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Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of microorganisms — Thermal performance testing of thermal cyclers

Microbiologie de la chaîne alimentaire — Réaction de polymérisation en chaîne (PCR) pour la recherche de micro-organismes — Essais de performance thermique des thermocycleurs

Reference number
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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition International Standard cancels and replaces the first edition Technical Specification (ISO/TS 20836:2005), which has been technically revised. The main changes compared with the previous edition are as follows:

— the Scope has been extended to include both thermal cyclers and real-time thermal cyclers;

— the physical performance testing method has been described in more detail, and the biochemical performance testing method has been taken out;

— information for laboratories regarding ISO/IEC 17025 has been included;

— the performance testing method has been aligned with ISO/IEC 17025;

— compliancy testing has been added;

— in Annex C, two procedures to set PCR-method-based specifications have been added.

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

This document is part of a family of International Standards under the general title *Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food borne pathogens*:

— ISO 22174, *General requirements and definitions*;

— ISO 20837, *Requirements for sample preparation for qualitative detection*;

— ISO 20836, *Thermal performance testing of thermal cyclers*;

— ISO 20838, *Requirements for amplifications and detection for qualitative methods*.

This document describes a method for performance testing for standard thermal cyclers and real-time thermal cyclers that allows laboratories to evaluate if the thermal cycler used is suitable for the intended use and meets the specifications set by the laboratory.

The described method is based on a physical method that measures directly in the thermal cycler block in block-based thermal cyclers and in tubes in heated-chamber-based thermal cyclers. The described method provides a measurement uncertainty that is sufficiently low to allow meaningful comparison to specifications.

Furthermore, the method does meet the criteria of a metrological traceable calibration method in case it is used by ISO/IEC 17025-compliant laboratories.

Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of microorganisms — Thermal performance testing of thermal cyclers

1 Scope

This document specifies requirements for the installation, maintenance, temperature calibration and temperature performance testing of standard thermal cyclers and real-time thermal cyclers. It is applicable to the detection of microorganisms as well as any other applications in the food chain using polymerase chain reaction (PCR)-based methods.

This document has been established for food testing, but is also applicable to other domains using thermal cyclers (e.g. environmental, human health, animal health, forensic testing).

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/IEC Guide 98-3:2008, *Uncertainty of measurement — Part 3: Guide to the expression of uncertainty in measurement (GUM:1995)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1 Polymerase chain reaction

3.1.1
polymerase chain reaction
PCR
enzymatic procedure that allows in vitro amplification of DNA

[SOURCE: ISO 22174:2005, 3.4.1]

3.1.2
PCR method
test method based on the *PCR* (3.1.1) technique

Note 1 to entry: Examples include, but are not limited to, PCR, quantitative real-time PCR (qPCR), reverse transcription PCR (RT PCR) and reverse transcription quantitative real-time PCR (RT qPCR).

3.2 Thermal cycler

3.2.1
thermal cycler
automatic device that performs defined heating and cooling cycles necessary for *PCR* (3.1.1) or real-time PCR

Note 1 to entry: The thermal cycler can be a block-based or (individual) reaction-chamber-based thermal cycler.

[SOURCE: ISO 22174:2005, 3.4.20, modified — "or real-time PCR" and Note 1 to entry have been added.]

3.2.2
reaction block
heated and cooled metal block in which PCR reaction vials, containing the PCR reaction mix, can be inserted

Note 1 to entry: The block can be heated and cooled by a number of technologies, among which Peltier heating and cooling is the most abundantly used.

3.2.3
reaction chamber
heated and cooled chamber in which PCR reaction vials, containing the PCR reaction mix, can be inserted directly or in a rotor

Note 1 to entry: The chamber can be heated and cooled by a number of technologies, among which air heating and cooling is the most abundantly used.

3.2.4
heated lid
heated cover of a *thermal cycler* (3.2.1), which is applied in block-based thermal cyclers onto reaction tubes to prevent condensate of reaction mix to collect inside the cap of the reaction tube or onto the seal, and evaporation from the reaction tube, and which applies pressure onto the tubes to ensure proper thermal contact

3.2.5
PCR temperature protocol
heating and cooling cycles required for *PCR* (3.1.1), typically consisting of denaturation, annealing and extension temperature steps, which are typically repeated 30 to 45 times

Note 1 to entry: In certain *PCR methods* (3.1.2), a two-step temperature protocol is used in which annealing and extension are combined to one step.

3.3 Temperature characteristics

3.3.1
thermal cycler temperature profile
graph of the course of the temperature by performing measurements at defined intervals

Note 1 to entry: See Annex D for an example graph of a thermal cycler temperature profile.

3.3.2
$T_{(i)}t$
temperature in °C of sensor *i* at time *t* in s

3.3.3
set temperature
T_{set}
target temperature programmed to be reached in °C

3.3.4
average temperature
T_{avg}

$$T_{avg}(t) = \sum_{i=1}^{N} \frac{T_i(t)}{N}$$

where

$T_{avg}(t)$	is average temperature in °C at time t;
i	is sensor i of N;
N	is total number of sensors.

average of measured values of all active temperature sensors in °C at a specific time in s

3.3.5
temperature deviation
T_{dev}
$T_{dev}(t) = T_{avg}(t) - T_{set}$
average temperature (3.3.4) minus *set temperature* (3.3.3) in °C at a specific time in s

3.3.6
minimum temperature
T_{min}
$T_{min}(t) = \min(T_i(t) ... T_N(t))$
minimum value of all active temperature sensors in °C at a specific time in s

3.3.7
maximum temperature
T_{max}
$T_{max}(t) = \max(T_i(t) ... T_N(t))$
maximum value of all active temperature sensors in °C at a specific time in s

3.3.8
temperature uniformity
$T_{uniformity}$
$T_{uniformity}(t) = T_{max}(t) - T_{min}(t)$
homogeneity of the temperature distribution within the *reaction block* (3.2.2) or chamber, defined as *maximum temperature* (3.3.7) minus *minimum temperature* (3.3.6) in °C at a specific time in s

3.3.9
temperature transition
$T_{transition}$
phase of fast temperature change from one set temperature to another set temperature

3.3.10
ramp rate
heat or cool rate of *thermal cycler* (3.2.1) in °C/s

3.3.11
average ramp rate
V_t

$$V_t = \sum_{i=1}^{N} = \left(\frac{T_{i,90\%} - T_{i,10\%}}{t_{i,90\%} - t_{i,10\%}} \right)$$

where

V_t is ramp rate in °C/s;

i is sensor i of N;

N is total number of sensors;

$T_{i,10\%}$ is T_i at 10 % temperature of the ramp slope in °C;

$T_{i,90\%}$ is T_i at 90 % temperature of the ramp slope in °C;

t is time in s.

heat or cool rate of *thermal cycler* (3.2.1) calculated between 10 % and 90 % time of the heating or cooling slope

Note 1 to entry: The heat rate is a positive *ramp rate* (3.3.10). The cool rate is a negative ramp rate.

