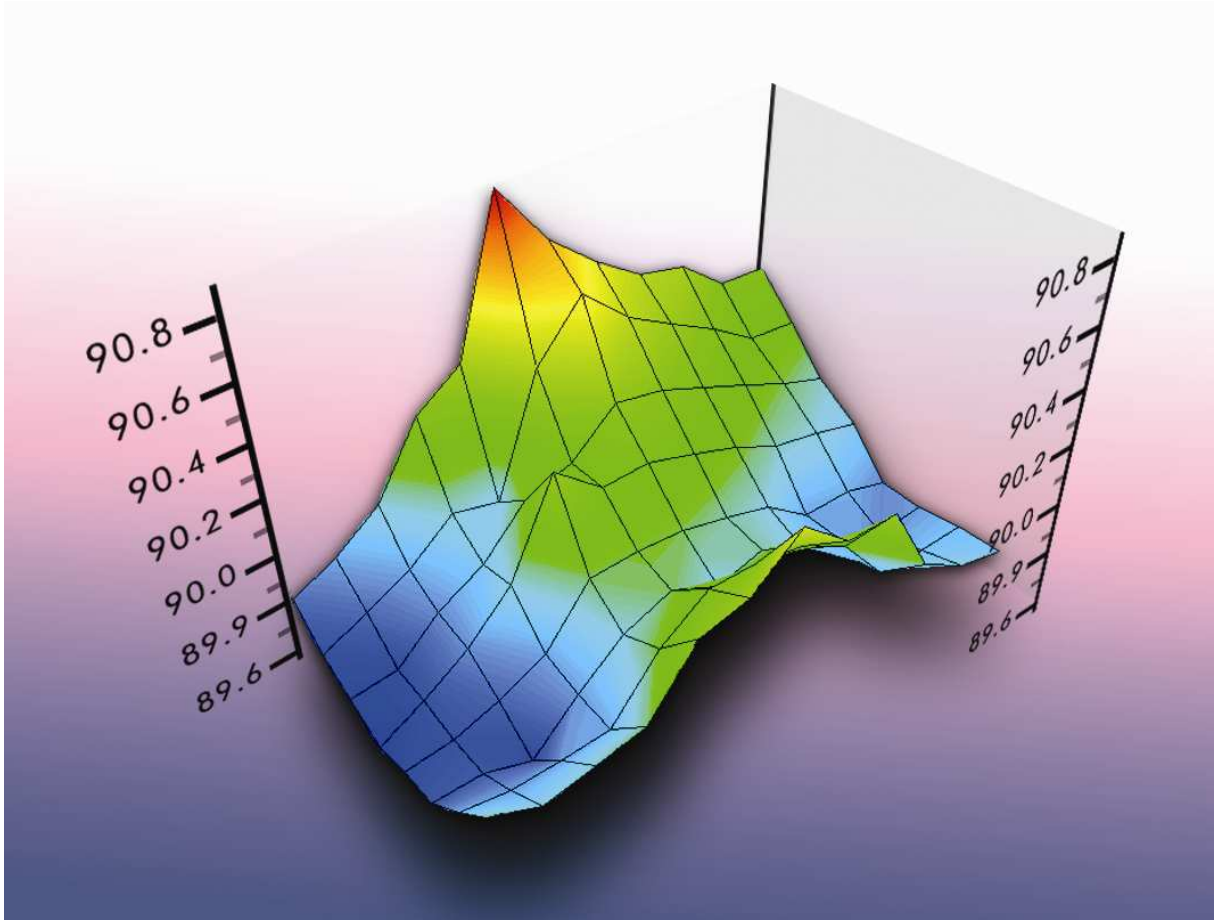
Thermocycler Calibration Guide



Introduction

PCR was invented over 25 years ago by Kary Mullis [Saiki, 1985], for which he received the Nobel Prize in chemistry in 1993 [Malmström, 1997]. PCR is considered to be the innovation which allowed molecular biology to evolve to the current level. It has become an indispensable technique in life science research and more recently in routine human and veterinary diagnostics.

PCR has evolved over the past decades from a technically complicated method to a simple and easy to apply method. There is a wide variety of ready-to-use reagents available that allows those with some basic training and who master the skill of pipetting to perform a PCR. Enzymes and instruments have been continously engineered to speed up the PCR process, so that a PCR can presently be performed in less than half an hour.

However, the simplicity of the method is its strength and weakness at the same time.

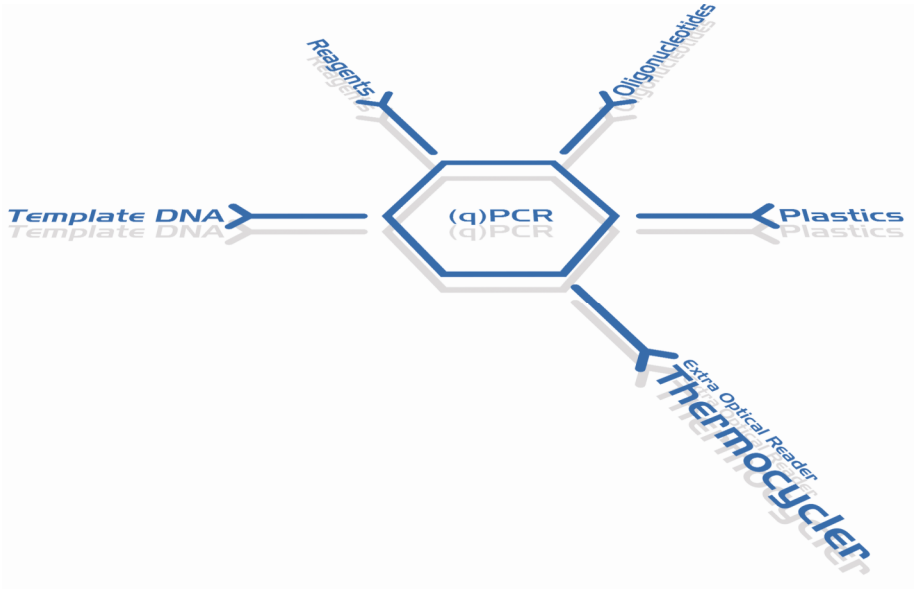
As it is relatively easy to generate a result many PCR users fail to appreciate the quality control that is required to generate reliable and meaningful results.

With the more recent use of PCR in diagnostics the call for quality control is increasing in accredited and quality aware laboratories. An increasing number of laboratories either elect or are required to obtain an ISO 17025 [CEN, 2005] or ISO 15189 [CEN, 2007] accreditation to guarantee the quality of the results generated.

At the same time, the research community's call for biologically meaningful conclusions is increasing in parallel. In 2009 a group of leading PCR scientists published guidelines (MIQE) [Bustin, 2009], that assist qPCR users to design a robust qPCR experiment that leads to trustworthy and biologically meaningful results which can be reproduced in any other laboratory.

The main variables of the (q)PCR reaction are the purity and quality of the template DNA or cDNA, the design, purity and concentration of the primers and probes, the concentration of the different reagents, the type of buffer and the type of enzyme, the tubes, strips or plates and the thermocycler used (figure 1). The vast majority of (q)PCR optimizations are performed on the variables of DNA, primers and template. Yet, very little attention is paid to the contribution of the variability of tubes and thermocycler to the (q)PCR result, as they are incorrectly considered to be constants rather than variables.

Figure 1. Main variables of (q)PCR process



The goal of this Thermocycler Calibration Guide is to illustrate which types of thermocycler variability do exist, show the impact of thermocycler variability on the outcome of PCRs or qPCRs, and offer practical solutions how to eliminate or control thermocycler variability.
The practical protocols allows us to put into practical use the data from CYCLERtest Calibration Certificates and Reports.
Examples will be given showing how thermocyclers can be aligned and programmed to mimic each other. Furthermore, examples will be given showing how calibration results can be used for validation purposes when working under ISO 17025, ISO 15189 accreditation and many other regulations.
This guide will allow PCR and qPCR users to explore the full potential of CYCLERtest thermocycler calibration data.

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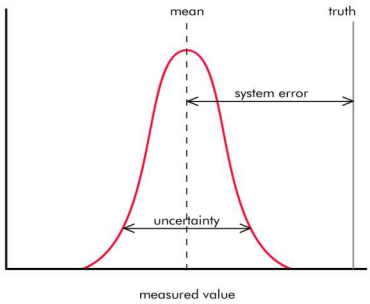
Terms and definitions

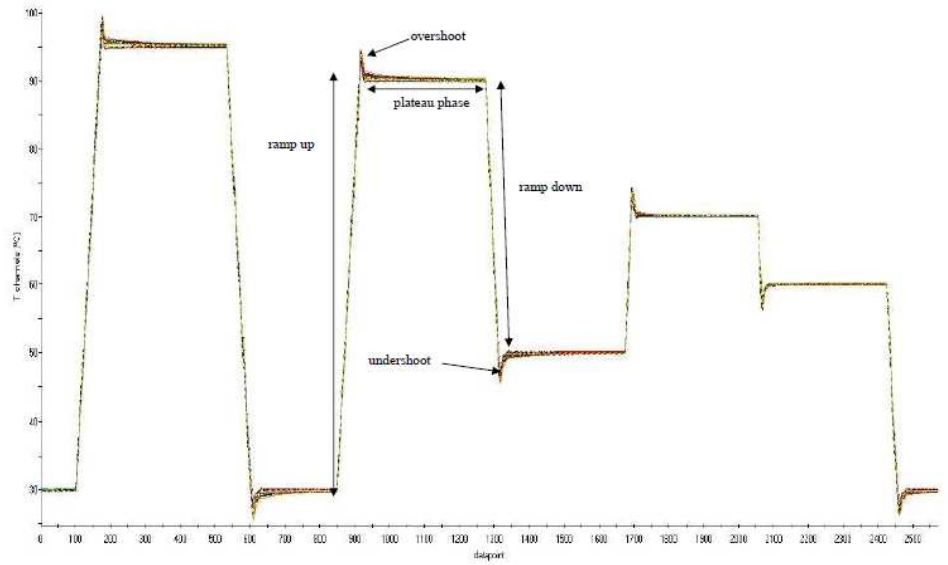
Calibration	The total set of operations that establish, under specified conditions, the relationship between values of quantities indicated by a measuring instrument or measuring system or values represented by a material measure, and the corresponding values realized by an international traceable reference standard. Traceability is guaranteed by the use of international traceable reference standards, by the calculated uncertainty of a calibration and the fact that calibrations are performed by technically competent calibration engineers. Calibration does not include adjustment.
Adjustment	Adjustment of an indicated value of the instrument back within given specifications or tolerances. Adjustments are always accompanied with an as found/as received calibration certificate and an as left/after adjustment calibration certificate.
Validation	Confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are met. For example if certain assays in combination with certain equipment generate the required results.
Verification	Quality control, via a defined procedure, to check if systems still meets the specifications.
Uncertainty	Parameter, associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the quantity intended to be measured
Error	Error is the difference between the measured value and the true value. Error can be divided in a systematic error and random error. The systematic error, also called bias, stays constant during repeated measurements; the random error, also called deviation, varies during repeated measurements. 
Traceability	Property of a measurement, whereby the result, can be related to a reference standard, through an unbroken chain of calibrations, each contributing to the measurement uncertainty. The result should preferably be expressed in SI units and a calculated uncertainty should be indicated.
ITS-90	International Temperature Scale of 1990. The international temperature standard.

MTAS	Professional dynamic thermocycler temperature calibration system, measuring accuracy, uniformity, overshoots and undershoots, ramp rates and hold times of thermocyclers. The MTAS system permits operation only by qualified calibration engineers.
DRIFTCON	End user dynamic thermocycler temperature calibration system measuring accuracy, uniformity, overshoots and undershoots, ramp rates and hold times of thermocyclers. The DRIFTCON system permits operation by end users.
Plateau phase/hold phase	Phase of the PCR process at which temperature is kept steady.
Ramp phase	Phase of the PCR process at which temperature is changing towards the next plateau phase.
Set temperature	The temperature which was programmed to be reached.
Accuracy	Difference between average reaction block temperature and set temperature at a defined moment in time (figure 2b).
Uniformity/Spread	Difference in temperature between hottest and coldest well in the reaction block at a defined moment in time (figure 2c).
Overshoot	Overshooting of temperature, above set temperature when ramping up (figure 2d).
Undershoot	Overshooting of temperature, below set temperature when ramping down. Note: an undershoot is defined as an overshoot going down
Ramp rate	Speed of heating (heat rate) or cooling (cool rate) while cycling up or down.
Hold time/plateau time	Time duration of the plateau phase (figure 2f).
Manufacturer specifications	Technical specifications of a particular brand and model thermocycler as provided by manufacturer. Typically uniformity, accuracy, maximum ramp rate and if applicable maximum gradient are specified. These specifications are typically stated without measurement uncertainty, measurement method and environmental conditions.
Market specifications	Specifications of a particular brand and model thermocycler based on calibrations for that type of thermocycler. The values represent average plus/minus 2 standard deviations and are based on measurements performed with a defined measurement uncertainty, a defined measurement method and under defined environmental conditions. Uniformity and accuracy at several moments in time, ramp rates, average and maximum overshoots plus hold times are specified.

