilmek için bütün cihazlarda reverse transkriptaz polimeraz zincir reaksiyonu testi (RT-PCR) eş zamanlı olarak yürütülmüştür. Gerçekleştirilen teşhis çalışmalarında, *Apple mosaic virus* (ApMV)'ü, *Zuchini yellow mosaic virus* (ZYMV)'ü, *Cherry leaf roll virus* (CLRV)'ü, ve *Potato spindle tuber viroid* (PSTVd)'i referans izolatlar olarak kullanılmıştır. PCR cihazları çoğunlukla üreticilerin belirttiği aralıklarda çalışmıştır. Elde edilen ürünlerde hafif farklılıklar oluşsa da, test edilen bütün cihazlar beklenen büyüklükte ve doğrulukta PCR ürünü çoğaltmışlardır. Her bir patojen için iki pozitif numunenin kullanıldığı testlerde, tekrarlanabilirlik her üç cihaz için gösterilmiştir. Testte kullanılan cihazların marka ve modeline bakılmaksızın, RT-PCR yöntemi ile elde edilen tüm PCR sonuçlarının tekrarlanabilirliğinin, benzer sonuçlar elde etmede kabul edilebilir sınırlar içinde olduğu sonucuna varılmıştır.

Anahtar kelimeler: Performans, PCR cihazları, RT-PCR

Introduction

PCR is designed to selectively amplify targeted DNA sequences to several billion-fold, and its remarkable selectivity and sensitivity of DNA amplification has led to its widespread application (Kim et al. 2008). Having been subjected to continuous improvements and modifications (Bootman and Kitchin 1992; Yang and Rothman 2004), PCR is now routinely applied in most bioanalytical and clinical laboratories, as well as in basic research fields (Yang and Rothman 2004; Klein**,** 2002; Kim et al. 2008). Numerous studies have reported the separate effects of altering different parameters, ratio of template DNA primers, concentration of *Taq* polymerase and Mg concentration on the bands obtained (Jones et al. 1997) in PCR (Innis and Gelfand 1990; Usta et al. 2005).

The breakthrough of PCR has been primarily due to the development of recombinant, thermostable DNA polymerases (Saiki et al. 1988) and the development of thermocyclers (Schoder et al. 2005). Thermal cyclers are the programmable heating blocks that control and maintain the temperature of the sample through the three temperature-dependent stages that constitute a single cycle of PCR: template

denaturation (~ 95 °C); primer annealing (~35–65 °C); and primer extension (72 °C). These temperatures are cycled up to 40 times to obtain amplification of the DNA target. If the optimum programmed temperatures are not met because the block overshoots or undershoots them or because they are not consistent across the block, reaction specificity and sensitivity can be compromised, leading to the consequences listed above and ultimately to the false interpretation and reporting of data (Saunders et al. 2001).

PCR protocols generated by one lab are hardly reproducible by another (Bootman and Kitchin 1992; Defer et al. 1992; Sheppard et al. 1991). Even under identical assay conditions, it was shown that there are difficulties in obtaining reproducible results (Sounders et al. 2001). The criticism raised against PCR concerns its level of reproducibility. Several factors have been identified, which are of importance regarding the stability of the method. PCR reagents (concentrations of primer, template and $MgCl_2$) and PCR conditions (duration of denaturation, annealing and extension, and type of thermal cycler) have been shown to influence PCR patterns (Yu and Pauls 1992; Ellsworth et al. 1993; MacPherson et al. 1993; Blixt et al. 2003).

The performance of thermal cyclers for polymerase chain reactions (PCR) is of great concern in terms of the reliability of PCR-based assays, particularly when rapid cycling conditions are applied to small volume reactions (Kim et al. 2008). Despite the striking importance of PCR, the literature on thermocyclers is scarce (Schoder et al. 2005). In the present study, an account is given of reproducibility testing of PCR in three different brands of commercial thermal cyclers.

Materials and Methods

Thermal cyclers. The thermal cyclers that took part in the reproducibility experiment are listed in Table 1. Three commercial thermal cyclers were tested in the current study. The selected instruments were: (*A*) Mastercycler personal (Eppendorf), (*B*) TC-3000 (Techne), and (*C*) Px2 Thermal Cycler (Thermo Electron Corporation). Each thermal cycler was programmed to perform the same specific cycling protocol (Table 2) for each pathogen.

