The Impact of the Temperature Performance of Thermal (PCR) Cyclers on the Generated Results, and the Obligation for Regular Validation of PCR Thermal Cyclers

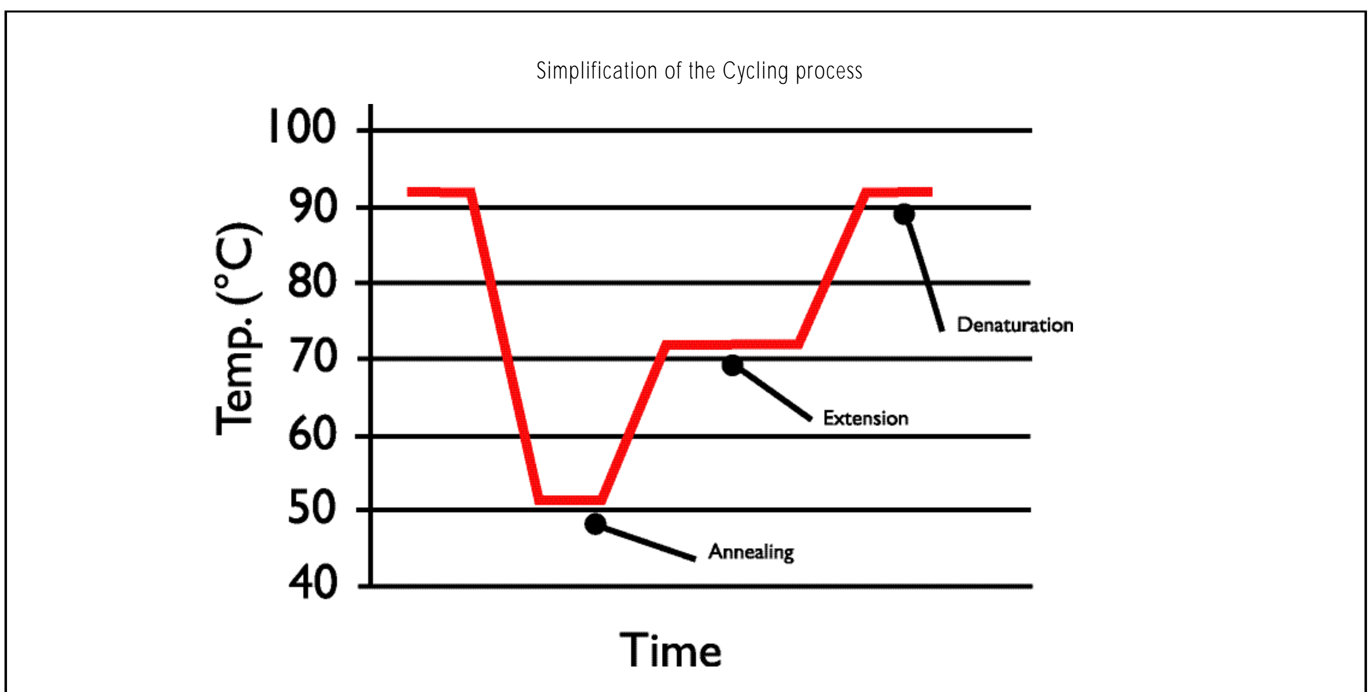
Tom Hendrikx, Marc Verblakt*, Roger Pierik****
*** GENO-tronics BV, Landgraaf, The Netherlands**
**** CYCLERtest BV, Landgraaf, The Netherlands**

Abstract

A fast, powerful, and extremely sensitive multi-channel temperature measurement system was developed for the dynamic temperature validation of thermal cylers. The results of temperature performance tests of several brands of PCR cyclers showed that most cyclers do not perform within the manufacturers' specifications. Furthermore, the measured temperature accuracies and spreads can have a substantial impact on the generated PCR results and (diagnostic) conclusions. None of these "out of specs" cylers indicated that the temperature performance was not as "expected", nor did any (warning) messages appear on the display of the cyclers. Our conclusion is that laboratories performing cycling reactions (PCR, Cycle-sequencing, LCR, PCR) should validate their cycler at least twice a year, or every 100 runs. However, a more frequent validation is preferable. The impact of sample block temperature in-uniformities and the lack of accuracy is not only proven by the used MTAS measurement system, but also supported by biological evidence.