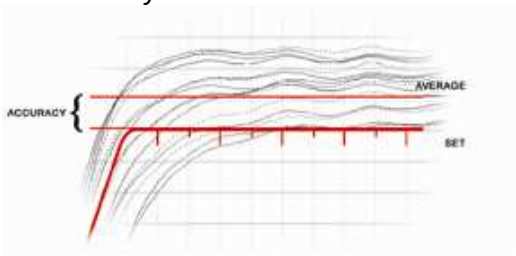
For more detailed metrology definitions please refer to International Vocabulary of Metrology [JCGM, 2008]

Figure 2. Graphical explanation of temperature parameters

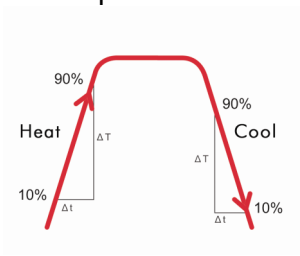
a. Plateau phase, ramp phase, overshoot and undershoot



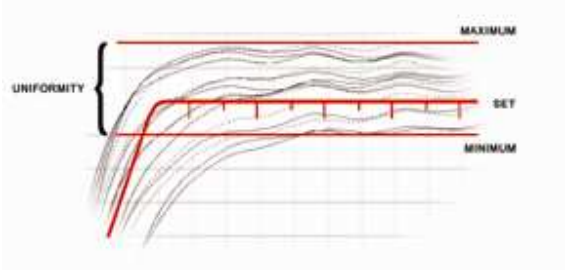
b. Accuracy



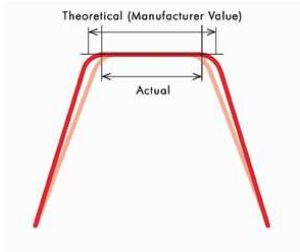
e. Ramp rates



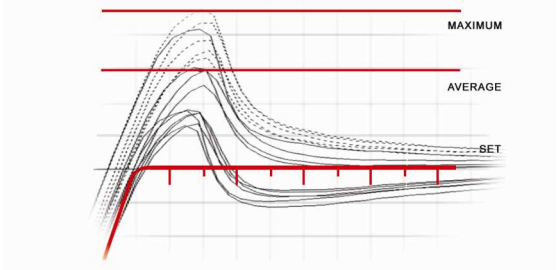
c. Uniformity



f. Hold time



d. Overshoot

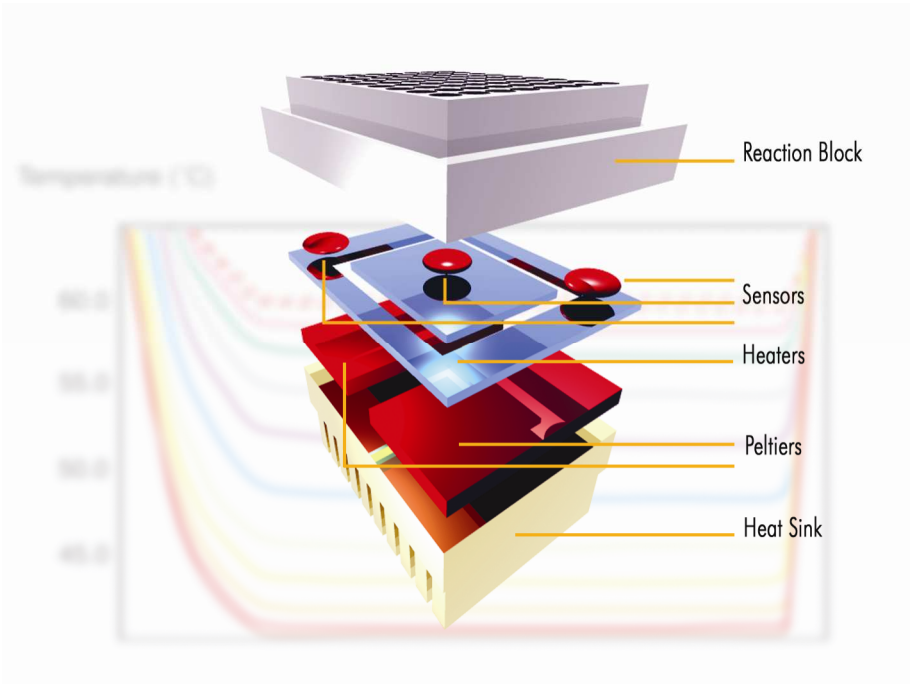


1 Thermocycler technical design

1.1 Various technologies

Since the invention of PCR many different types and models of thermocyclers have been designed and manufactured. Due to the continous development, a wide variety of heating and cooling techniques and temperature control mechanisms have been used in thermocyclers over the past decades.
In the early days of PCR a typicial thermocycler would use a heater combined with a liquid compression cooling system. With the need to design faster thermocyclers, Peltier based thermocyclers were introduced. The latest developments permit completing a PCR under 30 minutes, and use either minimized blocks, ceramic heaters or heated air. However, the Peltier based thermocycler is the most common type of thermocycler.
The principle of a Peltier element is that it either heats or cools dependent on how the electrical current is applied to the elements. As a result these elements can very quickly alternate from heating to cooling and reversely. Most Peltier based thermocyclers are designed according to the sandwich construction as shown in figure 3.

Figure 3. "Sandwich" construction of Peltier based thermocyclers



The reaction block that is visible to the user is the top of the sandwich construction. Underneath the block, one or more temperature sensors are positioned that monitor the reaction block temperature and provide input to the control mechanism that regulates the heat generated by the heater and Peltier element. During the heating phase the heat of the heater and the Peltier element is transferred to the reaction block and then to the reaction tubes and the reagents inside. During the cooling phase the Peltier element gets cold on one side and transfers this cold to the reaction block, on the other side the Peltier element generates heat, which is transfered to the heat sink and then ventilated to the environment via the fan.

1.2 Speed versus uniformity

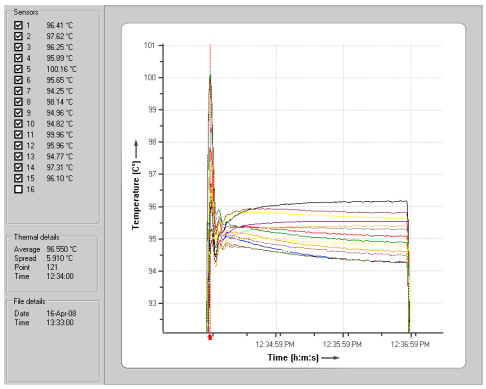
Thermocyclers are designed to function as instruments that can hold a defined steady temperature during a defined time, then change temperature, and then hold again a defined steady temperature during a defined time.
This requirement, as simple as it may seem, is the largest challenge to design and construct an accurate and uniform thermocycler.
To design a fast thermocycler, the mass that needs to be heated should be reduced to a minimum to allow fast energy transfer during heating and cooling. However, to design an instrument that can hold its temperature in a defined, steady and uniform way, a high mass is required. Thermocyclers with massive blocks are much more uniform, but also relatively slow (figure 4a).

Figure 4. Thermal profile of thermocycler with a high mass (a) and low mass (b) block

a. b.

Thermocyclers with low mass blocks are fast, but due to their speed also less controlled and therefore these blocks may easily overshoot the set temperature by several degrees before falling back to the set temperature (figure 4b). Depending on the control mechanism and the number of sensors that monitors the reaction block temperature these fast thermocyclers can not only show high overshoots, but also highly non uniform ramping phases, overshoots and plateau phases. This is especially the case in thermocyclers which are controlled by only a single sensor (figure 5).

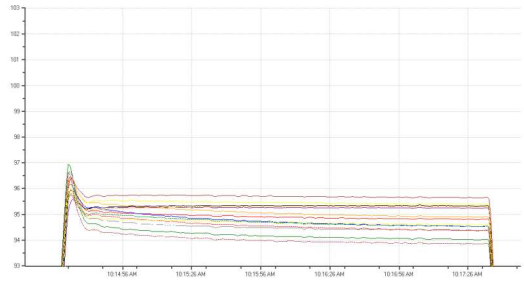
Figure 5. Poor controlled fast thermocycler which results in high overshoots plus high non uniformity, both during overshoot and plateau phase



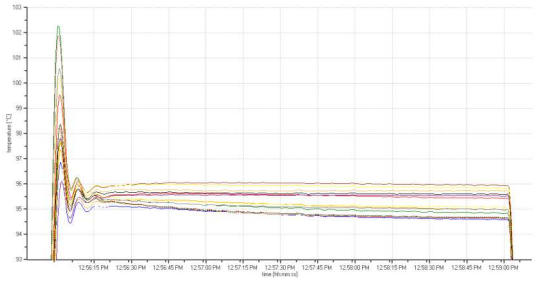
For those thermocyclers that can be programmed in “standard” or “fast” mode, the two different modes can lead to substantially different thermal profiles, which do not only differ in ramp rate, but also in height and duration of the overshoot and, uniformity of both overshoot and plateau phase (figure 6).

Figure 6. Thermal performance of the same thermocycler in standard (a) and fast (b) mode

a.



b.



Summarzing, in block based thermocyclers, high ramping speeds can be associated with high non-uniformities, especially during ramping, and high and poorly controlled overshoots. Therefore, when selecting thermocyclers for certain applications it is worth reviewing what is the more important criterium, speed or temperature uniformity.

2 Thermocycler variability and practical consequences

2.1 Inter and intra thermocycler variability

The thermal performance of a thermocycler is dependent on a number of variables. The main variables are the block “sandwich” construction, the different types and qualities of components used, the differences in technical design, the number of sensors and the temperature control mechanism.

Most PCR users know from experience that some PCRs provide good results on certain thermocyclers, but fail on others. When these thermocyclers are of different brands this is considered to be common sense as they are perceived to be different. However, different models of the same brand are often expected to function similarly while different serial numbers of the same model are considered to perform as identical copies.

As can be seen in the figures below, thermocyclers show a substantial variation, not only between brands (figure 7), but also between models (figure 7 and 8) of the same brand, between individual serial numbers of the same model and brand (figure 8) and even within one thermocycler (figure 9). Each thermocycler has a unique thermal “fingerprint”.

Figure 7. Average temperature accuracy and temperature uniformity of different models and brands of thermocyclers at 30 s at 95 ℃ (dots of t he same color represent thermocyclers of the same brand, size of the dots represent spread within subpopulation)

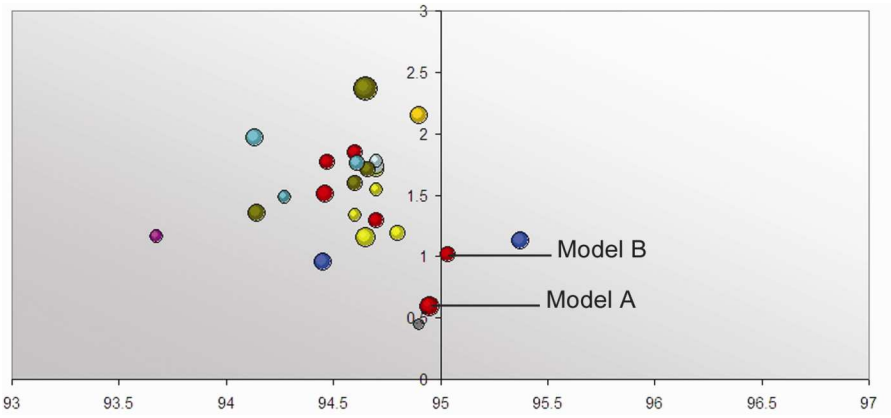


Figure 8. Individual temperature accuracy and non-uniformity at 30 s at 95 ℃ of thermocyclers of model A (▲) and model B (♦) of the same brand (dots represent individual serial numbers)

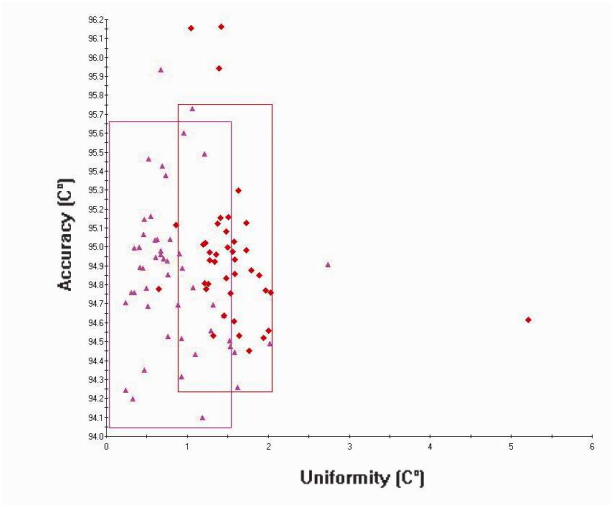
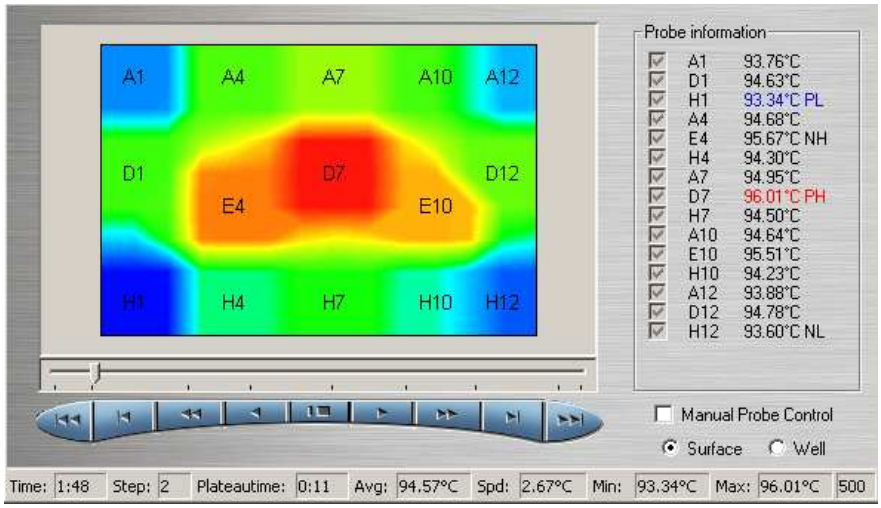


Figure 9. Temperature non uniformity within individual thermocycler block



2.2 Consequences of thermocycler variability

The inter thermocycler variability is the reason why PCRs function on certain thermocyclers and fail on others, or lead to different results on different thermocyclers [Vermeulen, 2009]. The intra thermocyclers variability is the reason why certain wells generate false negatives while in other wells positive results are generated during the same run on the same thermocycler [Adams, 2004].