3.3.12
maximum ramp rate
$V_{t\ \max}$
maximum heat or cool rate during heating or cooling slope in °C/s

3.3.13
maximum temperature overshoot
$T_{i,\text{ovs,max}}$

$$T_{i,\text{ovs,max}} = T_{i,\max}(t)\Big|_{thold=0s}^{thold=15s} - T_i(thold = 30s)$$

maximum temperature (3.3.7) value in °C of all active temperature sensors during temperature overshoot above the *average temperature* (3.3.4) of the *reaction block* (3.2.2) or chamber temperature at hold when heating up

Note 1 to entry: The maximum temperature overshoot is calculated between start and end of the overshoot and is expressed relative to the temperature at 30 s *hold time* (3.3.18).

Note 2 to entry: The overshoot occurs typically between 0 s and 15 s hold time. See Annex D for an example *thermal cycler temperature profile* (3.3.1).

3.3.14
minimum temperature undershoot
$T_{i,\text{uns,min}}$

$$T_{i,\text{uns,min}} = T_{i,\min}(t)\Big|_{thold=0s}^{thold=15s} - T_i(thold = 30s)$$

minimum temperature (3.3.6) value in °C of all active temperature sensors during temperature undershoot below the *average temperature* (3.3.4) of *reaction block* (3.2.2) or chamber temperature at hold when cooling down

Note 1 to entry: The maximum temperature undershoot is calculated between start and end of the undershoot and is expressed relative to the temperature at 30 s *hold time* (3.3.18). An undershoot is an overshoot in negative direction.

Note 2 to entry: The undershoot occurs typically between 0 s and 15 s hold time. See Annex D for an example *thermal cycler temperature profile* (3.3.1).

3.3.15
average temperature overshoot
$T_{\text{ovs,avg}}$

$$T_{\text{ovs,avg}} = \sum_{i=1}^{N}\left(\frac{T_{i,\text{ovs,max}}}{N}\right)$$

average value of *maximum temperature overshoots* (3.3.13) of all active block temperature sensors in °C

3.3.16
average temperature undershoot
$T_{\text{uns,avg}}$

$$T_{\text{uns,avg}} = \sum_{i=1}^{N} \left(\frac{T_{i,\text{uns,min}}}{N} \right)$$

average value of *minimum temperature undershoots* (3.3.14) of all active block temperature sensors in °C

3.3.17
overshoot duration
time elapsed between start and end of the overshoot in s

Note 1 to entry: The start of the overshoot is defined as the moment in time where the *average temperature* (3.3.4) exceeds the average hold temperature, calculated at 30 s hold, at the beginning of the overshoot. The end of the overshoot is defined as the moment in time where the average temperature reaches the average hold temperature at the finish of the overshoot.

3.3.18
hold time
time elapsed between start and end of a temperature hold in s

Note 1 to entry: See Annex D for an example to determine start and end of hold.

3.4 Temperature measurement

3.4.1
temperature measurement system
temperature measurement and data logging instrument

3.4.2
sampling frequency
number of samples per second taken from a time-continuous signal to make a time-discrete signal

3.4.3
response time
time required for the *temperature measurement system* (3.4.1), when subjected to a change in temperature, to react to this change

3.4.4
measurement 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

[SOURCE: ISO/IEC Guide 98-3:2008, B.2.18, modified — "quantity intended to be measured" and replaced "measurand" and the notes to entry have been deleted.]

3.4.5
performance test
test procedure that determines the performance of a *thermal cycler* (3.2.1)

3.4.6
calibration
operation that, under specified conditions, in a first step, establishes a relation between the quantity values with *measurement uncertainties* (3.4.4) provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication

Note 1 to entry: Calibration should not be confused with adjustment of a measuring system, nor with auto-check, self-verification test, verification, normalization, installation qualification (IQ), operational qualification (OQ) or performance qualification (PQ).

[SOURCE: ISO/IEC Guide 99:2007, 2.39, modified — Note 1 to entry has replaced the original Notes 1, 2 and 3 to entry.]

4 Installation of thermal cyclers

The manufacturer's instructions shall be followed.

The following should be taken into consideration.

a) Thermal cyclers should be installed and operated at suitable environmental conditions that do not invalidate the results or adversely affect the required quality of any test.

b) The environmental conditions that should at minimum be taken into account are room temperature and relative humidity.

See the manual of the thermal cycler for recommended room conditions.

Thermal cyclers shall be located in such a way that free circulation of air is permanently allowed.

See ISO 22174 for guidelines for contamination prevention and separation of incompatible laboratory activities.

5 Maintenance of thermal cyclers

The laboratory shall establish a maintenance programme, where appropriate, and keep records to ensure proper functioning and prevent deterioration of the thermal cyclers.

6 Performance testing of thermal cyclers

6.1 General

If the performance testing method of this document is used as a metrological traceable temperature calibration, as a conformity test or as a reference method, the performance test shall be carried out with a minimum number of sensors that represent at least 12,5 % of wells for reaction blocks or chambers < 96 wells or 12 wells for reaction blocks or chambers > 96 wells (see 6.4.5.1) and metrological traceability (see 6.3) shall be provided up to the level of the thermal cycler. If the performance testing method is used for other purposes, such as supplier's quality control or supplier's after sales service, the number of sensors may be reduced to a minimum number of sensors that represent at least 8 % of wells for reaction blocks or chambers < 96 wells or 8 wells for reaction blocks or chambers > 96 wells and metrological traceability shall be provided up to the level of the temperature measurement system.

In case of individual reaction chambers, each of the individual reaction chambers shall be tested.

The decision chart in Figure 1 can be used to determine if the performance test shall be a metrological traceable calibration or a performance test.