Table 1. Information on the assessed commercial thermal cyclers

Thermal Cycler (Models and Manufacturer)	Age (years)	Number of Wells	Max. Heating Rate[1] (°C/s)	Max. Cooling Rate[1] (°C/s)
THERMAL CYCLER A Mastercycler personol (Eppendorf)	7	25x0.2ml 16x0.5ml	3.0	2.0
THERMAL CYCLER B Thermal Cycler (Thermo Electron Corporation)	5	96x0.2ml	3.0	2.0
THERMAL CYCLER C TC-3000 (Techne)	3	25x0.2ml	3.6	2.0

[1]The values of the maximum heating/cooling rates were obtained from the manufacturers' catalogs.

Virus and viroid source. An apple isolate of *Apple mosaic virus* (ApMV), a melon isolate of *Zucchini yellow mosaic virus* (ZYMV), a walnut isolate of *Cherry leaf roll virus* (CLRV), and a potato isolate of *Potato spindle tuber viroid* (PSTVd) were used as reference isolates. Fresh leaf tissue of systemically infected apple (for ApMV), squash (*Cucumis pepo* L.) (for ZYMV), *Chenopodium amaranticolor* (for CLRV), and tomato (*Lycopersicon esculentum* Mill.) (for PSTVd) plants were used in RT-PCR assays.

Viral and viroid RNA isolation and reverse transcription. Total RNA extraction of virus/viroid isolates was performed according to silica-capture method described by Foissac et al. (2000). Purified RNA used as template to generate the single stranded cDNA for ApMV, ZYMV, CLRV, and, PSTVd. Reverse transcription was made according to Sipahioglu et al. (2006). The sequences of reverse complementary primers were used for all isolates in cDNA synthesis (Table 2).

PCR conditions. All reaction volumes were 25 µl, and each assay consisted of a total of two replicates of positive samples, one negative reaction, and one water control. A standard PCR protocol was adapted and applied for all tested pathogen (ApMV, CLRV, ZYMV, and PSTVd) as follows. A final volume of 25µl contained 1µl of cDNA, 2.5µl of 10X reaction buffer (200mM Tris-HCL pH: 8.4, 500mM KCl), 1.5µl of

$MgCl_2$ (25 mM), 0.5µl of dNTPs (10mM each), 0.5µl of each primer (100 pmol/µl), 0.2µl of *Taq* DNA polymerase, and 18.3µl of RNase free sterile water. Samples were amplified in selected thermo cyclers. The cycling conditions are given in Table 2. Aliquots of 10 µl PCR products were separated on 2% agarose gel in TAE buffer (40mM Tris pH 7.8, 20 mM acetic acid, 2 mM EDTA). The DNA was visualized by ethidium bromide staining (Sambrook et al. 1989).

Standardization of the assays. Every effort was made to minimize the introduction of variation not caused by thermal cycler performance. To ensure the homogeneity of the aliquots, the reaction units were prepared as a master mixture for the each pathogen. The whole mix was aliquoted to individual reaction tubes immediately. Four tubes (two positive, a negative, and a water control) of the PCR mixture belonging to each pathogen were placed in each thermal cycler. Because differences in the thickness of PCR tubes from different brands may affect the temperature that the sample reaches, standardized thin-walled microtubes (Axygene) of 0.2 ml size were also supplied. All experiments were performed with the lid temperature set to 105 °C.

Table 2. The primer pairs and cycling conditions used for various viruses and viroid in RT-PCR

Virus/ viroid	Primers	Amplified fragment	Reference	PCR Cycling Conditions
CLRV	CLRV-F 5'-TTGGCGACCGTGTAACGGCA-3' CLRV-R 5'GTCGGAAAGATTACGTAAAAGG-3'*	416 bp	Werner et al. (1997).	94^0C/2 min, 40X (94^0C/15s, 50^0C/30 s, 68^0C/1 min), 68^0C/10min
ZYMV	ZYMV-F 5'-TCAGGCACTCAGCCAACT-3' ZYMV-R 5-CTGCATTGTATTCACACCTAGT-3'*	837 bp	This publication	94^0C/3 min, 40 X (94^0C/30 s, 62^0C/30 s, 72^0C/45 s), 72^0C/10 min
ApMV	ApMV-F 5'-CAGTGGATCCGATGGTCTGCAAGTACTGCAAT-3' ApMV-R 5'-ATCGAAGCTTTCATAATTCTAACAAATC-3'*	690 bp	Kindly supplied from Prof. V. Pallas	94^0C/2 min, 40X (94^0C/30 s, 50^0C/30 s, 68^0C/1.5 min),68^0C/10min
PSTVd	PSTVd-F 3'-CGGAACTAAACTCGTGGTTCC-5' PSTVd-R 3'-AGGAACCAACTGCGGTTCCA-5'*	358 bp	This publication	94^0C/2 min, 40 X (94^0C/1min, 60^0C/1min, 72^0C/1min), 72^0C/10 min