But thermocycler variability can cause more than just false negatives. The effects move on a sliding scale from slightly less efficient PCRs, which still give a result, to complete failure.

The first category is often not noticed. In case of expression profiling via qPCR this category can lead to incorrect conclusions of gene up or down regulation. Less efficient PCRs with lower yields can, in case of minimal residual disease in leukemia, lead to lower cancer cell counts and a clinical decision to not administer a second chemotherapy, although in reality it is required.

False negatives are extremely risky in general as the can lead to incorrect conclusions and results, treatment or categorization. False negatives can lead , for example, to patients being diagnosed as healthy, whereas in reality they might be infected with a life threatening virus.

3 Effect of thermocycler variability on a PCR or qPCR

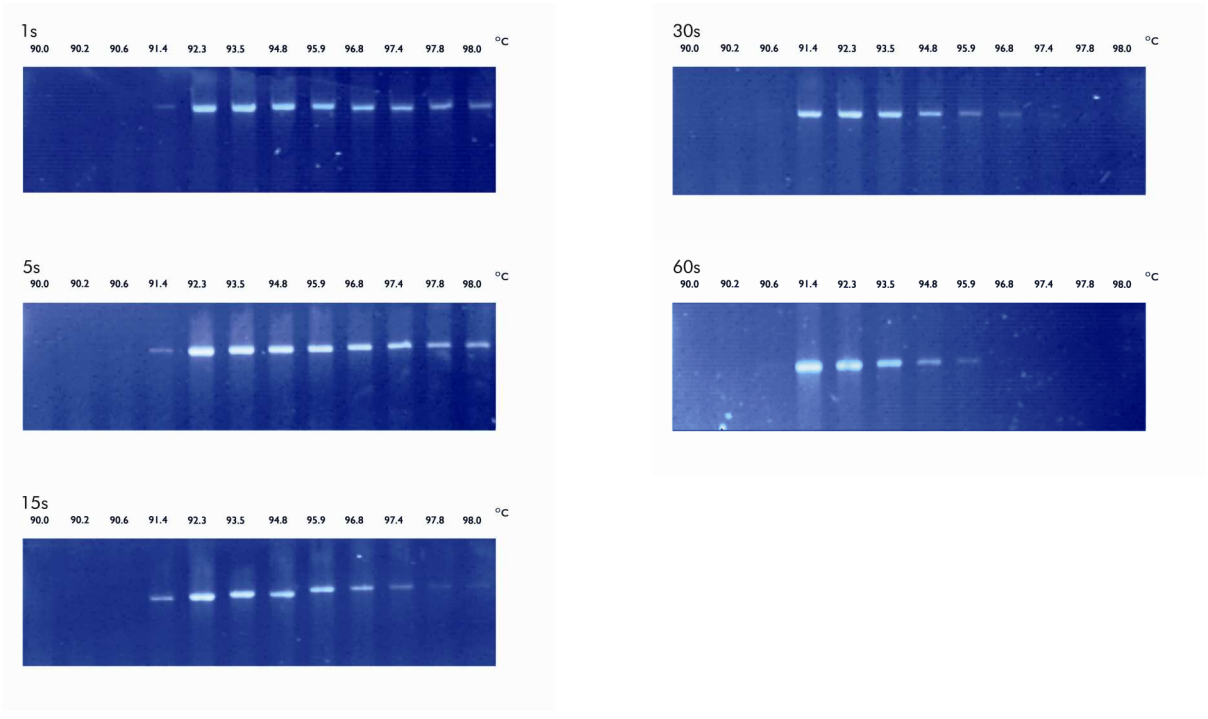
3.1 Different effects on different phases

Because PCR is a dynamic process with ramping phases of different rates and plateau phases of different temperatures, several types of variability can occur. The most common types of variability are: deviating plateau temperatures, high overshoots/undershoots, and non uniform plateau temperatures, ramping phases and overshoots/undershoots.
The effects of these types of variability are dependent on the phase of the PCR, as will be described in the paragraphs below.

3.2 Denaturation phase

The purpose of the denaturation phase is to denature the double stranded DNA, in order to obtain two single strands to which the primers can bind. Denaturation is typically performed at 94 ℃-95 ℃. However, at the same time the *Taq* polymerase is inactivated by the high temperature required for the denaturation. This inactivation is mainly temperature, but also time dependent (figure 10).

Figure 10. Influence of temperature and time (1 s, 5 s, 15 s, 30 s and 60 s) on PCR result during denaturation phase

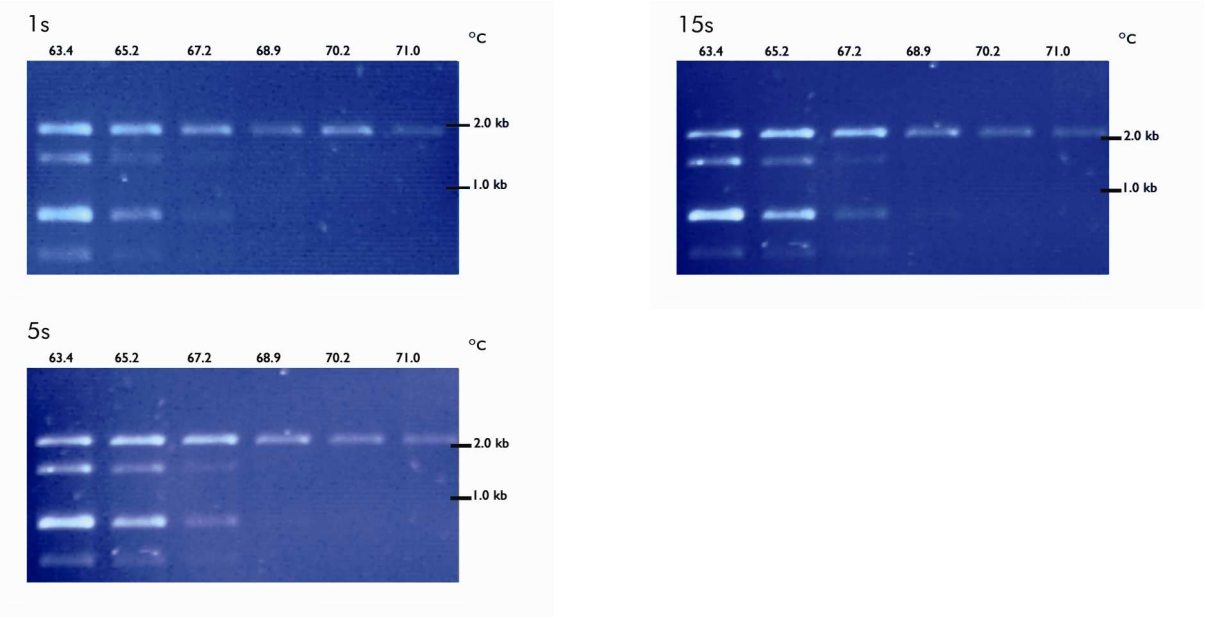


At 95 ℃ the half life time of *Taq* polymerase is 40 minutes, whereas at 98 ℃ it is already reduced to 5 minutes. Therefore incubating a PCR reaction long at high temperatures, or overshooting the target temperature by several degrees, can quickly lead to negative results, due to inactivation of the enzyme before detectable levels of amplicon are generated. Most false negative PCR results are caused by problems in the denaturation phase, especially by high overshoots or high plateau temperatures [Adams, 2004].
If the temperature of the denaturation phase is too low, no denaturation will occur and therefore also no amplification. The minimum denaturation temperature is mainly dependent on the GC content of the DNA and secondary structures. Typically denaturation functions over a temperature range of about 2-3 ℃ at 30 s de naturation time.

3.3 Annealing phase

The purpose of the annealing phase is to allow the primers that initiate the elongation to bind to the correct target sequence. This binding should preferably be as specific as possible. The specificity is highly temperature and salt concentration dependent, but hardly time dependent (figure 11). Annealing is typically performed between 45 ℃ and 70 ℃.

Figure 11. Influence of temperature and time (1 s, 5 s and 15 s) on PCR result during annealing phase



As temperature optimization of a PCR is typically only done for the annealing phase and not for the other phases, the annealing temperature is perceived as the most critical temperature step to allow a PCR to succeed. The annealing temperature is indeed critical for the specificity of a PCR [Uribe, 2004], but much less critical to produce a result at all.
Annealing functions over a much wider temperature range than denaturation. Typically annealing functions over a temperature range of about 5-7 ℃, albeit in varying degrees of specificity. Therefore the risk of missing a result, due to a false negative, is significantly higher in the denaturation phase. Thermal performance problems at annealing phase typically express themselves as non specificity or lower yields and are therefore more easily identified.

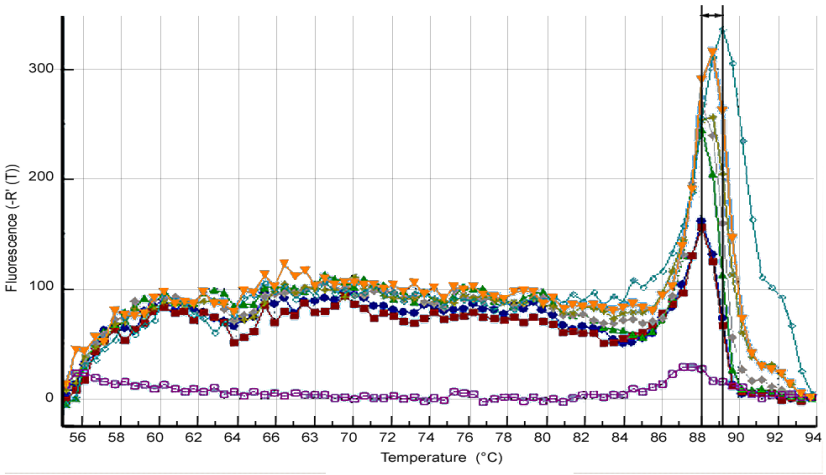
3.4 Elongation phase

The purpose of the elongation phase is to synthesize the new strand of DNA complementary to the template strand. The polymerization rate of *Taq* polymerase is temperature dependent (2000 bases/minute at 72 ℃), but elongation functi ons over a range of 55-85 ℃. To obtain highly efficient PCRs, many users design PCR products to be smaller than 200 bp, which means that elongations only take a few seconds, often much shorter than the protocol times programmed.
Elongation is generally insensitive to temperature variability at the level that occurs in thermocyclers. Non uniformity of the thermocycler could lead to different polymerization rates. However, in general excess times of elongation are used and therefore slower rates are compensated by longer times.

3.5 Meltcurve

Although the meltcurve, as post qPCR analysis step, is not part of the actual qPCR, it also is influenced by thermal variability.
The goal of a meltcurve is to identify if the correct amplicon has been amplified and if no non specific products or primer dimers are present in the reaction. In case of HRM (High Resolution Melting) the goal is genotyping or mutation scanning.
Temperature non uniformity during ramping can lead to shifts in meltcurves as shown in figure 12.