NOTE 1 Calibrations that meet the requirements of the ISO/IEC 17025 are considered to be metrological traceable. ISO/IEC 17025 describes when metrological traceability is required and how metrological traceability is established.

NOTE 2 The chemistry- or biochemistry-based normalization and verification kits available at the time of publication of this document offer no traceability to SI and are associated with high measurement uncertainties, and are therefore inapt as a performance testing method. The normalization kits are developed to optimize the optical detection and related software to enable the collection of fluorescent data in a real-time thermal cycler. These kits are not developed for calibration purposes.

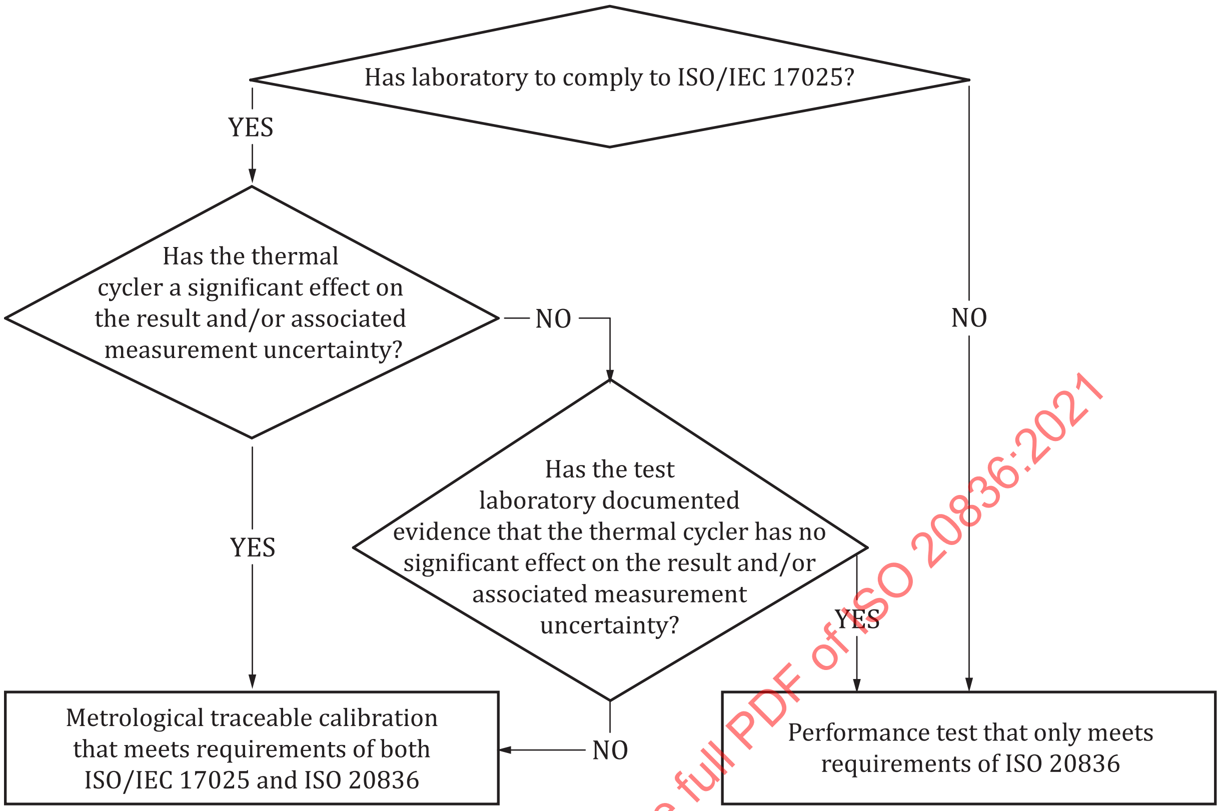


Figure 1 — Decision chart to determine the requirement for metrological traceable temperature calibration of thermal cyclers

6.2 Performance testing programme

The laboratory shall establish a performance testing programme, where appropriate, and keep records to ensure that the thermal cycler is capable of achieving the accuracy required and complies with the specifications relevant to the intended use.

ISO/IEC 17025-compliant laboratories are required to establish a planned calibration programme in order to maintain confidence in the status of calibration (see ISO/IEC 17025:2017, 6.4).

NOTE Guidance on how to determine calibration intervals, particularly when setting up a calibration programme, is provided in, for example, ILAC G24.

6.3 Metrological traceability

Performance testing shall be traceable to the International System of Units (SI).

Metrological traceability is established by considering, and then ensuring, the following:

a) the specification of the measurand (quantity to be measured);

b) a documented unbroken chain of calibrations going back to stated and appropriate references (appropriate references include national or international standards, and intrinsic standards);

c) measurement uncertainty for each step in the traceability chain is evaluated according to agreed methods;

d) each step of the chain is performed in accordance with appropriate methods, and the measurement results and associated, recorded measurement uncertainties;

e) the laboratories performing one or more steps in the chain supply evidence for their technical competence.

NOTE Calibration laboratories fulfilling the requirements of the ISO/IEC 17025 are considered to be competent. A thermal cycler calibration certificate bearing an accreditation body logo from a calibration laboratory accredited to the ISO/IEC 17025 is sufficient evidence of traceability of the calibration data reported.

6.4 Temperature performance testing method

6.4.1 General

This performance testing method is intended to determine the thermal cycler temperature parameters that influence the outcome of the PCR and RT PCR. It can be used to perform a temperature performance test on both PCR and real-time PCR thermal cyclers.

An example of a test and compliancy report is given in Annex E.

6.4.2 Principle

The temperature is measured by temperature sensors directly in the reaction block in block-based thermal cyclers or inside the reaction vials in reaction-chamber-based thermal cyclers, in order to achieve an adequately low measurement uncertainty. The measurement is performed over the complete reaction temperature range, including at least a minimum, maximum and middle temperature. If the thermal cycler has a heated lid, the measurement shall be performed with the heated lid closed and operating, when physically possible.

6.4.3 Equipment

6.4.3.1 Thermal cycler

The thermal cycler shall be checked, before the performance test, to be functional.

The ventilation openings shall be clean and not obstructed, allowing free air circulation.