Introduction

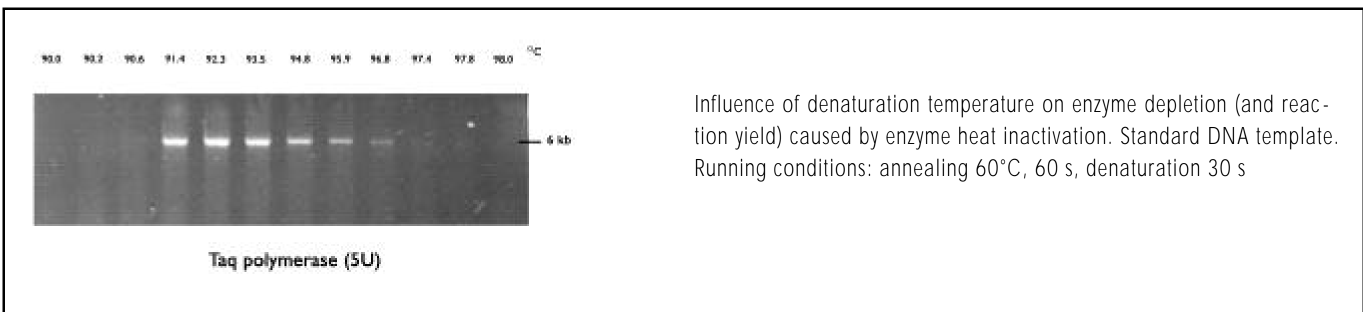
Although the PCR process is a dynamic reaction, which takes place once the first denaturation of the template is achieved (assuming that all needed ingredients are available in the reaction mixture), most of the PCR process variables are over-simplified by the majority of users, manufacturers and others, to basically three major steps. Unfortunately, at least half of the cycling-process takes place in the temperature changes between these three steps. The authors will, despite the availability of hard evidence within their companies, refrain from showing other cycler impacts on amplification results like: (1) heat-block -disposable fit characteristics, (2) Peltier hot and cold spots, (3) single sensor versus multi-sensor cycler technology, (4) partial block load effects, and other effects.



The PCR Cycling Process

Denaturation

During denaturation, the double stranded DNA melts open to single stranded DNA, and the enzymatic extension from a previous cycle comes to minimum. During denaturation partial heat inactivation of the polymerase enzyme occurs. During denaturation a subtle balance between enzyme inactivation and template denaturation is established. Most of the people performing PCR reactions within their laboratory have a (molecular) biology background. This background is the cause that most users (wrongly) prioritize denaturation over enzyme deactivation (heat inactivation). First, batch-to-batch variations of so called "heat stable enzymes" can be substantial. Secondly, the highest activity of these enzymes should be present during the last cycles of the process, when the largest amount of template is available. Because the enzyme deactivation (heat-inactivation) already took place this may result in enzyme depletion, and a significant decrease or even lack of yield.



Influence of denaturation temperature on enzyme depletion (and reaction yield) caused by enzyme heat inactivation. Standard DNA template. Running conditions: annealing 60°C, 60 s, denaturation 30 s

Denaturation time

1 second

5 seconds

15 seconds

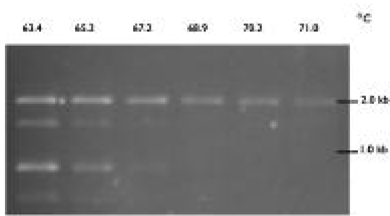
30 seconds

60 seconds

Influence of denaturation temperature and time on enzyme depletion, enzyme inactivation and reaction yield. In time the enzyme is heat inactivated due to a to high denaturation temperature. Notice that the denaturation temperature should not exceed 94 °C, preferable 93°C and the holding should normally not exceed 1minute. Standard DNA template. Running conditions: annealing 60°C, 60 s, and denaturation from 1 to 60 s. Taq Polymerase 5 units, elongation (extension) constant time.

