TECHNICAL SPECIFICATION

ISO/TS 20836

First edition
2005-08-01

Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Performance testing for thermal cyclers

Microbiologie des aliments — Réaction de polymérisation en chaîne (PCR) pour la recherche des micro-organismes pathogènes dans les aliments — Critères de performance pour les thermal cyclers

Reference number
ISO/TS 20836:2005(E)

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
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Published in Switzerland

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

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ISO/TS 20836 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

This Technical Specification is part of a series of International Standards under the general title *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens*:

— *General requirements and definitions* (ISO 22174);

— *Requirements for sample preparation for qualitative detection* (ISO 20837);

— *Performance testing for thermal cyclers* (ISO/TS 20836);

— *Requirements for amplification and detection for qualitative methods* (ISO 20838).

The International Organization for Standardization (ISO) draws attention to the fact that it is claimed that compliance with this document may involve the use of one or more patents concerning the PCR technology.

ISO takes no position concerning the evidence, validity and scope of these patent rights.

ISO has been informed that Applied Biosystems, Roche Molecular Systems, Inc. and F. Hoffman-La Roche Ltd. hold patent rights concerning the PCR technology. The companies have assured the ISO that they are willing to negotiate licences under reasonable and non-discriminatory terms and conditions with applicants throughout the world. In this respect, the statements of the holders of these patent rights are registered with ISO. Information may be obtained from:

Licensing Department
Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404
USA

and

Roche Molecular Systems, Inc.
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Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Performance testing of thermal cyclers

WARNING — The use of this Technical Specification may involve hazardous materials, operations and equipment. This Technical Specification does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this Technical Specification to establish appropriate safety and health practices and to determine the applicability of regulatory limitations prior to use.

1 Scope

This Technical Specification provides basic requirements for the installation, performance and maintenance of thermal cyclers. Although thermal cyclers are robust technical equipment, they do require regular maintenance. Their cooling/heating elements, either Peltier or other technology, have a limited lifetime. Proper functioning of the cooling/heating element depends both on the quality of the cooling/heating devices and proper use and care.

In addition to outlining the requirement for a defined maintenance programme, procedures are described for the determination of thermal cycler performance by biochemical or physical methods (see Annexes A and B).

2 Normative references

The following referenced documents are indispensable for the application 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 22174, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 22174 and the following apply.

3.1
heated lid
optional feature of a thermal cycler used to prevent evaporation from the reaction tube

3.2
temperature uniformity
homogeneity of the temperature within the thermally controlled unit (e.g. heating block)

3.3
biochemical performance test
test procedure which determines the performance of a thermal cycler by biochemical means (e.g. a temperature-sensitive PCR)

3.4
physical performance test
test procedure which determines the performance of a thermal cycler by physical means

3.5
non-robust PCR system
PCR system in which minor deviations from the prescribed PCR chemistry or PCR temperature protocol result in a less efficient amplification

3.6
critical position
position or area where a deviation of the sample temperature from the displayed temperature is more likely to occur than in other areas of the thermal cycler sample holder

4 Installation of thermal cyclers

The manufacturer's instructions should be followed.

The following should be taken into consideration:

a) thermal cyclers should be installed and run at an appropriate temperature and humidity;

b) thermal cyclers should be located

- so they can be inspected visually,
- in such a way as to allow a steady exchange of heat with the environment and free circulation of air, and
- in a constant environment.

5 Maintenance of thermal cyclers

The laboratory should establish and employ a defined maintenance programme for each thermal cycler machine, based on the documented hours of use or number of runs.

6 Performance tests

6.1 General

The performance testing of each thermal cycler should be carried out and documented, either indirectly using a biochemical performance test or directly using a physical performance test.

6.2 Biochemical performance test

A biochemical procedure for thermal cycler performance testing should be carried out, which may be a temperature-sensitive non-robust PCR method.

An example of a suitable method is described in Annex A. Any other PCR methods that can be shown to fulfil the requirements may also be used.

The performance test frequency should be based on the documented hours of use or the number of runs of each thermal cycler machine.

6.3 Physical performance test

A physical test procedure should be carried out to measure the actual sample temperature within each well during the temperature cycle, and to compare it to the displayed temperature.