6.4.3.2 Temperature measurement system

The temperature measurement system shall meet at least the following criteria:

a) multi-sensor system with an adequate number of temperature sensors to measure simultaneously in at least the number of required wells (see 6.4.5.1), allowing to measure uniformity;

b) capable of recording the heated lid temperature with at least one temperature sensor (when physically possible);

c) capable of recording temperatures dynamically with a sampling frequency of at least one time per second in order to measure correctly the ramp rate and overshoot; for thermal cyclers with heat rates above 4 °C/s, a sampling frequency of at least four times per second per temperature sensor is recommended;

d) capable of recording temperature over the complete reaction temperature range;

e) capable of being calibrated, traceable to SI, over at least the complete reaction temperature range;

f) resolution ≤ 0,1 °C;

g) expanded combined measurement uncertainty ($k = 2$) ≤ 0,15 °C/sensor (determined in accordance with ISO/IEC Guide 98-3:2008);

h) total mass of the sensor head approximately equal to load of thermal cycler when the heating block or heating chamber is completely filled with tubes containing reagents;

i) response time of the sensors ≤ 1 s.

The temperature performance test unit shall be calibrated in a metrological traceable manner at regular intervals according to a predefined programme.

6.4.4 Environmental conditions

The performance test shall be performed at suitable environmental conditions that do not invalidate the results or adversely affect the required quality of the performance test.

NOTE The performance testing environmental conditions typically correspond to the environmental conditions required for operation of the thermal cycler.

6.4.5 Procedure

6.4.5.1 Temperature sensor locations

If the performance testing method of this document is used as a metrological traceable temperature calibration, as a conformity test or as a reference method the following combination of criteria shall apply.

For each heating block-based or heating-chamber-based thermal cycler the following criterion shall be met:

— the temperature sensors shall be located evenly distributed over the reaction block or chamber in at least 12,5 % of the wells for reaction blocks or chambers ≤ 96 wells or 12 wells for reaction blocks or chambers > 96 wells.

For rectangular heating block-based thermal cyclers the following additional combination of criteria shall be met:

a) the locations shall include four corner locations for heating blocks > two columns or rows of wells or two corner locations for heating blocks ≤ two columns or rows of wells;

b) the locations shall include at least one edge location for each edge of the heating block ≥ six wells;

c) the locations shall include at least two central locations for heating blocks > 18 wells and at least four central locations for heating blocks > 48 wells.

For thermal cyclers that contain independent heating and control units, each of the units shall be tested.

If the performance testing method is used for other purposes, such as supplier's quality control or supplier's after sales service, the number of sensors may be reduced to a minimum of eight sensors and metrological traceability shall be provided up to the level of the temperature measurement system.

NOTE Guidelines for sensor locations are described in Annex A.

6.4.5.2 Recording

The data acquisition frequency shall be sufficiently high to allow a dynamic measurement of ramp rates and overshoots.

The thermal cycler shall be programmed with a temperature protocol that shall meet the following criteria:

a) the temperature protocol shall be representative for the used reaction temperature range and include at least a minimum, middle and maximum temperature;

b) the temperature protocol shall include a preheat of at least 60 s at minimum temperature, 60 s at maximum temperature and 60 s at minimum temperature to preheat the thermal cycler and allow the thermal cycler and the temperature measurement system to reach an equilibrium;

c) the hold times for each temperature shall be representative for PCR applications in which temperatures quickly alternate, but be at minimum 30 s long to allow stabilization of the temperature;

d) the temperature protocol shall include an up-going ramp from minimum to maximum temperature in order to determine the maximum heat rate;

e) the temperature protocol shall include a down-going ramp from maximum to minimum temperature in order to determine the maximum cool rate;

f) the temperature protocol shall include an up-going ramp towards the temperature hold representative of the denaturation temperature in order to determine the overshoot for denaturation;

g) the temperature protocol shall include a down-going ramp towards the temperature hold representative of the annealing temperature in order to determine the undershoot for annealing;

h) the temperature protocol may include an up-going ramp towards the temperature hold representative of the elongation temperature in order to determine the overshoot for elongation;

i) in case of block-based thermal cyclers: the set temperature of the heated lid shall be higher than the set temperature of the block at any time during the temperature protocol.

It is recommended to use universal temperature protocols that allow comparisons between thermal cyclers and comparisons to specifications, rather than temperature protocols specific to certain PCR methods that require a performance test of each thermal cycler for each PCR method used.

NOTE An example of a universal temperature protocol is described in Annex B.

6.4.6 Performance test results

For each of the temperature holds measured at least the following parameters shall be calculated:

a) temperature uniformity after 30 s hold time at the different temperature holds;

b) temperature uniformity at maximum overshoot or undershoot at the different temperature holds;

c) average temperature overshoot or undershoot at the different temperature holds;

d) maximum temperature overshoot and undershoot at the different temperature holds;

e) overshoot duration;

f) average temperature after 30 s hold time at the different temperature holds;

g) temperature deviation after 30 s hold time at the different temperature holds;

h) average ramp rate and maximum ramp rate between each temperature hold;

i) hold time at the different temperature holds.

For fast thermal cyclers, the parameters may be calculated at hold times shorter than 30 s.

6.4.7 Performance test report

The test report shall contain at least the following information:

a) a title;

b) the name and address of the laboratory, and the location where the performance tests were carried out;

c) unique identification of the performance test report and on each page an identification in order to ensure that the page is recognized as a part of the performance test report;

d) identification of the method used;

e) a reference to this document, i.e. ISO 20836:2021;

f) an unambiguous identification of the thermal cycler tested;

g) the date of performance of the performance test;

h) performance test results with the units of measurement;

i) the name, function and signature or equivalent identification of person authorizing the performance test report;

j) the operation modus of the thermal cycler;

k) the programmed sample volume;

l) operation of thermal cycler with opened or closed lid;

m) if applicable, the quantity of contact medium (thermal oil or thermal paste) used.

Furthermore, it is recommended to record the environmental conditions under which the performance test was made.

The following requirements apply if the performance testing method of this document is used as a metrological traceable calibration, as a conformity test or as a reference method:

— uncertainty of measurement of the performance test [expanded combined uncertainty (k = 2)];

— evidence that performance test is traceable to SI.

NOTE Examples of the operation modi of thermal cyclers are block mode, calculated mode, tube mode, simulated mode, emulated mode, etc.

6.4.8 Compliancy testing

The thermal cycler can be qualified as suitable for intended use in different ways:

a) comparison to manufacturer specifications;

b) comparison to PCR-method-based specifications;

c) if, no specifications may be obtained, by a functional test with low positive controls in the wells with the most extreme temperatures.