*= Reverse complementary primer

Results

Evaluation of the performance of thermal cyclers. All PCR experiments were performed using the adapted PCR protocol. Duplicates of positive samples of the different pathogens were run at the same time. It was shown that the reproducibility of the designed PCR protocol was satisfactory with high similarity degree for individual pathogens (Fig. 1). The results from three instruments exhibited very slight variations in the yield of PCR product generated by genome specific primers. Of these, in the detection of PSTVd, only the thermal cycler B exhibited substantial variations in the yield from the replicate reactions. The instrument generated very little product (Fig. 1c).

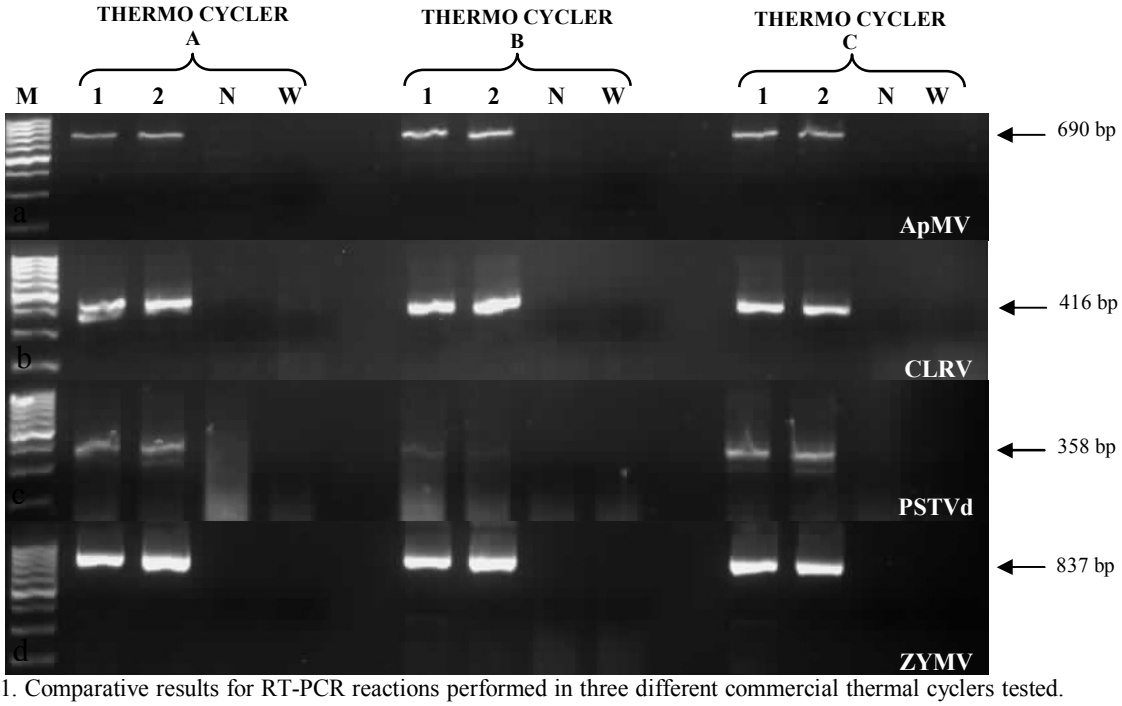

Figure 1. Comparative results for RT-PCR reactions performed in three different commercial thermal cyclers tested. Lane M; 100-bp molecular weight maker, 1 and 2; RT-PCR results of all thermal cyclers tested as two repetition, N; negative control, W; Water control

An interesting finding, but not the main focus of the present study, was observed in the electricity cut took place in the course of thermo cyclers run. Only thermal cycler B (96 well block) generated PCR products in the event of electricity cut occurred at the half stage of PCR run (Fig. 2). This was attributed to the 96 well block which has relatively slower heating and cooling capacity respect to 25-well cyclers A and C. Therefore, to further investigate the reason for the production of PCR products was investigated by using extended step lengths in cycling conditions.