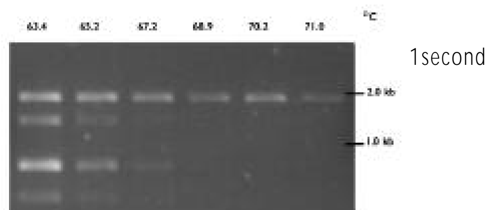
Annealing

The primers are jiggling around. Hydrogen bonds are constantly formed and broken between the single stranded primers and the single stranded template. More stable bounds last a little bit longer (primers that fit exactly) and on that little double-stranded piece the polymerase can attach and start copying the template. Annealing times and temperatures are important to optimize signal and to minimize non-specific binding of primers to the template.



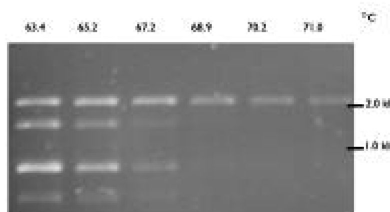
Influence of annealing temperature on results using standard DNA template. Running conditions : denaturation 93°C, 15 s, annealing 15 s, Taq Polymerase 5 Units, elongation (extension) constant time.

Annealing time

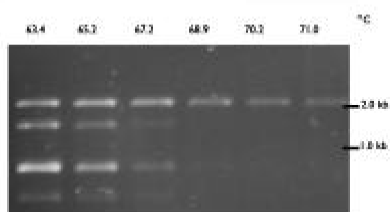


1second

Influence of time on annealing temperature. Standard DNA template, running conditions: denaturation 93°C, 15 seconds, Taq Polymerase 5 units, elongation (extension) constant time..



5 seconds



15 seconds

Extension

During extension the primer binds to the single stranded DNA template on the 3' side. The polymerase adds dNTP's complementary to the tem - plate from 5' to 3', reading the template from 3' to 5' side. The ideal working temperature (mostly 72°C) for the polymerase is used.

PCR Temperature Measurement - The MTAS System

The above-mentioned biological tests show that that both time and correct temperature are of vital importance to generate reproducible PCR results. It is therefore obvious that a cycler should perform accurate and precise, as well as in respect to targeting to the correct temperature in short time with limited overshoots and undershoots, as well as performing as uniform as possible.

We have measured substantial amounts of PCR cyclers in the market. The used measurement system (MTAS) is developed by GENO-tronics, Landgraaf, The Netherlands. It is the most sensitive, accurate and independent PCR cycler temperature measurement system, available world -wide. The system acquires temperature data with a software sampling rate of 2Hz, with 16 independent channels simultaneously. The data is captured dynamically and the measurements are traceable to international temperature standards (ITS-90/NIST). The MTAS systems are accredited by national and international councils of temperature accreditation.

The MTAS system has the following specifications:

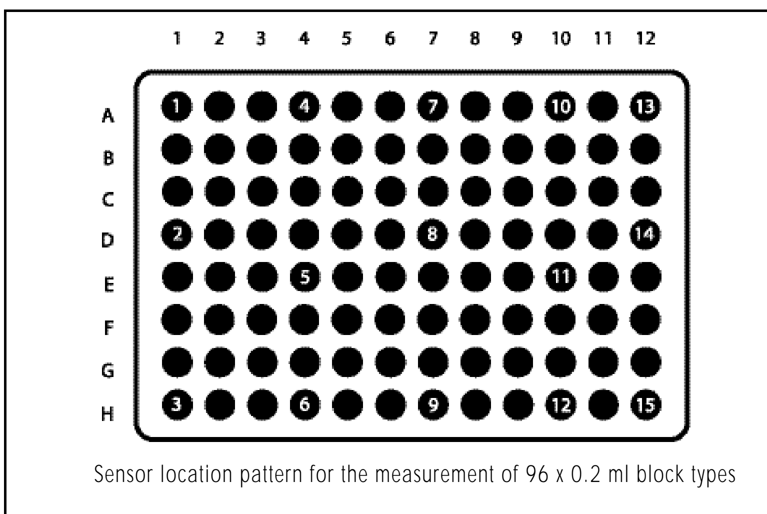
Temperature reference	ITS-90 scale (traceable)
Number of channels	16 channels
Measurement resolution	16 bits
Measurement range	10 to 105 °C
Accuracy per channel	Better than ±0.1 °C
Maximum deviation between channels	Better than 0.05 °C (uncalibrated
Temperature measurement speed (software)	(16 channels x 2 samples) per second
Sensor self-heating	Eliminated by hardware
Independent calibration steps for	- Hardware - Probe sets
Calibration data storage	- In hardware - In each multiprobe set
Accreditation	- CE-mark - Temperature accuracy validation by Dutch Council of Accreditation