See Annex C for more information on compliancy testing.

6.5 Optical performance testing method

Optical performance testing is intended to determine the real-time thermal cycler optical parameters that influence the outcome of the real-time PCR. Optical parameters that have an effect are optical non-uniformity, nonlinearity, detection sensitivity, and detection saturation.

At the time of publication of this document, no metrological traceable optical calibration methods for real-time thermal cyclers exist. Therefore, it is recommended to check the optical unit of real-time thermal cyclers in addition to the temperature unit with a biological, biochemical or physical test. The checking of the optical unit should be performed around the moment of the performance testing of the temperature unit.

NOTE The existing biochemistry-based optical kits are designed for normalization or verification purposes, not for calibration purposes, although their name might suggest so. The kits available at the time of publication of this document are not traceable to SI and are associated with large measurement uncertainties. The physical methods available at the time of publication of this document offer a higher level of traceability, but are also at the moment not fully traceability to SI.

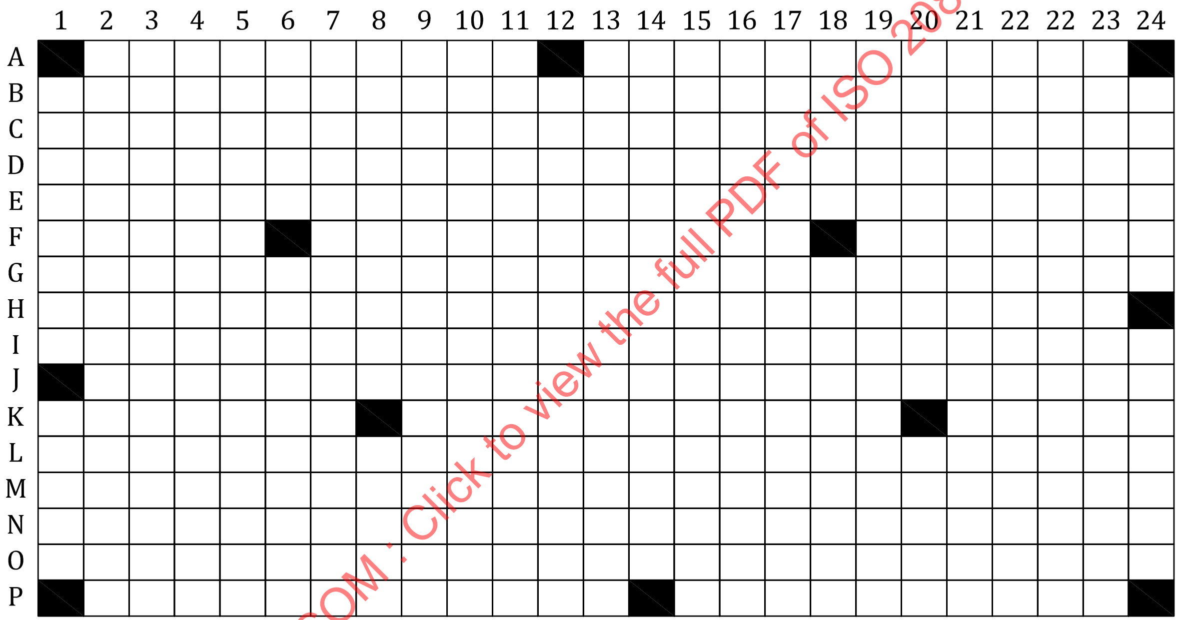
Annex A
(informative)

Sensor locations

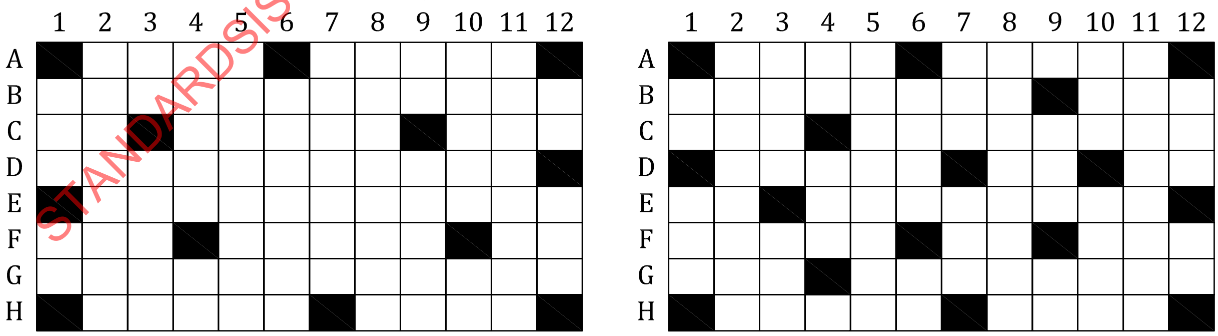
The sensor locations described in Figure A.1 are guidelines for sensor locations for different thermal cycler block formats that meet the requirements of this document.

For 96 well blocks, the guidelines contain both a 12 sensor layout (minimal) and a 16 sensor layout (optimal).

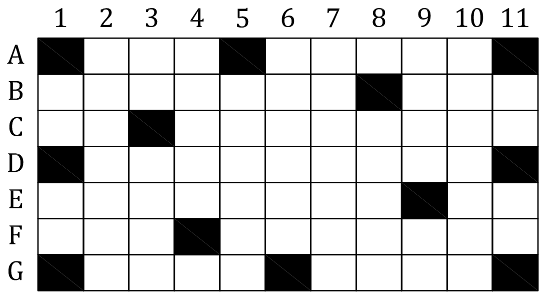
Other relevant sensor layouts meeting the requirements of 6.4.5.1 can also be used.