Figure 12. Shifted meltcurves on non uniform thermocycler



What seem to be amplicons of different lenghts and/or sequences, due to their differing Tm (melting temperature), are in reality identical amplicons melted at different moments in time.
The meltcurve plots the 1st negative derivative of the relative fluorescence versus the temperature, but in reality relative fluorescence versus time instead of temperature is plotted. If a thermocycler is non uniform, one well after the other will reach the Tm and therefore a shift in time, depicted as temperature, occurs.

4 Thermocycler calibration methods

4.1 How to calibrate a thermocycler?

With the increasing need to calibrate thermocyclers an increasing number of groups have views on how a thermocycler should be calibrated. These views range from “the molecular biologists view” of mimicking a PCR reaction to “the metrologists view” of calibrating in a defined and traceable manner, by qualified personel, at defined environmental conditions circumstances, with the lowest achievable and known measurement uncertainty. The paragraphs below will discuss the pros and cons of the different views and describe which method of calibrating a thermocycler is the most certain.

4.2 Calibration by mimicking the PCR process

From a molecular biologist perspective the best way to calibrate a thermocycler is by mimicking the PCR process. In other words, put tubes in all wells of the block, fill them with reaction mix and put sensors in, closed the tubes lids, close the thermocyclers heated lid and run the PCR protocol normally used in the laboratory. This would come as close as possible to the real temperature inside a particular tube, filled with a particular reaction mix, during a particular PCR protocol, in a particular instrument. The phrasing already illustrates the major shortcoming of the method in that it can not be used in a standardized way. For each combination of tube, mix, protocol and instrument this “calibration” should be repeated, requiring a tremendous workload, generating results that can neither be compared to each other nor to any kind of standard, nor to specifications of the thermocycler manufacturer.
Furthermore, this way of “calibrating” would introduce many variables which are uncontrolled and therefore add a large component to the measurement uncertainty, ending up with uncertainties well over 2 ℃.
Practically, when the uncertainty would be for example 2 ℃, while measuring 96 ℃, the thermocycler’s temperature could be anywhere between 94 ℃ and 98 ℃. As described in paragraph 3.2 this difference in temperature does have tremendous effects on the inactivation of the *Taq* polymerase. Therefore this level of uncertainty is not acceptable for thermocycler calibration, although mimicking the PCR process may seem a simple and attractive choice.

4.3 Calibration representative of the process

From a clinical chemist perspective the best way to calibrate a thermocycler is by measuring in a standardized method representative of the PCR process. This standardized method allows one calibration for all the different types of PCRs used and also allows comparison to a standard.
For a calibration to be representative of the PCR process it needs to take into account both the dynamic and the static part of the PCR process, as certain problems can not be diagnosed by just checking the static part of a PCR [Adams, 2004]. Furthermore, a number of different temperatures, preferably close to the denaturation, annealing and elongation temperatures, should be evaluated since effects can vary with temperature (see chapter 3).

4.4 Calibration conforming to international standards

From a metrologist perspective the best way to calibrate a thermocycler is measuring in a defined and traceable manner, by qualified personel, at defined environmental conditions, with the lowest achievable and known measurement uncertainty, excluding as many non controlled variables as possible.
In metrology, calibration is defined as the total set of operations that establish, under specified conditions, the relationship between values of quantities indicated by a measuring

instrument or measuring system or values represented by a material measure, and the corresponding values realized by an international traceable reference standard.
Traceability is guaranteed by the use of international traceable reference standards and the expression of the calibration values in SI units, but also by the calculated uncertainty of a calibration and the fact that calibrations are only performed by technically competent and qualified calibration engineers. The uncertainty should be as small as technically possible.
Only if the requirements above are met a calibration is considered to be fully traceable to the ITS-90 (International Temperature Standard). To be compliant to the ISO 10725 standard [ISO, 2005], additional requirements need to be met.
Any PCR test laboratory that is accredited under ISO 17025 or ISO 15189 or any CE-IVD PCR kit manufacturer that is certified under ISO 13485, should, according to these international standards, calibrate their thermocyclers in a traceable way and therefore conform to ISO 17025.

4.5 Most certain method of calibrating a thermocycler

For a calibration to be certain, compliant to ISO 17025, and representative of the PCR process, a number of criteria should be met.
The calibration should be (1) measuring all thermal characteristics that influence the outcome of a PCR, including accuracy, uniformity, overshoot/undershoot, heat/cool rate and hold time, (2) performed in a dynamic way, as PCR is a dynamic and not a static process, (3) performed simultaneously with multi-channels to exclude any time effects between wells, (4) performed under controlled environmental conditions, (5) performed by qualified and trained personal and (6) traceable to the ITS-90 and expressing calibration values in SI units with a calculated uncertainty.
To be able to meet these criteria a typical thermocycler calibration system will be a physical sensor-based multichannel system that measures dynamically with a frequency of more than once per second and that can be calibrated traceable to the ITS-90 via a temperature reference standard (Hendrikx, 2003). To minimize the measurement uncertainty, the influence of poorly controlled variables like reaction tubes and thermocycler heated lids is excluded.
By calibrating with traceably calibrated equipment, under defined environmental conditions, with qualified personal, directly in the block, in a statistically relevant number of the wells, it is possible to obtain total measurement uncertainties as low as 0.1 ℃ for a multichannel system. This allows making a certainty statement about the measured value and allows comparison to specifications, either lab defined or manufacturer defined.

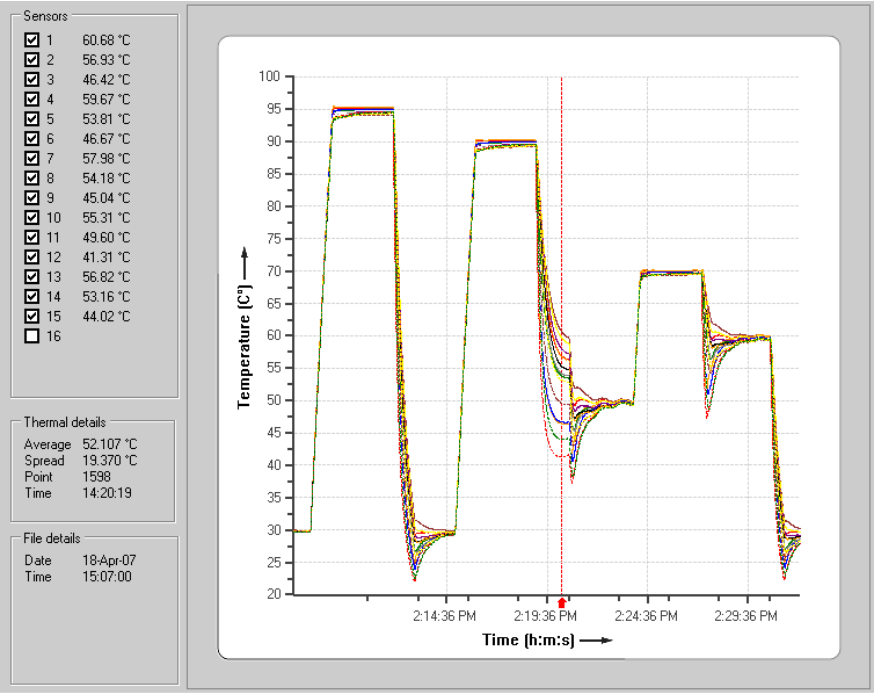
The following methods are either uncertain, not ISO 17025 compliant or not representative of the PCR process due to the reasons discussed.
Any method based on PCR assays, qPCR assays, TLCs (thermochromic liquid crystals), single channel thermometers (analog and digital) and multichannel static measurements will not show all of the characteristics that determine the thermal profile of a thermocycler and are therefore these methods are not representative of the PCR process. Any method based on in-tube measurements is linked to large uncertainties due to contact errors between tubes and reaction block, and errors due to non uniformity in wall thickness and heat conductivity of the tubes. Results are therefore connected to high uncertainties, disallowing certainty statements about the thermocyclers' performance. Any method based on measuring all wells of the reaction block simultaneously will require the addition of a substantial mass to the block, and will lead to biased dynamic values since ramp rates and overshoots can be strongly influenced by the addition of mass, leading to incorrect results.

5 Evaluating calibration results

5.1 Introduction

Calibration results are often evaluated by categorizing a thermocycler to be “in” or “out” of manufacturer specifications. However, evaluating the thermal performance of a thermocycler goes far beyond categorizing a thermocycler to be in or out of specifications. For example, a thermocycler can be well within manufacturer specifications for accuracy and uniformity at 95 ℃ and, at the same time show severe cooling pro blems and high non uniformity at annealing temperatures (figure 13). Therefore, to evaluate the performance of a thermocycler it is necessary to review the complete data in a calibration report.

Figure 13. Thermocycler that is in specifications, but has severe cooling problems



5.2 CYCLERtest calibration reports

CYCLERtest offers a calibration service using the MTAS system, and an end user calibration system that is called the DRIFTCON system.

Both systems measure the same thermal characteristics of accuracy, uniformity, overshoot/undershoot, heat/cool rate and hold time, but present them differently in the reports.

DRIFTCON provides information about uniformity at defined moments in time during plateau phases, whereas MTAS provides information about uniformity during the complete calibration, including uniformity during ramping and overshooting/undershooting.

DRIFTCON provides graphical presentation of the results in a thermal map in the software whereas MTAS reports contain overview graphs of all data, average data and uniformity data, plus detailed graphs of every static step. For more detailed information please refer to either DRIFTCON or MTAS reports.

5.3 MTAS calibration certificates and reports

MTAS calibration certificates and reports are very detailed and contain an extensive amount of information.
The calibration certificates contain the thermal performance categories that categorize a thermocycler to function in or out of manufacturer and market specifications.
The reports contain a data overview chart, average chart and uniformity chart that are highly informative and give a good overview of the thermocycler's performance. In addition to the graphs, the reports contain static data tables with detailed information of all channels at defined moments in time.
To evaluate the thermocycler's performance it is advisable not just be review the certificate, but also the complete data in the report, especially the overview graphs.
The guidelines in table 1 can be used as a check list to verify thermocyclers performance based on MTAS reports. The guidance criteria in table 3 can be used to categorize a thermocycler based on objective universal values.

5.4 DRIFTCON calibration reports

DRIFTCON reports are less detailed than MTAS Certificates and Reports, but still contain a large amount of information. The data in DRIFTCON reports are presented by default as values after 30 seconds at plateau phase. However, the DRIFTCON software provides a setting to report data after 15 and 90 seconds plateau phases.
DRIFTCON reports contain recommended control positions. The recommended control positions are the hottest and coldest spots of the thermocycler during the first 10 seconds of the 95 ℃ step. By putting the positive and negativ e controls of an assay on these positions it is possible to determine if an assay functions in the wells which represent the temperature extremes of a thermocycler.
In DRIFTCON reports the calibration data are only compared to market specifications if the default protocol is selected. By a green check, blue exclamation mark, or red cross the thermocycler is categorized to be in or out of market specifications for heat rate, hold time, maximum overshoot, average overshoot, accuracy and uniformity.
To evaluate the thermal performance of the thermocycler it is not advisable to just categorize the thermocycler to be in or out of market specifications, but also to evaluate the complete data in the calibration report to assure the thermocycler is suitable for the assay. This is called assay validation. Chapter 7 describes how these assay validations can be performed for PCRs and qPCRs, taking into account the thermocycler variability.
The guidelines in table 2 can be used as a check list to verify thermocycler performance based on DRIFTCON reports. The guidance criteria in table 3 can be used to categorize a thermocycler based on objective universal values. These guidance criteria are based on uniformity and overshoots/undershoots, as these characteristics of a thermocycler can in most cases not be directly modified by programming the thermocycler differently. The average temperature (accuracy) can be adjusted by modifying the set temperature and therefore is not the most crucial criterium for categorizing a thermocycler.
Chapter 6 discusses how thermocyclers can be adjusted and aligned by modifying programmed protocols.