The protocol

The MTAS protocol used for testing is described as follows:

MTAS DYN-STAT I Test Protocol:

30 °C	1 minute
95 °C	3 minutes
30 °C	2 minutes
90 °C	3 minutes
50 °C	3 minutes
70 °C	3 minutes
60 °C	3 minutes
30 °C	1 minute



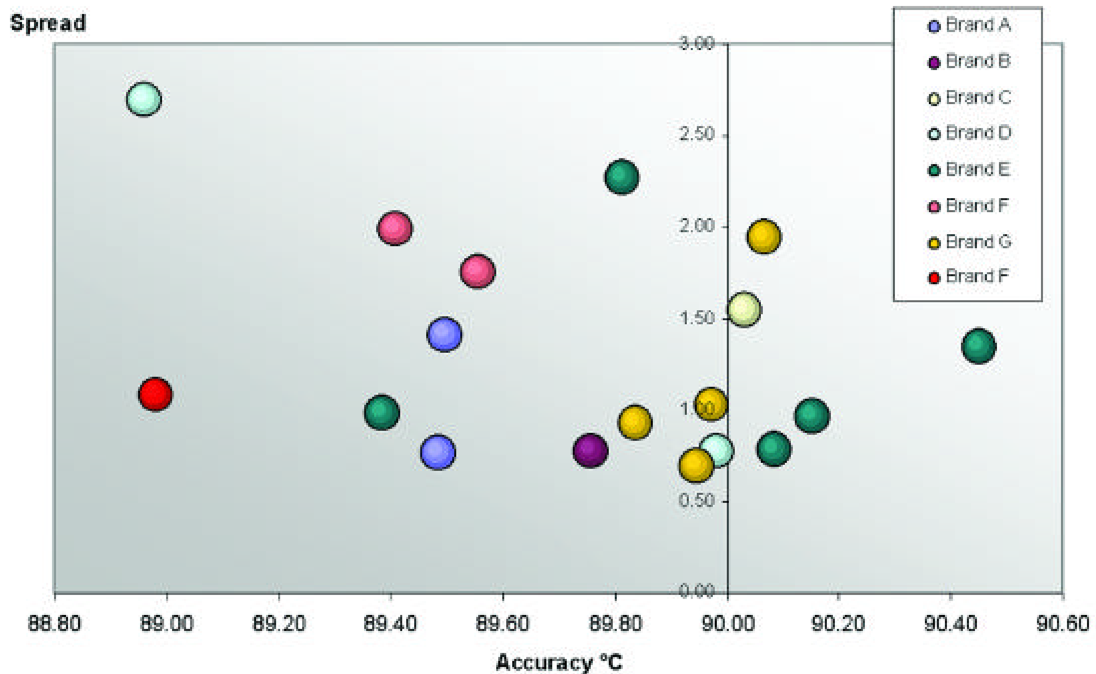
Sensor location pattern for the measurement of 96 x 0.2 ml block types

The test protocol covers all important temperatures of a standard PCR reaction as well as the temperatures that cycler manufacturers use to specify their equipment. In some cases cycler manufacturers are incomplete in providing exact specification data. All measured cyclers were running in block mode.

Tested Cyclers

The following brands and types of cyclers were tested with respect to the accuracy of the block temperature, and the spread of the temperature within the block. Notice that the results showed in the graph are mean values of number of measurement of cyclers from the same brand and block type!