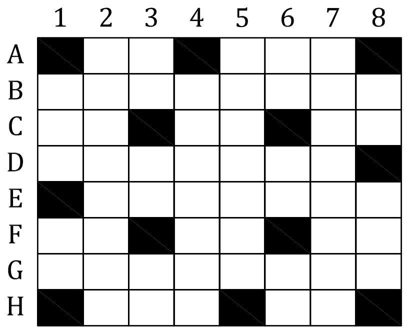
a) 384 well block – 12 sensors



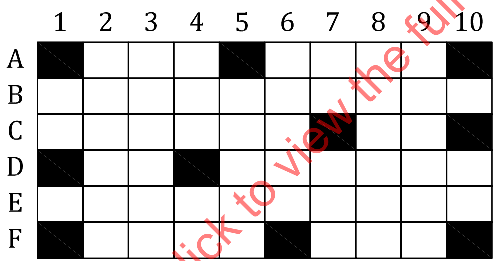
b) 96 well block – 12 sensors (minimal) or 16 sensors (optimal)



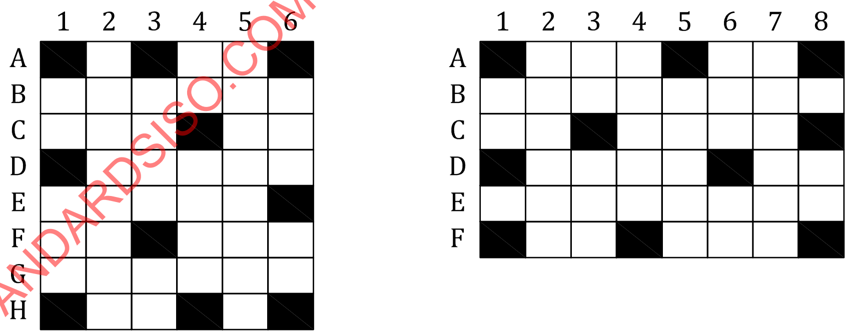
c) 77 well block – 12 sensors



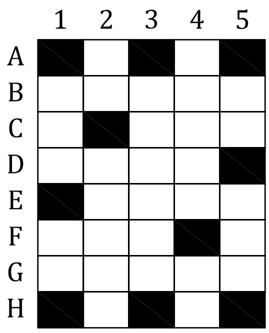
d) 64 well block – 12 sensors



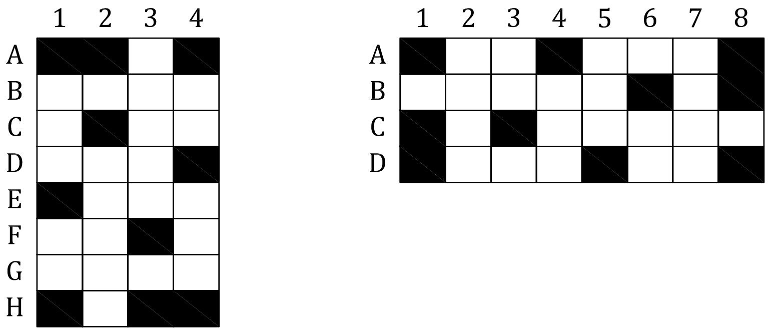
e) 60 well block – 10 sensors



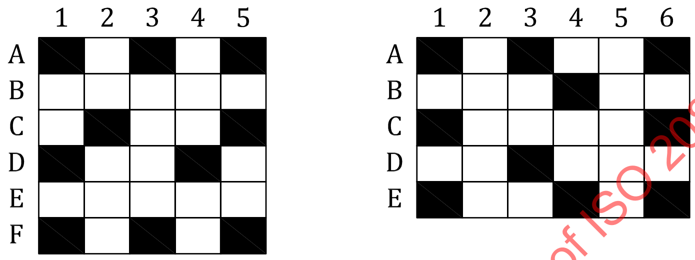
f) 48 well block – 10 sensors



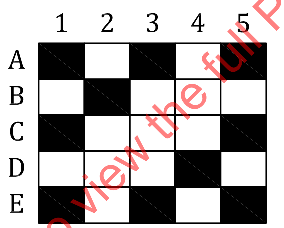
g) 40 well block – 10 sensors



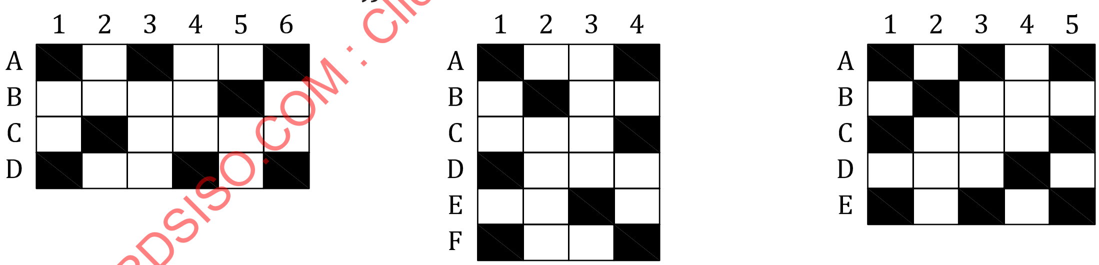
h) 32 well block – 10 sensors



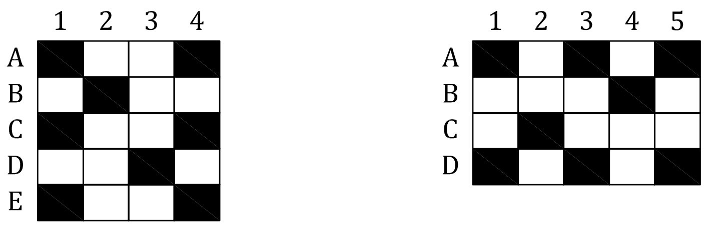
i) 30 well block – 10 sensors



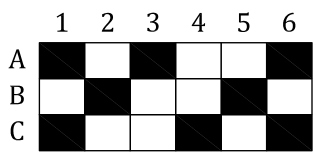
j) 25 well block – 10 sensors



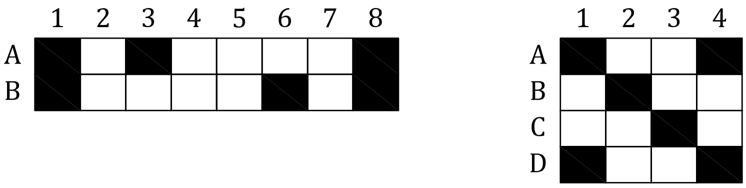
k) 24 well block – 8 or 10 sensors



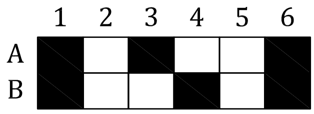
l) 20 well block – 8 sensors



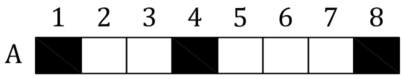
m) 18 well block – 8 sensors



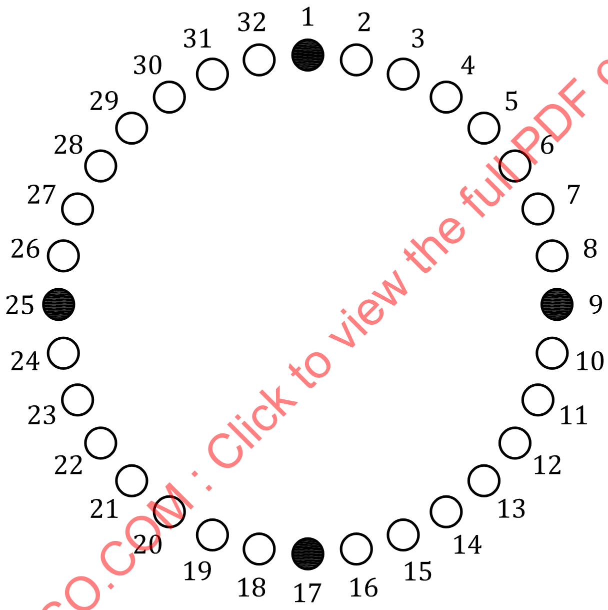
n) 16 well block – 6 sensors



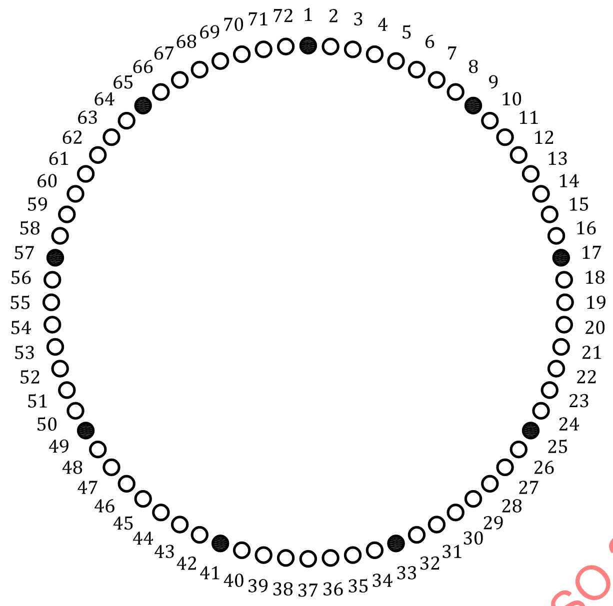
o) 12 well block – 6 sensors



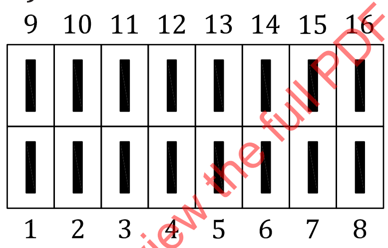
p) 8 well block – 3 sensors



q) 32 well rotor – 4 sensors



r) 72 well rotor – 9 sensors



s) Individual units – 1 sensor/unit

Figure A.1 — Sensor locations meeting the requirements of this document in different thermal cycler block formats

Annex B
(informative)

Universal temperature protocol

The protocol given in Figure B.1 meets the requirements of this document and can be used on heating block based-thermal cyclers. For heating-chamber-based thermal cyclers, it is recommended to increase the minimal temperature to 40 °C or 45 °C as this type of thermal cycler is typically not able to cool to 30 °C.

30 °C for 60 s 95 °C for 60 s 30 °C for 60 s	pre-heat step
30 °C for 60 s 95 °C for 60 s 30 °C for 60 s 90 °C for 60 s 50 °C for 60 s 70 °C for 60 s 60 °C for 60 s 30 °C for 60 s	calibration step

Figure B.1 — Universal temperature protocol

The goal of the pre-heat step is to reach a stable starting point in which the thermal cycler is pre-heated, and the thermal cycler and the temperature measurement system have reached equilibrium. Depending on the temperature measurement system used, the times and temperatures of the pre-heat step may need to be adjusted.

This universal temperature protocol is a universal performance testing protocol that can be applied to all thermal cyclers for the purpose of performance testing. It is not a PCR temperature protocol.

Annex C
(informative)

Compliancy testing

C.1 Comparison to specifications

C.1.1 General

The thermal cycler can be qualified as suitable for intended use in different ways:

a) comparison to manufacturer specifications;

b) comparison to PCR-method-based specifications;

c) if no specifications can be obtained, by a functional test with low positive controls in the wells with the most extreme temperatures.