Table 1. Thermocycler performance guidelines for MTAS calibration reports

Item calibration report	Comment	Performance criterium to check
Results overview		
Manufacturer specifications	Manufacturer specifications are specifications as provided by the thermocycler manufacturer. These specifications are stated without any measurement uncertainty, measurement method and environmental conditions. The manufacturer specifications allow categorizing a thermocycler to be in or out of manufacturer specifications.	Check if thermocycler is specified at 90 ℃ or 95 ° C as thermocyclers specified at 90 ℃ or 95 ℃ are typically more unif orm than those specified at 70 ℃ or 50 ℃. Check if thermocycler is in or out of specifications for accuracy, uniformity, heat and cool rate.
Market specifications	Market specifications are specifications based on all calibrations done on a particular brand and model thermocycler. The value represents average ± 2 standard deviation and data are based on measurements performed with a defined uncertainty. The market specifications allow categorizing a thermocycler as being representative for the total population or not.	Check if thermocycler is specified at 90 ℃ or 95 ° C as thermocyclers specified at 90 ℃ or 95 ℃ are typically more unif orm than those specified at 70 ℃ or 50 ℃. Check if thermocycler is in or out of specifications for accuracy, uniformity, heat and cool rate.
Static results	Static results at 30 seconds after set plateau temperature was reached. This table provides a summary of minimum temperatures, maximum temperatures, maximum overshoots, average overshoots, average temperatures, deviation from set temperatures and uniformities at all plateau temperatures. This table allows a good overview of the thermocycler at all measured temperatures.	Check for extreme values in all columns, especially in maximum overshoot, average overshoot and uniformity. See table 2a and b for categorization.
Data chart		
Curve morphology	The curve morpohology is dependent on brand and model of thermocycler, either a curve in which the plateau temperatures are approached, or a curve with overshoots after which the plateau temperatures are achieved.	Check if thermocyclers of the same brand and model show similar curve morphologies.
Outlying channels	Outlying channels are an indication of extreme hot or cold spots in block.	Check if one or more channels lie above or below the rest of the curves.
Large temperature non uniformities	Large non uniformities are an indication for suboptimal thermal control of the reaction block and are more frequently found in thermocyclers monitored by a single sensor or in worn out blocks.	Check if no large non uniformities are present during ramping, overshoot or plateau. See table 3a for categorization.
Divergation/convergation of channels at plateau phase	Divergation of channels at plateau phase is an indication for suboptimal thermal control of the reaction block. Typically plateau uniformity improves (converges) when a thermocycler is programmed for a longer time at plateau phase.	Check if divergation/convergation of channels at plateau phase occurs.
Height of overshoots	High overshoots are an indication for suboptimal thermal control of the reaction block and can be typically found in fast or air driven thermocyclers. High average overshoots at 95℃ lead to increased *Taq* polymerase inactivation. High average overshoots at 50℃ and 60℃ can cause mispriming during annealing.	Check if overshoots do not exceed plateau temperatures by more than 5 ℃ for all temperatures.
Slow ramping going down	Slow ramping going down can be and indication of cooling problems, either caused by the environment or the instrument. In air driven thermocycler that cool by convection the lowest achievable temperature is typically 10-15 ℃ above ambient temperature.	Check if no slow ramping going down occurs. If slow ramping occurs check if room temperature is within range as indicated by manufacturer, if the cycler-to-cycler or cycler-to-wall distance is more than 20 cm and/or if fan is not clogged by dust or malfunctioning.
Average chart		
Average temperature	Large deviations in plateau temperatures from set temperatures can already be identified in the average chart.	Check visually if average temperature does not strongly deviate from set temperature.

Curve morphology	The curve morphology of the average chart can differ from the data chart. Sometimes clusters with overshoots and clusters without overshoots can be observed. This can indicate left-right or top-bottom effects. The effects can be caused by variations in the heating mechanism or by wearing of the reaction block over time.	Check if curve morphology of average chart is different from data chart. If different, analyze data per cluster of curves with identical curve morphology.
Uniformity chart		
Uniformity during ramping	Large non uniformities during ramping are an indication for suboptimal thermal control of the block. Fast thermocyclers typically show larger non uniformities during ramping than standard thermocyclers. Typically the ramping uniformity improves when the ramping speed is reduced. Furthermore, the ramping uniformity improves when the difference in temperature between two plateaus decreases, i.e. the ramping non uniformity while heating from 30℃ to 95℃ is larger than while heating from 50℃ to 70℃.	Check if no large non uniformities are present during ramping. See table 3a for categorization. Check if ramping uniformity improves with decreasing delta temperature.
Uniformity during overshoot	Large non uniformities during overshoots are an indication for suboptimal thermal control of the block. Fast thermocyclers typically show larger non uniformities during overshoots than standard thermocyclers. Typically, the overshoot uniformity improves as the set temperature gets closer to the environmental temperature.	Check if no large non uniformities are present during overshoots. See table 3a for categorization. Check if overshoot uniformity improves with decreasing set temperature.
Uniformity during plateau	Large non uniformities during plateau phases are an indication for suboptimal thermal control of the block. Fast and single sensor thermocyclers frequently typically show larger non uniformities during plateau phases than standard and multi sensor thermocyclers. Typically the uniformity improves as the set temperature gets closer to the environmental temperature.	Check if no large non uniformities are present during plateaus. See table 3a for categorization. Check if plateau uniformity improves with decreasing set temperature.
Ramp results		
Average ramp rate	The average ramp rate is determined between 10% and 90% of the ramp. The ramp rate is strongly dependent on brand and model of thermocycler. Typically cooling rates are slower than heating rates, especially in air driven thermocyclers. Thermocyclers with fast ramp rates typically show higher overshoots, especially in air driven thermocyclers.	Check if thermocycler is in or out of specifications.
Maximum ramp rate	The maximum ramp rate is determined between 10% and 90% of the ramp and represents the point during the ramp where the thermocycler heats or cools the fastest. Fast thermocyclers typically show larger non uniformities during ramping than standard thermocyclers. Furthermore, the ramping uniformity improves when the difference in temperature between two plateaus decreases, i.e. the ramping non uniformity while heating from 30℃ to 95℃ is larger than while heating from 50℃ to 70℃.	Check if thermocycler is in or out of specifciations. Check if ramping uniformity improves with decreasing delta temperature.
Room conditions		
Temperature	Room temperature is not a characteristic of the thermocycler, but does influence its performance. At room temperatures above 28℃ slower cooling can be observed. In air driven cyclers also failure to reach set temperatures of 50℃ and lower can be observed.	Check if room temperature does not exceed 28 ℃ in case of slow cooling (see data chart and ramp results). Check if environmental conditions are within limits of operation conditions as recommended by the thermocycler manufacturer.
Relative humidity	Relative humidity is not a characteristic of the thermocycler, but does influence its long term performance. High relative humidities can lead to condensation inside the thermocycler during cooling, which may reduce the life span of a thermocycler, and are often induced by frequent overnight 4℃ steps.	No direct influence on thermocycler visible, only increased wearing over time. Can only be checked by comparing calibration reports over time. Check if environmental conditions are within limits of operation conditions as recommended by the thermocycler manufacturer.

Static data at all temperatures		
Hold time	The hold time is the duration of the plateau phase.	Check if hold time corresponds to protocol of instrument (page 4 of report)
Temperature per channel per time point	Values at 30s are most representative as thermocycler has completed overshoot or approach of plateau and has had some time to reach equilibrium.	Check for values strongly deviating from set temperature
t90(avg), st. dev.	t90(avg) ± st.dev. is the average temperature of all active channels ± standard deviation. Typically t90(avg) shows less deviation from set temperature with increasing hold times.	Check if t90(avg) approaches better tset with increasing hold time
Uniformity	Uniformity improves with increasing hold times.	Check if uniformity improves with increasing hold times See table 3a for categorization.
t90(avg)-tset	t90(avg)-tset is the deviation between average temperature and set temperature. If thermocycler needs to be adjusted because it is too low or high in temperature take the 30 s value and correct by programming the thermocycler respectively -(t90(avg)-tset)℃ higher.	Check value of t90(avg)-tset and if necessary correct for the plateau temperatures of the assay protocols by –(t90(avg)-tset)℃ to obtain the required reaction block temperatures.
Uncertainty	Uncertainty of measurement is not a characteristic of the thermocycler, but a requirement for a calibration to be ISO 17025 compliant calibration. In MTAS reports the uncertainty is not taken into account for the categorization.	
Minimum	Minimum temperature. Minimum temperatures that strongly deviate from the average temperature are an indication for cold spots.	Check if minimum is not strongly deviating from average temperature. In case of extreme cold spots over all moments in time avoid using the well.
Maximum	Maximum temperature. Maximum temperatures that strongly deviate from the average temperature are an indication for hot spots.	Check if maximum is not strongly deviating from average temperature. In case of extreme hot spots over all moments in time avoid using the well.
Average overshoot	High average overshoots at 95℃ lead to increased *Taq* polymerase inactivation. High average overshoots at 50℃ and 60℃ can cause mispriming during annealing.	Check if overshoots do not exceed plateau temperatures by more than 5℃ for all temperatures. See table 3b for categorization.
Maximum overshoot	High maximum overshoots at 95℃ lead to increased *Taq* polymerase inactivation. High maximum overshoots at 50℃ and 60℃ can cause mispriming during annealing.	Check if overshoots do not exceed plateau temperatures by more than 5℃ for all temperatures. See table 3b for categorization.
Detailed graphs at all temperatures		
Overshoot	High and long overshoots at 95℃ lead to increased *Taq* polymerase inactivation. If the overshoot at 95℃ is highly non uniform the *Taq* polymerase inactivation can vary substantially per well and can lead to positive results in certain reactions and false negative in others. High and long overshoots at 50℃ and 60℃ can cause mispriming during annealing. If the overshoot at 50℃ and 60℃ is highly non uni form mispriming can occur in certain wells and not in others, leading to non specific results in certain wells and not in others.	Check if overshoots do not exceed plateau temperatures by more than 5 ℃ for all temperatures. Check if overshoot does not last longer than 10 seconds for all temperatures. Check if all channels go through overshoot for all temperatures. Check non uniformity during overshoot for all temperatures. See table 3b for categorization.
Plateau	Oscillation and divergation during the plateau phase at 50℃ and 60℃ can cause mispriming during annealing. Oscillation and divergation during the plateau phase at 95℃ can cause variation in *Taq* polymerase inactivation and therefore variation in yields.	Check for oscillation at all temperatures. Check for divergation at all temperatures.

Table 2. Thermocycler performance guidelines for DRIFTCON reports

Item calibration report	Comment	Performance criterium to check
Recommended control positions		
Positive/Negative	Positive indicates the hottest and coldest spot of the reaction block during the first 10 seconds of the 95℃ step. Negative indicated the second hottest and second coldest spot of the reaction block during the first 10 seconds of the 95℃ step During the denaturation phase false negatives can be caused by a too low temperature or a too high temperature. If the temperature is too low no denaturation will occur and therefore also no annealing. If the temperature is too high, fast inactivation of the *Taq* polymerase will occur leading to premature stopping of the reaction, before the detection threshold is exceeded. By positioning positive controls on the most extreme position it is possible to control for both these effects. Negative controls are positioned on the second hottest and second coldest spot.	Check if hot and cold spots give abberant results in a PCR test or put a positive control on the hottest and coldest spot and a negative control on the second hottest and second coldest spot. Take a positive control of the same order of magnitude as the samples, as strong positive controls might still function while lower copy number samples might start to fail.
Environmental conditions		
Temperature	Room temperature is not a characteristic of the thermocycler, but does influence its performance. At room temperatures above 28℃ slower cooling can be observed. In air driven cyclers also failure to reach set temperatures of 50℃ and lower can be observed.	Check if room temperature does not exceed 28℃ in c ase of slow cooling (see step results of 30 ℃, cool rate). Check if environmental conditions are within limits of operation conditions as recommended by the thermocycler manufacturer.
Relative humidity	The relative humidity is not a characteristic of the thermocycler, but does influence its long term performance. High relative humidities can lead to condensation inside the thermocycler during cooling, which may reduce the life span of a thermocycler, and are often induced by frequent overnight 4℃ steps.	No direct influence on thermocycler visible, only increased wearing over time. Can only be checked by comparing calibration reports over time. Check if environmental conditions are within limits of operation conditions as recommended by the thermocycler manufacturer.
Pressure	The air pressure is not a characteristic of the thermocycler, but does influence its performance. At altitudes above 4000 m the measurement uncertainty can be higher than stated.	No direct influence on thermocycler performance visible.
Values after 30 seconds at all temperatures		
Measured	Measured indicates the measured temperature after 30 seconds into plateau phase. + and – indicate hottest and coldest channel after 30 seconds. These channels can differ from the channels indicated under Recommended control positions, as they are calcualted in a different way.	Check for extreme temperatures. Check if minimum/maximum is not strongly deviating from average temperature. In case of extreme cold/hot spots over all moments in time, at all temperatures, avoid using the well.
T(meas-set)	T(meas-set) indicated the difference between set temperature and measured temperature after 30 seconds into the plateau phase.	Check for temperatures strongly deviating from set temperature.
Status	Status indicates if the channel was active during the measurement. This is based on deviation of the channel from the average measured temperature. If this deviation is too wide this can be an indication that the channel is not functioning correctly. However, it can also be the thermocycler causing this large deviation. To identify the cause repeat the measurement with the fixture turned 180°, so se nsor 1 ends up in well H12. If the problem moves with the sensor, the sensor is malfunctioning. If the problem occurs on a different sensor, the thermocycler is causing the deviation, which is then real.	Check if all channels stay active during the whole measurement. If sensors become inactive and are not visbily broken turn fixture 180° and repeat measurement to determine if channel is broken or thermocycler has certain largely deviating well temperatures.