An example of a suitable method is described in Annex B. A physical performance test should be undertaken if a biochemical performance test is not performed, or if the biochemical performance test indicates inefficient amplification.

The performance test frequency should be based on the documented hours of use or the number of runs of each thermal cycler machine.

7 Test report and documentation of irregularities

The test report shall conform to ISO/IEC 17025 and should contain at least the following:

— identification number of the thermal cycler;

— a reference to this Technical Specification and the Annex used for the test;

— the date of testing;

— person responsible for the analysis;

— test results;

— any particular points observed during testing;

— any deviation, additions to or exclusions from the test specification;

— any other information relevant to the specific test.

The laboratory should have documented procedures in place to identify and address any irregularities concerning thermal cycler performance (see also ISO/IEC 17025).

Annex A
(informative)

Biochemical performance test — PCR method for testing the temperature accuracy

A.1 General

This method verifies the accuracy of the control of the annealing temperature of thermal cyclers. The test is sensitive to an increase in the real annealing temperature relative to the displayed temperature during a defined temperature programme cycle.

A.2 Principle

The accuracy of the programmed/displayed temperature is tested using a specifically designed PCR. The PCR samples are highly sensitive to an overshoot of the annealing temperature during the temperature cycles.

A 362 base pair (bp) DNA sequence, which contains the target site for the PCR, is generated by amplifying the flanking region of the cloning site of the pGEM vector with a standard M13 sequencing primer pair. The cloned sequence is shown in Figure A.1.

CAGGAAACAG CTATGACCAT GATTACGCCA AGCTATTTAG GTGACACTAT AGAATACTCA AGCTATGCAT
CCAACGCGTT GGGAGCTCTC CCATATGGTC GACCTGCAGG CGGCCGCAC AGTGATTAGC AACCTCGGTA
CCATATACTA ACTCGATACA GAAACATCGG TTGGTTGATCG ATCGAGGTTT TTAAAAACCC CCTCTAGCTA
GCTAGCTAGC GATTGCTTCA CCAAGAAGAG CTCCAACAGC CTGATGGCAT CAAGTTACAC AATCCCGCGG
CCATGGCGGC CGGGAGCATG CGACGTCGGG CCCAATTCGC CCTATAGTGA GTCGTATTAC AATTCACTGG
CCGTCGTTTT AC

Figure A.1 — Template and primer sequences used in a thermal cycler performance test

The sequence of the PCR product was derived by using the M13-primers ('double'-underlined) in the PCR amplification of the target sequence from the modified pGEM plasmid (pSC17[1)]). The primers used in the test system for the thermal cycler validation are highlighted and shown separately (VAL1 and VAL2, see A.3.5.3 and A.3.5.4). The sequence of the PCR product generated in this thermal cycler performance test is underlined. A mismatch is shown in bold.

The primers VAL1 and VAL2 produce a 116 bp PCR product, which is detected by agarose gel electrophoresis. Due to the mismatch at the 3'-end of primer VAL1, the PCR is sensitive to an overshooting of the in-sample annealing temperature compared to the defined temperature. An overshooting from 63 °C to 66 °C leads to less efficient amplification and to a non-detectable PCR product of 116 bp.

The samples shall be placed at positions that are representative of the thermally controlled area, including critical positions. When the critical positions are not known, the samples should cover central and corner positions [1].

An example of a 96-well thermal cycler is given in Figure A.2.

1) This is an example of a suitable product available commercially. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

A.3 Reagents

Use only analytically pure reagents suitable for molecular biological detection methods.

A.3.1 Water.

For the amplification reaction, use water that is DNase- and RNase-free at all times. Suitable ultrapure water is available commercially.

A.3.2 PCR-buffer (without $MgCl_2$), 10× (150 mmol/l TRIS, pH 8; 500 mmol/l KCl).

A.3.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

A.3.4 dNTP solution, c(dNTPs) = 10 mmol/l (each).

A.3.5 Oligonucleotides.

Oligonucleotides require purification after synthesis.

A.3.5.1 Forward primer for the construction of the 362 bp target sequence

M13mp8 phage cloning vector (GenBank accession No. M77826.1).