PCR-method-based specifications are in general much wider than thermal cycler manufacturer specifications, which only apply to the thermal cycler but not to the method. The PCR-method-based specifications are strongly dependent on the robustness of the PCR method. If no method-based specifications are provided by the laboratory that has developed the PCR method, one of the methods mentioned in C.1.3 can be applied for setting PCR-method-based specifications.

C.1.2 Comparison to manufacturer specifications

Check if the thermal cycler is within manufacturer specifications by comparing the measured values with the specification limits, taking the measurement uncertainty into account (see Figure C.1).

C.1.3 Comparison to PCR-method-based specifications

C.1.3.1 General

To qualify if a thermal cycler is suitable for the intended use, the temperature robustness of the PCR method can be determined. Determining the robustness of a PCR can be done via one of the following methods; by gradient PCR or by programming suboptimal temperatures[6][7]. The goal is to identify the highest and lowest denaturation and annealing temperature at which a qualitative PCR yields a positive result or a quantitative PCR provides quantification with an acceptable difference. Depending on the availability of a gradient thermal cycler, the gradient PCR method (see C.1.3.2) or the suboptimal temperature method (see C.1.3.3) can be selected.

C.1.3.2 Gradient PCR method

In the gradient PCR method, the exact temperature limits of a PCR method are identified via the following method.

a) Calibrate the thermal cycler on which the PCR-method-based specifications will be determined in accordance with this document and determine the accuracy and deviation at the denaturation, annealing and elongation temperature.

b) Optimize the annealing and denaturation temperature of the PCR method.

c) Run a gradient PCR at denaturation temperature with a gradient window of 4 °C around the optimal denaturation temperature, using a weak positive control. Keep the annealing and elongation temperature at optimum.

d) Determine the minimum and maximum denaturation temperature that yield a correct PCR result.

e) Run a gradient PCR at annealing temperature with a gradient window of 4 °C around the optimal annealing temperature, using a weak positive control. Keep denaturation and elongation temperature at optimum.

f) Determine the minimum and maximum annealing temperature that yield a correct PCR result.

g) Set the PCR-method-based specifications based on the minimal and maximum denaturation and annealing temperatures.

h) Correct these temperatures with the deviation measured during the calibration in step a).

i) Calibrate each thermal cycler to be qualified in accordance with this document.

j) Check if each thermal cycler is within the PCR-method-based specifications, taking the measurement uncertainty into account (see Figure C.1).