- Biometra T3 Thermocycler 96 x 0.2ml
- Biometra T-gradient 96 x 0.2ml
- Biorad I-Cycler 96 x 0.2ml
- Eppendorf MasterCycler Gradient 96 x 0.2ml
- Hybaid Ltd. Omnigene TR3 CM220 96 x 0.2ml
- Hybaid Ltd. PCR Express 96 x 0.2ml
- MJ Research Inc. PTC-100 96AgV 96 x 0.2ml
- MJ Research Inc. PTC-100 96v 96 x 0.2ml
- MJ Research Inc. PTC-150 25 x 0.2ml
- MJ Research Inc. PTC-200-220-225 48-48 Alpha Unit 48 x 0.2ml
- MJ Research Inc. PTC-200-220-225 96v Alpha Unit 96 x 0.2ml
- MWG Biotech Primus 96 Legal PCR System 96 x 0.2ml
- MWG Biotech Primus HT 9600 96 x 0.2ml
- Perkin Elmer Applied Biosystems GeneAmp PCR System 2400 24 x 0.2ml
- Perkin Elmer Applied Biosystems GeneAmp PCR System 480 48 x 0.5ml
- Perkin Elmer Applied Biosystems GeneAmp PCR System 9600 96 x 0.2ml
- Perkin Elmer Applied Biosystems GeneAmp PCR System 9700 384 microplate
- Perkin Elmer Applied Biosystems GeneAmp PCR System 9700 96 x 0.2ml
- Thermolyne Amplitron II



The displayed results are mean values of multiple measurements of cyclers. Each balloon represents a certain brand and type of cycler.

Conclusions and discussion

The results of the accuracy, spread of temperatures in a "homogeneous sample block", undershoots and overshoots of temperatures in cyclers, are beyond most users' imagination. The impact of the measured temperature performance of cyclers could be substantial to the generated results and conclusions, and is not even depending on brand and type of cycler but also is depending on the position of the investigated sample in the block.

A temperature validation and a detailed report can be used to determine not only the temperature quality of a thermal cycler but also for understanding and explaining cycler-to cycler (same brand) differences and cycler brand-to-brand differences. It can be used for protocol to protocol, as well for cycler-to-cycler transitions. Detailed report values of dynamic temperatures at different locations in the sample block (including undershoots) may be used to adjust the annealing temperature and to minimize non-specific binding of primers to the template. Although not mentioned in this report, multi-sensor equipped cyclers are performing better than single sensor models. The quality of the used Peltiers (hot-cold spots), the "block-sandwich" construction, the quality of heating/cooling regulation, as well as the capability
to "recognize" mass differences has direct effect on the temperature performance of cyclers.

The use of biological positive, negative and threshold controls are insufficient to guarantee trustable PCR results. Temperature validation for cyclers is a necessity. Validation of cyclers should not be limited to laboratories that comply with ISO, GLP, GMP, FDA, Ster-Lab, Bell-test and other norms and regulation but should be useful and recommended for all cyclers and their users in the market. Cycler validation is required for accreditation by several American Associations including e.g. the college of American Pathologists. Furthermore, soon it will be incorporated in European regulations.

At the moment, one temperature validation every half a year or every 100 runs, whatever comes first, is strongly recommended. Validation should be done dynamically with at least 15 channels for a 96-well block. Furthermore it is strongly recommended to check batch to batch quality of the used thermo stable enzymes, since small differences in thermal stability may substantially influence PCR yields and results.

Users using denaturation temperatures over 94 °C, and a denaturation time of 60 seconds or longer, are substantial heat-inactivating their "heat stable" enzyme resulting in possible enzyme-depletion. Generally enzymes depletion can
be avoided by using the correct denaturation temperature. Enzyme depletion caused by "heat inactivation" of the enzyme can and may also be avoided by adding more enzyme (units) to the reaction mixture. However one has to consider that this option is not only extremely expensive, but also greatly increases the chance of enzyme (reaction) inhibition in the first cycles of a run. The described inhibition occurs more often at low target copy numbers.

At the moment, the MTAS system from GENO-tronics is the most accurate and dynamic system worldwide available. Several cycler manufacturers showed interest to incorporate the MTAS systems at their production facilities for internal QC; however all of them are aware that higher "fall outs" during the manufacturing process and Quality Control will occur. The validation service using the MTAS and MTAS DAS systems is offered in the Benelux by CYCLERtest. CYCLERtest is increasing their worldwide distributor network.

MTAS and MTAS-DAS and GENO-tronics are trademarks of GENO-tronics BV The Netherlands
CYCLERtest is a trademark of CYCLERtest the Netherlands
Copyright, CYCLERtest, The Netherlands, August, 2001.