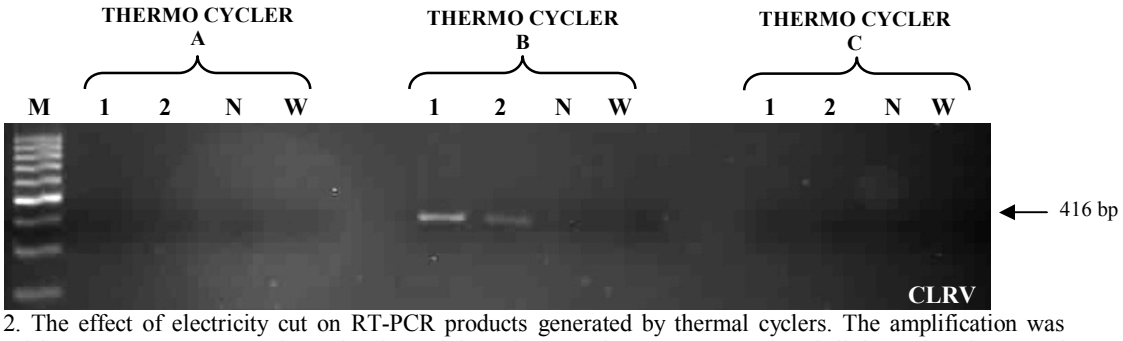

Figure 2. The effect of electricity cut on RT-PCR products generated by thermal cyclers. The amplification was conducted for CLRV. Lane M; 100-bp molecular weight maker, 1 and 2; RT-PCR results of all thermal cyclers tested as two repetition, N; negative control, W; Water control

Effects of extended step lengths. Extended step lengths during PCR run have been investigated in only ApMV detection. Intensities of the 690 bp bands of ApMV gradually intensified as the step lengths were increased 2 to 3 fold (Fig. 3).

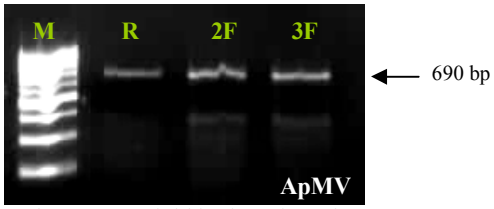

Figure 3. The effects of extended step lengths on PCR yield in detection of ApMV. Lane M; 100-bp molecular weight maker, R; RT-PCR result of regular cycling condition, 2F and 3F; RT-PCR results of 2 to 3 fold extended step lengths respectively.

Discussion

The performance of a thermocycler has a critical influence on PCR efficiency. Interlaboratory trials comparing several thermocyclers of various makes and models reported a failure to obtain reproducible banding patterns by RAPD assays (Jones et al. 1997; Rezendez-Perez and Barrera-Saldana 1990). Use of inappropriate cyclers was mentioned as one of the main reasons for these results; this was perhaps attributable to differences in age, temperature control options, and calibration status (Saunders et al. 2001).

The results were analyzed both on the presence of a single amplification product of the expected size and on the relative yield of each product within a single thermal cycler. In general, single-product PCR results showed apparent similarities between different thermal cyclers. Typical examples are given in Fig. 1. Almost all of the instruments were able to reproduce the same amplification pattern when working with the same genomic RNA extracted from the same plant. The performance of a thermocycler has a critical influence on PCR efficiency. It is well known that PCR protocols need to be optimized for different instruments and that a cycling program that works on one brand of instrument may not necessarily work on another. (Schoder et al. 2005). Soon after the introduction of the first commercial instrument in 1987 (Perkin-Elmer Cetus DNA thermal cycler), other suppliers offered similar devices. Today the thermocycler market is dominated by a few major manufacturers. Thermocyclers of the previous generations differ widely from the contemporary models. Developments of heating/cooling systems (circulating water versus Peltier technology), internal temperature control options (block control versus in-sample probe control and calculated temperature control), and evaporation control concepts (oil overlay versus heated lid) have been changing the technical architecture of the cyclers completely (Saiki et al.1988; Saunders et al. 2001).

During the first 20 cycle, the thermal inhomogeneities became most evident in the electricity cut occurred in the detection of CLRV. The Cyclers A and C, both failed to achieve a temperature plateau for the cycling conditions (denaturation, annealing, and elongation steps) to have elongated fragments (Fig. 2)

The extended the step length has resulted an increase in the generation of RT-PCR products. When ApMV was tested with elongated step-times (2 and 3 fold for each step), however, strong PCR product bands were obtained. This particular finding strongly supports the fact that the slower thermal cyclers can still be functional by applying relatively slow cycling to allow sufficient time to reach the programmed temperatures. The observation is not only in accordance with the report by Kim et al. 2008, but also illustrates a general issue in temperature arrangement of cycling conditions. However, small variations in amplification efficiency during early rounds of the PCR can result in disparate quantities of PCR product during the exponential phase of amplification (Saunders et al. 2001). Consequently, short cycling conditions may not supply adequate denaturation, annealing, and elongation temperatures during short cycling holds. We concluded that false-negative PCR results would most likely be caused by insufficient cycling holds.