Primer M13(-26): 5'-CAg gAA ACA gCT ATg AC-3'.

A.3.5.2 Reverse primer for the construction of the 362 bp target sequence

M13mp8 phage cloning vector (GenBank accession No. M77826.1).

Primer M13(-20): 5'-gTA AAA CgA Cgg CCA gT-3'.

A.3.5.3 Forward primer for testing the annealing temperature of thermal cyclers

Synthetic construct:

Primer VAL1: 5'-gAT ACA gAA ACA TCg gTT ggC-3'.

A.3.5.4 Reverse primer for testing the annealing temperature of thermal cyclers

Synthetic construct:

Primer VAL2: 5'-gTg TAA CTT gAT gCC ATC Agg-3'.

A.3.6 Plasmid pSC17[1)].

A.3.7 Thermostable DNA-polymerase (for hot-start PCR), 5 U/µl.

A.3.8 Agarose, suitable for DNA electrophoresis and for the intended size separation.

A.3.9 Boric acid (H_3BO_3), for the TBE buffer system only.

A.3.10 Bromophenol blue ($C_{19}H_9Br_4O_5SNa$) and/or **xylene cyanole FF** ($C_{25}H_{27}N_2O_6S_2Na$).

A.3.11 Glacial acetic acid (CH_3COOH), for the TAE buffer system only.

A.3.12 Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

A.3.13 Ethidium bromide (EtBr) ($C_{21}H_{20}N_3Br$).

A.3.14 Glycerol ($C_3H_8O_3$).

A.3.15 Sodium acetate (CH_3COONa), for the TAE buffer system only.

A.3.16 Hydrochloric acid (HCl).

A.3.17 Sodium hydroxide (NaOH).

A.3.18 Tris (hydroxymethyl) aminomethane (TRIS) [$NH_2C(CH_2OH)_3$]

A.3.19 TAE buffer solution, 1×, c(TRIS) = 0,050 mol/l, c(CH_3COONa) = 0,020 mol/l, c(Na_2-EDTA) = 0,001 mol/l.

Adjust the pH to 8,0 with glacial acetic acid or sodium hydroxide.

TAE buffer may be prepared as a 10-fold concentrated stock solution. It should be discarded if a precipitate forms. The concentrated solution should be diluted with water (A.3.1).

A.3.20 TRIS/borate (TBE) buffer solution, 0,5×, c(TRIS) = 0,055 mol/l, c(H_3BO_3) = 0,055 mol/l, c(Na_2-EDTA) = 0,001 mol/l.

Adjust the pH to 8,0 with hydrochloric acid or sodium hydroxide.

The TBE buffer may be prepared as a 10-fold concentrated stock solution. It should be discarded if a precipitate forms. The concentrated solution should be diluted with water (A.3.1).

A.3.21 Sample loading buffer solution, 5×, ϕ(glycerol) = 50 %, ρ(bromophenol blue) = 2,5 g/l and/or ρ(xylene cyanole FF) = 2,5 g/l, dissolved in electrophoresis buffer solution (A.3.19 or A.3.20).

A.3.22 Ethidium bromide solution, ρ(EtBr) = 0,5 mg/l.

Ethidium bromide solution should be stored as a concentrate (e.g. 10 mg/ml) at 5 °C in the dark.

WARNING — EtBr is potentially mutagenic, carcinogenic and teratogenic. Wear gloves and a mask while handling EtBr. If possible, work in a chemical hood. EtBr should be disposed of properly as chemical waste.

A stock solution should be prepared by adding an appropriate amount of water to a vessel already containing the EtBr powder, or pre-weighed EtBr tablets.

Protect the EtBr solution from light during solubilization. Dissolve slowly at room temperature by gentle mixing. Do not heat.

A.3.23 DNA molecular weight standard, e.g. a commercial preparation containing DNA fragments which cover the range from 100 bp to 200 bp.

A.4 Apparatus

A.4.1 Thermal cycler.

A.4.2 Microwave oven or **boiling water-bath**.

A.4.3 Equipment for agarose gel electrophoresis, with accessories and power supply.

A.4.4 Ultraviolet (UV) transilluminator or **lamp**, working wavelength should include 312 nm.