C.1.3.3 Suboptimal temperature method

In the suboptimal PCR method, the temperature limits of a PCR method are approximated by programming the thermal cycler off optimum. The limits are identified via the following method.

a) Calibrate the thermal cycler on which the PCR-method-based specifications will be determined in accordance with this document and determine the accuracy and deviation at the denaturation, annealing and elongation temperature.

b) Optimize the annealing and denaturation temperature of the PCR method.

c) Run several PCRs with varying denaturation temperatures (e.g. optimal denaturation temperature −1 °C, +1 °C, −2 °C and +2 °C), using a weak positive control. Keep the annealing and elongation temperature at optimum.

d) Determine the minimum and maximum denaturation temperature that yield a correct PCR result.

e) Run several PCRs with varying annealing temperatures (e.g. optimal annealing temperature −1 °C, +1 °C, −2 °C and +2 °C), using a weak positive control. Keep the denaturation and elongation temperature at optimum.

f) Determine the minimum and maximum annealing temperature that yield a correct PCR result.

g) Set the PCR-method-based specifications based on the minimal and maximum denaturation and annealing temperatures.

h) Correct these temperatures with the deviation measured during the calibration in step a).

i) Check if each thermal cycler is within the set PCR-method-based specifications, taking the measurement uncertainty into account (see Figure C.1).

C.1.4 Functional testing using extreme temperature positions

If no specifications are available and compliancy reporting is not possible, the thermal cycler can be still qualified as suitable for intended use by a functional test on the most extreme temperature positions as described in the procedure below.

To test a thermal cycler without defining specifications the laboratory can do the following.

a) Based on the calibration results, determine the hottest, second hottest, coldest and second coldest position of the thermal cycler during the maximum overshoot at denaturation temperature and maximum undershoot at annealing temperature. In cases where the temperature profile does not show overshoots, the hottest and coldest temperatures at 30 s hold time can be determined.

b) Put weak positive PCR controls in the hottest and coldest wells and negative PCR controls in the second hottest and second coldest wells and run the PCR temperature protocol belonging to the PCR method.

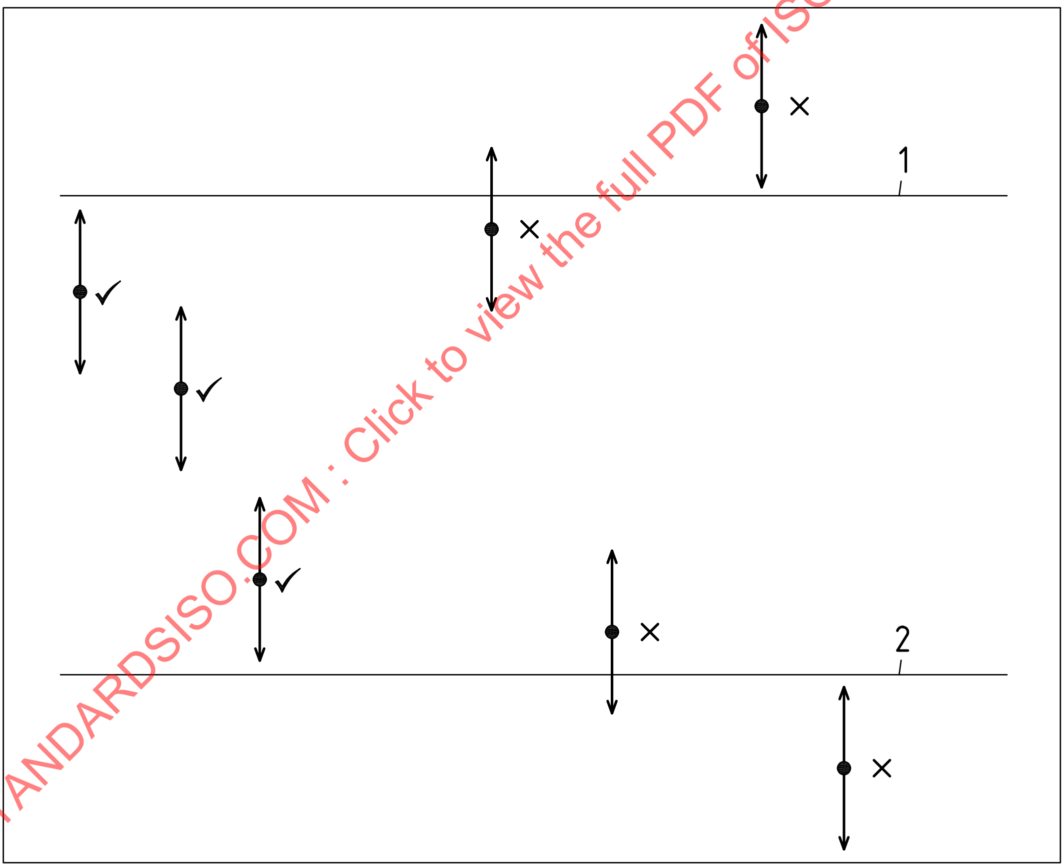
c) Check if all positive controls yield results in case of a qualitative analysis or if all positive controls yield quantities with an acceptable difference in quantity, in case of a quantitative analysis.

It is recommended to take weak positive controls close to the limit of detection (LOD) or limit of quantification (LOQ) of the PCR method itself.

When it is not feasible to perform the functional test for all methods in use, a laboratory may decide, based on a risk-based analysis, to perform the functional test with the least robust method.

Figure C.1 shows compliance with specifications for an upper and lower limit.

Thermal cyclers meet specifications (✓) when the measured value plus or minus the expanded combined uncertainty lies within the specification limits. When the uncertainty interval lies completely or partially above the upper limit or below the lower limit, the thermal cycler does not meet specifications (✗).



Key

1 upper specification limit

2 lower specification limit

Figure C.1 — Compliance with specifications for an upper and lower limit