This study demonstrates that RT-PCR fragments can be successfully reproduced among different brands of thermal cyclers under the same reaction conditions. Tested instruments supply the similar temperature profiles inside tubes to achieve reasonable results.

References

Blixt Y, Knutsson R, Borch E, Radstrom P (2003). Interlaboratory random amplified polymorphic DNA typing of *Yersinia enterocolitica* and *Y. Enterocolitica* like bacteria. International Journal of Food Microbiology 83: 15-26.

Bootman JS, Kitchin PA (1992). An international collaborative study to assess a set of reference reagents for HIV-1 PCR. J. Virol. Methods 37:23–41.

Defer C, Agut H, Garbarg-Chenon A, Moncany M, Morinet F, Vignon D, Mariotti M, Lefrere JJ (1992). Multicenter quality control of polymerase chain reaction for detection of HIV DNA. AIDS 6:659–663.

Ellsworth DL, Rittenhouse KD, Honeycutt RL (1993). Artifactual variation in randomly amplified polymorphic DNA banding patterns. BioTechniques 14: 214-216.

Foissac X, Savalle-Dumas L, Gentit P, Dulucq MJ, Candresse T (2000). Polyvalent detection of fruit tree Tricho, Capillo and Faveaviruses by nested RT-PCR using degenerated and inosine containing primers (PDO RT-PCR), Acta Hort. 357 pp. 52–59.

Ginny C, Saunders, Juliet Dukes, Helen C. Parkes, Johanne H. Cornett (2001). Interlaboratory Study on Thermal Cycler Performance in Controlled PCR and Random Amplified Polymorphic DNA Analyses. Clinical Chemistry 47: 47-55.

Innis MA, Gelfand DH (1990). Optimization of PCRs. In: PCR Protocols. (Innis AI., Gelfand DH., Sninsky JJ., White TJ., ed.), Academic Press, San Diego, CA, USA, 3–12.

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, Van de Weil C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A, Karp A (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol Breed 3:381–390.

Kim Young Ho, Inchul Yang, Young-Seuk Bae, Sang-Ryoul Park (2008). Performance evaluation of thermal cyclers for PCR in a rapid cycling condition, BioTechniques Vol.:44, No:4 495-505.

Klein D (2002). Quantification using real-time PCR technology: applications and limitations. Trends Mol. Med. *8*:257-260.

MacPherson JM, Eckstein PE, Scoles GJ, Gajadhar AA (1993). Variability of the random amplified polymorphic DNA assay among thermal cyclers, and effects of primer and DNA concentration. Mol. Cell. Probes 7, 293–299.

Rezendez-Perez D, Barrera-Saldana HA (1990).Thermocycler temperature variation invalidates PCR results. Biotechniques 1990;9:286–92.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.

Saunders GC, Dukes J, Parkes HC, Cornett JH (2001). Interlaboratory study in thermal cycler performance in controlled PCR and random amplified polymorphic DNA analyses. Clin. Chem. 47:47–55.

Sheppard HW, Ascher MS, Busch MP, Sohmer PR, Stanley M, Luce MC, Chimera JA, Madej R, Rodgers GC,. Lynch C (1991). A multicenter proficiency trial of gene amplification (PCR) for the detection of HIV-1. J. Acquir. Immun. Defic. Syndr. 4:277–283.

Sipahioglu HM, Usta M, Ocak M (2006). Use of dried high-phenolic laden host leaves for virus and viroid preservation and detection by PCR methods. Journal of Virological Methods.137, 120–124.

Usta M, Sipahioglu HM, Polat B (2005). Studies on parameters influencing the performance of reverse transcriptase polymerase chain reaction (RT-PCR) in detecting prunus necrotic ringpot virus (PNRSV), Phytopathol. Mediterr. 44 pp. 189–194.

Werner R, Mühlbach HP, Büttner C (1997). Detection of cherry leaf roll nepovirus (CLRV) in birch, beech and petunia by immunocapture reverse transcription-PCR using a conserved primer pair. European Journal of Forestry and Pathology 27:309-318.

Yang S, Rothman RE (2004). PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect. Dis. 4:337-348.

Yu K, Pauls KP (1992). Optimization of the PCR program for RAPD analysis. Nucleic Acids Res. 20: 2606.