Step results		
N	N indicates the number of data sets on which the market specifcations have been based. The way of calculating the standard deviation is different with smaller numbers and less certain. Market specifications become more certain with increasing numbers of measurements and can also change over time. Therefore it is possible that a thermocycler which has given close to identical measured values over time, has been in specifications over many years and can get out of specifications because of the narrowing of the specifications. Take into account that specifcations based on N<50 are wider than specifications based on N>250 data sets.	Check if market specifications have stayed identical when comparing actual DRIFTCON reports with historical DRIFTCON reports as specifications do narrow with increasing numbers of measurements.
Heat rate	In DRIFTCON reports heat rates are only calculated in the 30-95°C ramp. The way of determining differs slightly from MTAS and therefore the MTAS and DRIFTCON heat rates are not directly comparable.	Check if measured result is within market specification.
Cool rate	In DRIFTCON reports cool rates are only calculated in the 95-30°C ramp. The way of determining differs slightly from MTAS and therefore the MTAS and DRIFTCON heat rates are not directly comparable.	Check if measured result is within market specification. If slow cooling occurs check if room temperature is within range as indicated by manufacturer, if the cycler-to-cycler or cycler-to-wall distance is more than 20 cm and/or if the fan is not clogged by dust or malfunctioning.
Plateau start	Moment of plateau start	
Hold time	Duration of plateau phase	Check if measured result is within market specifications. Check if measured result is corresponding with programmed time.
Max. overshoot	Max. overshoot is maximum overshoot during heating and maximum undershoot during cooling. High maximum overshoots at 95°C lead to increased *Taq* polymerase inactivation. High maximum overshoots at 50°C and 60°C can cause mispriming during annealing.	Check if measured result is within market specification. Check if overshoots do not exceed plateau temperatures by more than 5 °C for all temperatures. See table 3b for categorization.
Avg. overshoot	Avg. overshoot is average overshoot during heating and average undershoot during cooling. High average overshoots at 95°C lead to increased *Taq* polymerase inactivation. High average overshoots at 50°C and 60°C can cause mispriming during annealing.	Check if measured result is within market specification. Check if overshoots do not exceed plateau temperatures by more than 5 °C for all temperatures. See table 3b for categorization.
Accuracy results		
N	N indicates the number of data sets on which the market specifcations have been based. The way of calculating the standard deviation is different with smaller numbers and less certain. Market specifications become more certain with increasing numbers of measurements and can also change over time. Therefore it is possible that a thermocycler which has given close to identical measured values over time and has been in specifications over many years, can get out of specifications because of the narrowing of the specifications. Consider specifcations based on N<50 as less certain than specifications based on N>250.	Check if market specifications have stayed identical when comparing actual DRIFTCON reports with historical DRIFTCON reports as specifications do narrow with increasing numbers of measurements.
Measured	Measured indicates accuracy results at a particular moment or within a certain time interval during a particular plateau phase. By comparing the values of 15, 30 and 90 seconds it is possible to check for divergation or convergation during the plateau phase. Divergation during the plateau phase at 50°C and 60 °C can cause mispriming during annealing. Divergation during the plateau phase at 95°C can ca use variation in *Taq* polymerase inactivation and therefore variation in yields. If a thermocyclers should be alligned take the 30 seocnds value.	Check if measured results are within market specifications. Check if no drift occurs during plateau phase by comparing 15, 30 and 90 seconds values. Check if measured accuracy approaches better set temperature with increasing hold time

Spread results		
N	N indicates the number of data sets on which the market specifcations have been based. The way of calculating the standard deviation is different with smaller numbers and less certain. Market specifications become more certain with increasing numbers of measurements and can also change over time. Therefore it is possible that a thermocycler which has given close to identical measured values over time and has been in specifications over many years, can get out of specifications because of the narrowing of the specifications. Consider specifcations based on N<50 as less certain than specifications based on N>250.	Check if market specifications have stayed identical when comparing actual DRIFTCON reports with historical DRIFTCON reports as specifications become more narrow with increasing numbers of measurements.
Measured	Measured indicates spread (uniformity) results at a particular moment or within a certain time interval during a particular plateau phase. By comparing the values of 15, 30 and 90 seconds it is possible to check for divergation/convergation during the plateau phase. Divergation of channels at plateau phase is an indication for suboptimal thermal control of the reaction block and is frequently found in blocks monitored by a single sensor. Typically plateau uniformity improves (convergation) when a thermocycler is programmed for a longer time at plateau phase.	Check if measured results are within market specifications. See table 3a for guidance criteria. Check if no divergation/convergation occurs during plateau phase by comparing 15, 30 and 90 seconds values. Check if uniformity improves with increasing hold times

Table 3a. Guidance criteria for uniformity

Phase	Excellent cycler	Good cycler	Moderate cycler	Poor cycler
Ramping	<3 ℃	3-4 ℃	5-8 ℃	>8 ℃
Overshoot	<2 ℃	<2 ℃	2-3 ℃	>3 ℃
Plateau (95℃ 30s)	<0.6 ℃	0.6-1 ℃	1-2 ℃	>2 ℃

Table 3b. Guidance criteria for overshoots at 95 ℃ – durati on of maximum overshoot

Max overshoot @ 95 ℃	Good cycler	Moderate cycler	Poor cycler
105 ℃	< 0.3 s	0.3 - 0.5 s	> 0.5 s
102 ℃	< 0.7 s	0.7 - 1 s	> 1 s
100 ℃	< 2 s	1 – 4 s	> 4 s
97.5 ℃	< 7 s	7 – 10 s	> 10 s
96.5 ℃	< 12 s	12 – 15 s	> 15 s

6 Modifying thermocycler performance

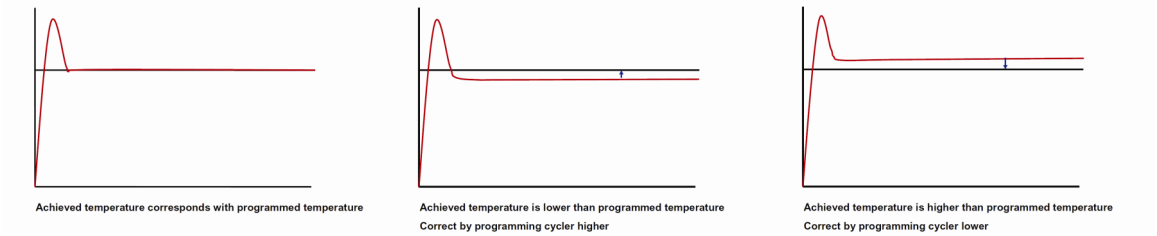
6.1 Introduction

Objective evaluation of thermocycler performance leads to the insight that each thermocycler has a unique thermal fingerprint. No thermocycler is an identical copy of another thermocycler, even when comparing cyclers with different serial numbers of the same model and brand.
Currently, most cyclers can only be calibrated and not adjusted, neither by the user, nor by the manufacturer. However, by modifying the programmed protocol any user can modify the performance. The programmed protocols will differ by thermocycler, but the resulting thermal profiles will be identical or close to identical.
The parameters that can be modified by programming are accuracy, ramp rate, overshoot and hold time. Uniformity can be indirectly influenced by modifying other parameters, but can not be modified directly and is therefore the most difficult parameter in a thermocycler to adjust and to control.

6.2 Modifying average plateau temperature

Average plateau temperatures (accuracies) can be modified by adapting the set temperature. Thermocyclers with average temperatures below/above the required temperature can be programmed at higher/lower set temperatures so that the required temperature will be achieved (Figure 14). Take the accuracy of 30 seconds at plateau and increase/decrease by the difference between average and set temperature to obtain the required temperature. For example, if a thermocycler reaches 94.5 ℃ when it is programmed at 95 ℃, change the set temperature to 95.5 ℃ to allow the thermocycler to reach 95 ℃. Verify the result of the adapted progr amming by a calibration and check if the correct result has been achieved. If not, fine tune the correction required.
Register modified program and resulting temperatures in the labjournal.

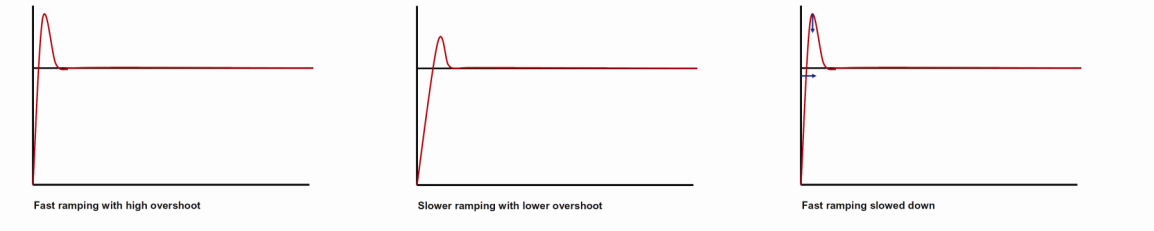
Figure 14. Modifying accuracies



6.3 Modifying ramp rates

Ramp rates can be modified by adapting the heat and cool rates (figure 15). When maximum ramp is the default setting be aware that the ramp rate can only be reduced and not increased. So, if two thermocyclers should function alike, adapt to the thermocycler with the lowest ramp rate or to a fixed ramp rate. Register modified program and resulting ramp rates in the labjournal.
If adapted to a fixed ramp rate, the total run time of a PCR will, under defined environmental conditions, always be the same. The total run time can be used as a routine check to control for incorrect protocols, strongly deviating environmental conditions and daily QC of the thermocycler. Register total run time in the labjournal.
Reducing the ramp rate will in general also result in an improved uniformity during ramping and overshooting. Furthermore, the height of the overshoot will be reduced.

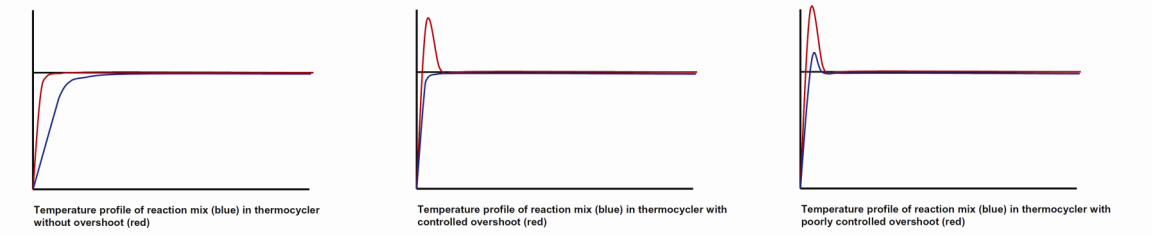
Figure 15. Modifying ramp rates



6.4 Modifying temperature overshoots

Thermocyclers have a thermal profile which is inherent to model and brand. Certain thermocyclers have a thermal profile with overshoots, others without overshoot. The advantage of an overshoot at denaturation is that plateau times can be drastically reduced as the reaction mix heats up much faster (figure 16). The disadvantage is that, in case of high and poorly controlled overshoots, fast inactivation of the *Taq* polymerase occurs as also the reaction mix goes through the overshoot.

Figure 16. Effect of reaction block temperature (red curve) on reaction mix temperature (blue curve) in thermocycler without overshoot, with controlled overshoot and with poorly controlled overshoot



Depending on the requirement, overshoots can be programmed in or out. If the requirement is fast cycling and fast results it is recommendable to leave overshoots at denaturation in or program them in. If the requirement is minimal *Taq* polymerase inactivation, maximum number of cycles and sensitive detection, it is recommendable to leave overshoots at denaturation out, minimize them or program them out. Overshoots at denaturation can be programmed out by adding a short plateau at a slightly lower temperature to the program. For example, 30 s 95 ℃, 30 s 62 ℃, 30 s 72 ℃ can be modified into 1 s 90 ℃, 30 s 95 ℃, 30 s 62 ℃, 30 s 72 ℃. In this way the thermocycle r ramps up with maximum speed to 90 ℃, overshoots it, continues to 95 ℃ in a slower m ore controlled way and therefore hardly overshoots 95 ℃, saving the *Taq* polymerase (figure 17a). Depending on brand and model of thermocycler this requires fine tuning.
Programming overshoots in is also possible. For example 30 s 95 ℃, 30 s 62 ℃, 30 s 72 ℃ can be modified into 1 s 98 ℃, 30 s 95 ℃, 30s 62 ℃, 30 s 72 ℃ (figure 17b). In this way the plateau times of the denaturation phase can be reduced, reducing the total run time.

Figure 17a. Programming overshoots out

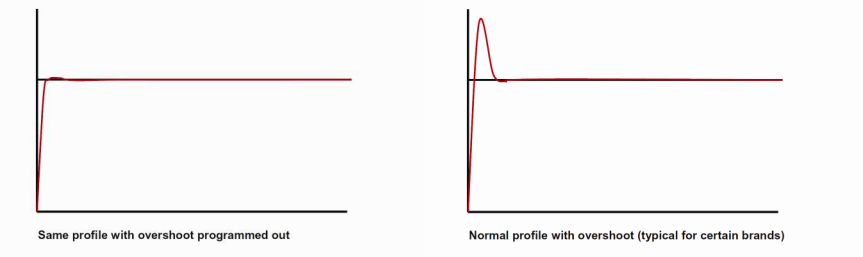
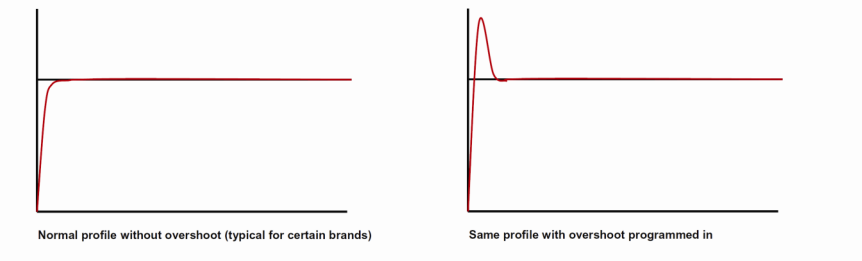
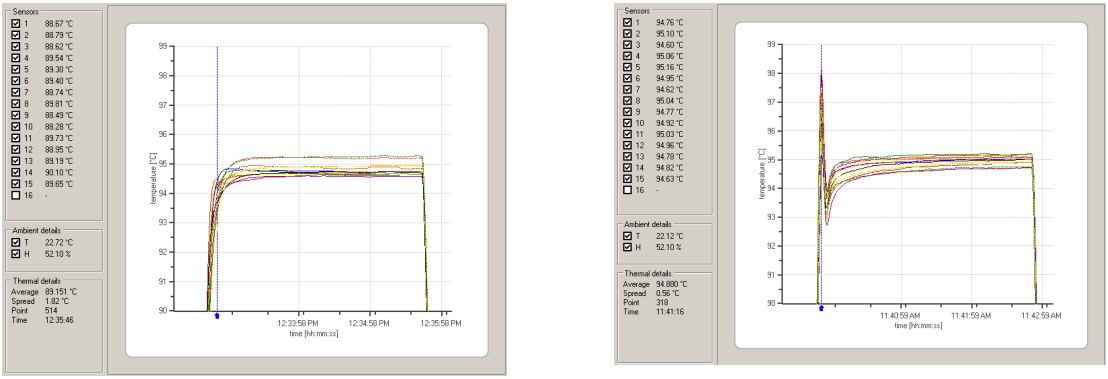


Figure 17b. Programming overshoots in



To control the overshoots well also the duration of the overshoot should be well defined. It is advisable to also define the ramp rate. Certain thermocyclers allow to program the reaction volume. In these thermocyclers the overshoot can also be reduced/increased by programming a volume that is lower/higher than the volume used (figure 18). The effect is not linear and should be verified by a calibration.

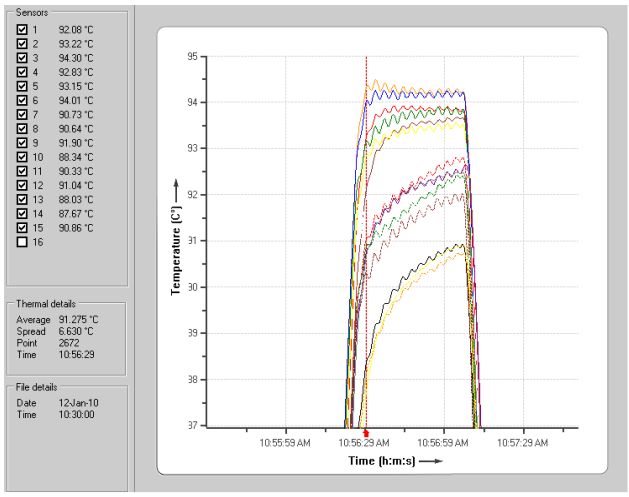
Figure 18. Effect of programming 0 µl or 20 µl reaction volume on height of overshoot



6.5 Modifying temperature uniformity

The only parameter that, unfortunately, can not be modified is the uniformity of a thermocycler. Uniformity can only be indirectly influenced by modifying the ramp rate or by adding mass to the reaction block and loading it evenly. It is therefore the most difficult parameter in a thermocycler to modify and to control. The effect of modifying the ramp rate is depending on the design and construction of the thermocycler, mainly on the number of Peltier elements, the number of sensors and the control mechanism. Slowing down the ramping rate typically leads to lower overshoots and improved uniformity during both the ramping phase and plateau phase (figure 15).
The same phenomenon can in certain thermocyclers also be obtained by filling all unused wells with tubes with water (same volume as the samples). In this way more mass is added to the reaction block and the mass is distributed evenly, slowing the thermocycler down, forcing it to heat evenly and therefore improving its uniformity. In well regulated thermocyclers adding mass does have little effect on the uniformity. It will mainly increase the height of the overshoot.
Uneven loading can lead to deteriorated thermocycler uniformity over time (figure 19). The left side of thermocycler blocks is often more worn out than the right side due to left to right loading when using single tubes or strips. The best thermal performance of a block is obtained if it is loaded evenly. In other words, distribute the tubes or strips evenly over the block, starting from the middle of the block.

Figure 19. Example of worn out reaction block due to assymetric use of block



6.6 *Aligning thermocyclers*

Adapted thermocycler protocols can be used to bring the thermocycler's performance back to the required performance. However, adapted protocols can also be used to align several thermocyclers. This can be done between different thermocyclers of the same model, but also between models and even between brands. It is straightforward to put in place, and once all thermal characteristics are known it allows a laboratory to use its full thermocycler capacity, it the sense that each assay can be ran on each thermocycler.
Be aware that the uniformity can not be directly adapted and that ramp rates can only be decreased and not increased. Therefore initial validation (see chapter 7) should always be done on the least uniform thermocycler.

Protocol aligning thermocyclers

1. Calibrate all thermocyclers to be aligned using DRIFTCON or MTAS.
2. Select thermocycler with the lowest ramp rate or define a ramp rate.
3. Adjust the ramp rates of all thermocyclers to the slowest thermocycler or to the defined ramp rate.
4. Adjust the accuracy either to an absolute temperature or to a selected thermocycler (take 30 s plateau values from calibration data).
5. Program overshoots in or out to obtain desired thermal profile (take overshoot value from 95 ℃ step).
6. Verify by a second calibration the effects of adapting the protocol.
7. Finetune if necessary and verify again.
8. During assay validation (chapter 7), validate the assay on the thermocycler with the highest non uniformity. If the reaction produces a positive result in all 96 wells it will also function on all aligned thermocyclers and does not require additional validation on these instruments.

NOTE:
In case of a defined ramp rate the total run time can be used as daily QC to monitor the thermocycler performance and to check if no modifications to the protocol have been made

Alignment example

All thermocyclers are aligned for accuracy to 95 ℃ and ramp rate to model A, as this thermocycler has the slowest ramp rate. The uniformities are not adjusted as they can not be adjusted.

Data as measured	Model A	Model B	Model C
Accuracy	96.14 ℃ (30 s)	95.12 ℃ (30 s)	95.42 ℃ (30 s)
Uniformity	1.37 ℃ (30 s)	0.85 ℃ (30 s)	0.40 ℃ (3 0 s)
Ramp rate	2.43 ℃/s	3.50 ℃/s	4.53 ℃/s
Target accuracy	95.00 ℃	95.00 ℃	95.00 ℃
Program after alignment	Model A	Model B	Model C
Accuracy	93.86 ℃	94.88 ℃	94.58 ℃
Uniformity	Can not be programmed	Can not be programmed	Can not be programmed
Ramp rate	2.43 ℃/s	2.43 ℃/s	2.43 ℃/s

7 Assay validation

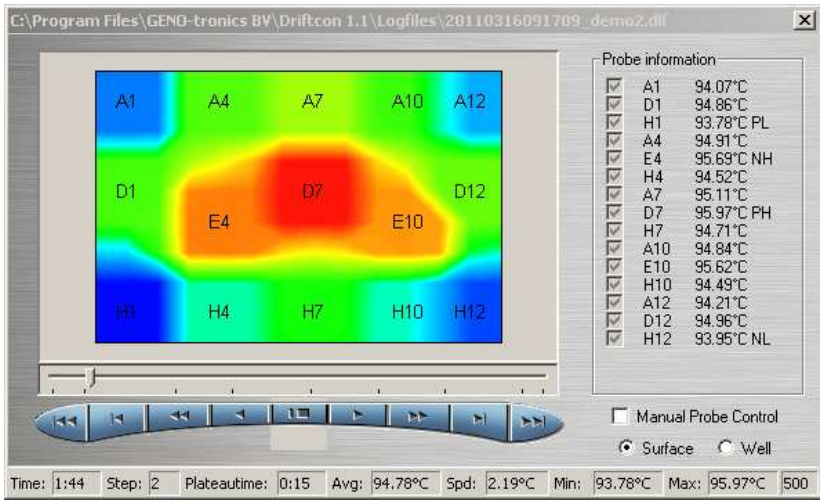
7.1 Introduction

Both the ISO 17025 and ISO 15189 standard and also many other regulations do require (q)PCR assay validation in addition to thermocycler calibration. The principle is to prove that the thermocycler is suitable to run a particular assay, as being within specifications is not a guarantee that a certain kit will function on a particular thermocycler, even when initially validated by the manufacturer on that particular model and brand. As all thermocyclers are more or less non uniform, a validation done in just one row or column of the reaction block is not sufficient as it might not cover the most extreme positions, the hot and the cold spots of the block. An assay might function in the validation test wells and not function in others as they are too cold to denature or too hot, leading to *Taq* polymerase inactivation before a detectable result is generated.
Therefore, intra thermocycler variability should be taken into account during the validation process, not only by the laboratory, but also by the kit manufacturer.
The methods below describe several ways to perform assay validation, taking intra thermocycler variability in account. Depending on the equipment available, the workload involved and the type of laboratory one of the methods described below will be most suitable.

7.2 Hot-cold spot method

In the hot-cold spot method the hottest and the coldest positions in the reaction block are determined via a calibration (figure 20).

Figure 20. Hot and cold spots of a 96-well block thermocycler



Two positive controls and two negatived controls are used. The two positive controls are positioned on the hottest spot and the coldest spot. The two negative controls are positioned on the second hottest and second coldest spot. If the (q)PCR assay functions correctly on the temperature extremes it will also do at all temperatures inbetween and hereby the proof is provided that the assay will function in all wells.
The advantage of this method is that it can be put very easy and quickly into place on any thermocycler and therefore is the recommended method for labs doing many different assays over time, like research laboratories. The disadvantages are that controls can end up in the middle samples series or that the hot and cold spots can move through the reaction block over time.

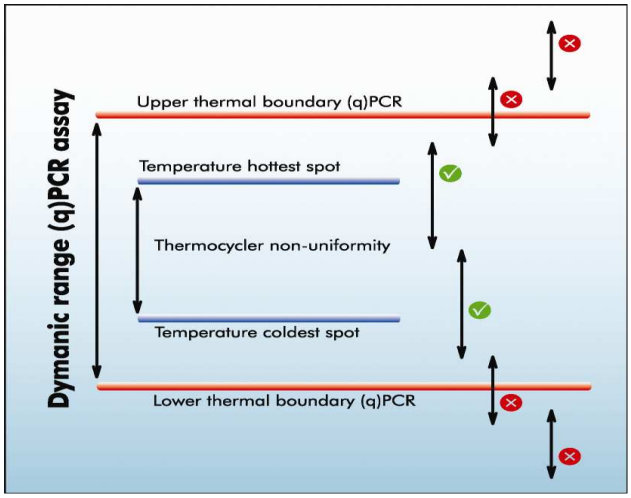
Protocol hot-cold spot method

1. Calibrate the thermocycler using DRIFTCON or MTAS (include a 95 ℃ step).
2. DRIFTCON: take the PH, PL, NH, NL positions from the report in section Recommended control positions.
   MTAS: take the highest and second highest temperature and the lowest and second lowest temperature at 10 s into the 95 ℃ st ep in section Static data step 2: 95 ℃.
3. Position positive and negative controls in recommended wells.
4. Run PCR protocol as usual.
5. Check if positive and negative controls give correct result.
6. If yes, from a temperature perspective, the assay will give reliable results in all other wells.
   If no, avoid hottest/coldest wells and position positive and negative controls on second hottest/coldest and third hottest/coldest well and start from step 4 or decrease/increase set temperature thermocycler closer to target temperature and start from step 1.

7.3 Thermal boundary method

The thermal boundary method comes down to determining the temperature extremes at which the (q)PCR assay still gives a correct result. The minimum and maximum denaturation, annealing and elongation temperatures are determined and then a thermocycler is qualified as being within these thermal boundaries or not. If the thermocycler, including its full non uniformity, lies within the thermal boundaries the cycler is qualified as suitable (figure 21).

Figure 21. Thermal boundaries of a (q)PCR assay



The advantage of this method is that it is very exact and allows laboratory technicians to position controls wherever they like. It also allows kit manufacturers to specify the thermal boundaries of a kit, instead of protocols for particular models and brands of thermocyclers on which the kit has been validated by the manufacturer.
The thermal boundary method is a solution for CE-IVD kits that do not function correctly in the hands of end users and do not achieve the sensitivity and reproducibility claimed, although used exactly according to protocol, on the thermocycler on which the kit has been validated. The cause for this is in many cases limited validation by the manufacturer, not taking thermocycler variability into account (see chapter 7.5).

Laboratories that use CE-IVD kits can also determined these thermal boundaries themselves. In this way kits can be used on alternative thermocyclers, than on which the kit was orginally validated. According to the ISO 17025 and ISO 15189 standard, this is called use of a standardized method outside its intended scope. The thermal boundaries method allows using the laboratory's full thermocycler capacity and allows universal thermocycler use. The disadvantage of this method is that it is initially labour intensive as the thermal boundaries need to be sorted out for the denaturation, annealing and elongation phase. Sometimes, it is also necessary to verify the define the ramp rates and the height and length of overshoots. But once sorted out, it can be used for a long time. This method is, therefore, recommended for PCR and qPCR kit manufacturers and diagnostic laboratories that repeat the same test frequently over a long period of time. The workload of this initial validation by thermal boundaries can be reduced by designing all assays to function at the same denaturation, annealing and elongation temperature. On other words, in the design phase the thermal boundaries can already be defined and then only a verification needs to be done.

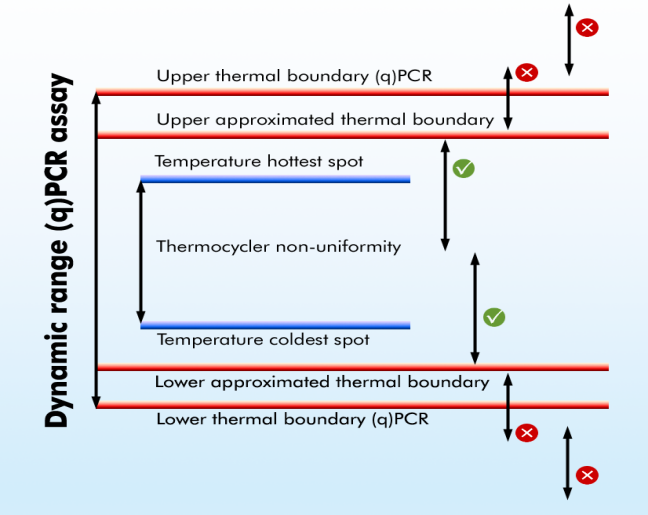
Protocol thermal boundaries method

1. Calibrate the used thermocycler in non gradient mode and gradient mode (if available) with MTAS or DRIFTCON, as the performance in gradient mode can differ from performance in non gradient mode, and determine the real temperatures the thermocycler reaches, in both modes.
2. If two different thermocyclers are used, in case the normally used thermocycler does not have a gradient, check the relation between both cyclers and align them or take the difference into account in the calculations as made below.
3. Set up 2.5 ml mastermix of the selected (q)PCR assay, pipet 25 µl in each well of a BIOplastics 96 well plate
4. Run PCR with a gradient at the denaturation temperature (90-100 ℃)
5. Determine the lowest (D_L) and highest (D_H) denaturation temperature at which the PCR still gives a result, based on the calibration data, not based on thermocycler program
6. Determine optimal denaturation temperature ($D_O=(D_L+D_H)/2$)
7. Run PCR with D_O as denaturation temperature and gradient at annealing temperature (±5 ℃ theoretical annealing temperatur e)
8. Determine the lowest (A_L) and highest (A_H) annealing temperature at which the PCR still gives a result, based on the calibration data, not based on thermocycler program
9. Determine optimal annealing temperature ($A_O=A_L+A_H/2$)
10. Elongation less critical, but can also be done
11. Optimal protocol: D_O, A_O, E_O
12. Thermal boundaries: D_L- D_H, A_L-A_H, E_L-E_H
13. Calibrate unknown cycler, check if within D_L- D_H, A_L-A_H and E_L-E_H
14. If within boundaries the cycler can be used without any adjustment
15. If completely or partially outside boundaries either adjust the protocol to bring the thermocycler within boundaries (see paragraph 6.6) or do qualify as not suitable
16. Optional: Use recommended + and – control positions (based on calibration) to check for drifting in time
17. Optional: Define ramp rate and height and length of overshoots. In case of a defined ramp rate the total run time can be used as daily QC to monitor the thermocycler performance and to check that no modifications to the protocol have been made

7.4 Approximated thermal boundary method

In the approximated thermal boundary method the thermal boundaries are not determined exactly, but are approximated by programming the thermocycler a few degrees off-plateau. The resulting approximated temperature boundaries are more narrow then with the thermal boundary method, but for the rest the method is comparable. If the thermocycler, including its full non uniformity, lies within the approximated thermal boundaries the cycler is qualified as suitable (figure 22).

Figure 22. Approximated thermal boundaries of a (q)PCR assay



The advantage of this method is that it is exact and allows laboratory technicians to position controls wherever they like. It also allows kit manufacturers to specify the approximated thermal boundaries of a kit, instead of protocols for particular models and brands of thermocyclers on which the kit has been validated by the manufacturer.
This method is also suitable for laboratories that do not have a gradient thermocycler in their instruments portfolio.
The disadvantage of this method is that it is initially labour intensive as the approximated thermal boundaries need to be sorted out for the denaturation, annealing and elongation phase. Sometimes, it is also necessary to verify the effect of the ramp rates and the overshoots. But once sorted out, it can be used for a long time. This method is, therefore, recommended for PCR and qPCR kit manufacturers and diagnostic laboratories that repeat the same test frequently over a long period of time.
The workload of this initial validation by thermal boundaries can be reduced by designing all assays to function at the same denaturation, annealing and elongation temperature. In other words, in the design phase the thermal boundaries can already be defined and then only a verification needs to be done.

Protocol approximated thermal boundary method

1. Calibrate the used thermocycler with MTAS or DRIFTCON and determine the real temperatures the thermocycler reaches.
2. Set up 2.5 ml mastermix of the selected (q)PCR assay, pipet 25 µl in each well of a BIOplastics 96 well plate
3. Run PCR with denaturation temperature 1 or 2 ℃ below and above normally used temperature.
4. Check if PCR still gives a result at lowest programmed denaturation temperature (D_{AL}) and at highest programmed denaturation temperature (D_{AH}), based on the calibration data, not based on thermocycler program
5. Run PCR with annealing temperature 1 or 2 ℃ below and above normally used temperature.
6. Check if PCR still give a result at lowest programmed annealing temperature (A_{AL}) and at highest programmed annealing temperature (A_{AH}), based on the calibration data, not based on thermocycler program
7. Elongation less critical, but can also be done
8. Thermal boundaries: D_{AL}-D_{AH}, A_{AL}-A_{AH}, E_{AL}-E_{AH}
9. Calibrate unknown cycler, check if within D_{AL}-D_{AH}, A_{AL}-A_{AH} and E_{AL}-E_{AH}
10. If within boundaries the cycler can be used without any adjustment
11. If completely or partially outside boundaries either adjust the protocol to bring the thermocycler within boundaries (see paragraph 6.6) or qualify as not suitable
12. Optional: Use recommended + and – control positions (based on calibration) to check for drifting in time
13. Optional: Define ramp rate and height and length of overshoots. In case of a defined ramp rate the total run time can be used as daily QC to monitor the thermocycler performance and to check if no modifications to the protocol have been made

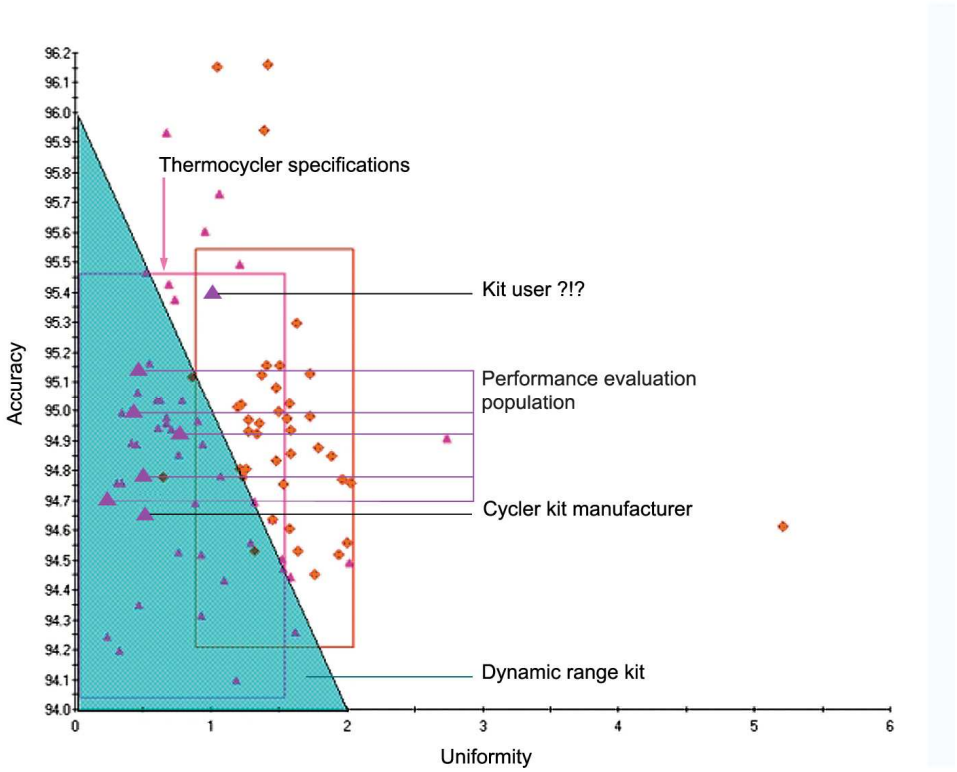
7.5 CE-IVD kit validation, verification and revalidation

Most CE-IVD kits are validated on a large number of samples to check for matrix effects. However, the number of thermocyclers on which the kits are validated are in general very modest, often not more than 10 thermocyclers. Given the substantial variability present in the population of a particular model, low numbers of thermocyclers are not statistically representative for the total population. Therefore the “coldest”, “hottest”, and “least uniform” thermocyclers, although within specifications, are often missing in these validation studies by the manufacturer (figure 22). As a result the kit will produce results in most laboratories, but a small percentage of the laboratories are not capable of obtaining positive results in all wells or no results at all with a particular kit.

Specifying thermal boundaries in a kit manual would allow an end-user to use any model of thermocycler, as long as the thermocycler lies with its full non uniformity, within these thermal boundaries.

However, the laboratory which uses the kit can also perform an initial verification to check if the kit produces the result as claimed. If not, the laboratory can also determined the thermal boundaries themselves and revalidate the kit to meet the requirements of the ISO 17025 or ISO 15189 standard in order to guarantee the human diagnostic results they produce.

Figure 22. Thermocycler variability versus dynamic range of a diagnostic kit



8 Conclusions

Thermocyclers show a substantial variation, not only between brands, but also between models of the same brand, between individual serial numbers of the same model and brand and even within one thermocycler.
The effects of this variation differ by phase of the PCR. Increased plateau temperatures and high and long overshoots typically cause problems during the denaturation phase due to premature inactivation of the *Taq* polymerase. In the annealing phase deviating temperatures, either too high or too low typically cause problems due to non specific or inefficient priming. The effects on the final PCR results move on a sliding scale from slightly less efficient PCRs, which still give a result, to complete failure. The first category is often not noticed and in case of quantitation can lead to incorrect counts.
Due to thermocycler variation it is necessary to calibrate thermocyclers and validate thermocycler – assay combinations.
To perform a calibration that is representative of the process and measures all parameters of thermal performance, including uniformity, accuracy, overshoot, ramp rate and hold time, a thermocycler calibration should be performed in a dynamic and multichannel manner. To be ISO 17025 compliant the temperature calibration should furthermore be traceable by comparison to the internal reference standard ITS-90, performed by trained and qualified person, under controlled environmental conditions and with a calculated measurement uncertainty.
The calibration results can be compared to manufacturer specifications, but should also be analyzed in an objective manner.
Thermocyclers can be adjusted or synchronized by adapting the programmed protocol.
They fullfill the requirement of validation under ISO 17025 and ISO 15189 accreditation and many other regulations. Either the hot-spot method, thermal boundary method or approximated thermal boundary method can be used, depending on the type of laboratory.
Most important is to realize that thermocyclers do vary and that solutions must be sought to manage this variation in daily use to ensure that correct and reliable data are produced.

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Offices

THE NETHERLANDS

CYCLERtest BV (Headquarters)
Rötscherweg 61
6374 XW Landgraaf
The Netherlands
T +31 45 533 8733
F +31 45 533 8794
www.cyclertest.com
support@cyclertest.com

USA

BPCTi Inc
2933 South Miami Blvd. Suite 121
Durham, NC 27703
USA
T +1 919 806 8811
F +1 919 806 2014
www.bpcti.com
info@bpcti.com

CHINA

Hendrikx Inc. BPCTi Inc.
Jin Sha Jiang Road
Room 803, 8/F, No. 5, Lane 1628#
200333, Shanghai
China
T +86 21 3251 3173
F +86 21 3251 3651
www.bpcti.com.cn
info@bpcti.com.cn

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