A.4.5 Recording instrument, e.g. photo documentation system with 3 000 ASA film and UV filter suitable for EtBr fluorescence, or video-documentation system with CCD camera, UV filter and (optional) quantitative analysis software.

A.5 Procedure

A.5.1 Preparation of the 362 bp target sequence

A.5.1.1 PCR setup

Suitable final concentrations of reagents are given in Table A.1. The method uses a final PCR volume of 50 µl per reaction.

Table A.1 — Composition of Mastermix (PCR-mix)

Reagent	Final concentration	Volume per sample (µl)
Plasmid pSC17	10 000 copies ~ 6 pg	2,0
Water		31,8
10× PCR-buffer (without $MgCl_2$)	1×	5,0
$MgCl_2$ solution[a], 25 mmol/l	2,5 mmol/l	5,0
dNTP solution, 10 mmol/l	0,2 mmol/l	1,0
Primer M13(-26), 5 µmol/l	0,25 µmol/l	2,5
Primer M13(-20), 5 µmol/l	0,25 µmol/l	2,5
Taq-DNA-polymerase, 5 U/µl	1 Unit per reaction	0,2

[a] If the PCR-buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture should be adjusted to 2,5 mmol/l.

A.5.1.2 Temperature-time programme

The temperature-time programme is given in Table A.2.

The programme may be modified depending on the cycler model and/or enzyme used.

Table A.2 — Temperature-time programme

Initial denaturation	2 min/95 °C
Amplification	10 s/95 °C 30 s/45 °C 20 s/72 °C
Number of cycles	40
Final extension	2 min/72 °C

A.5.1.3 Purification of the PCR product and determination of the yield

A 5 µl aliquot of the PCR product is separated by agarose gel electrophoresis and stained with ethidium bromide to visualize the PCR product. The remaining PCR product is purified and the yield is determined spectrophotometrically or by any other suitable method [2].

A.5.1.4 Preparation of the template concentrations

From the yield of the PCR product, the number of copies of the 362 bp fragment is calculated using standard conversion procedures.

Three solutions of the PCR product are prepared in water at a concentration of 1 000 copies/µl, 100 copies/µl and 10 copies/µl.

The code for the concentration of target DNA is the following:

— a = 1 000 copies/µl (corresponds to 5000 copies per PCR);

— b = 100 copies/µl (corresponds to 500 copies per PCR);

— c = 10 copies/µl (corresponds to 50 copies per PCR).

A.5.2 Thermal cycler test

A.5.2.1 PCR setup

This method uses a final PCR volume of 50 µl per reaction. The reagents and final concentrations are listed in Table A.3.

A minimum of 16 PCR reactions are required for a 96-well block thermal cycler.

Set up the PCR as follows:

— prepare the PCR-mix;

— divide the PCR-mix into 3 aliquots and 1 negative control;

— add the target DNA at different concentrations to the 3 aliquots to create the PCR target mix;

— divide each of the PCR target mix into 5 replicates and place the replicates in the thermal cycler sample tray at defined positions.

Table A.3 — Addition of reagents

Reagent	**Final concentration**	**Volume per sample** (µl)	**Recommended volume per 16 samples** (µl)
Water		24,78	401,48[b]
10× PCR-buffer (without $MgCl_2$)	1×	5,0	80,0
$MgCl_2$ solution[a], 25 mmol/l	3,0 mmol/l	6,0	96,0
dNTP solution, 10 mmol/l	0,2 mmol/l	1,0	16,0
Primer VAL1, 5 µmol/l	0,4 µmol/l	4,0	64,0
Primer VAL2, 5 µmol/l	0,4 µmol/l	4,0	64,0
Taq-DNA-polymerase, 5 U/µl	1,1 Units per reaction	0,22	3,52

[a] If the PCR-buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture should be adjusted to 3 mmol/l.

[b] Refers to water for 16 reactions and 5 µl for the NTC (5 µl template replaced by 5 µl water).

Divide the PCR-mix into 3 aliquots of 225 µl and 1 sample of 50 µl negative control (NTC). Add to the first aliquot 25 µl of target DNA at concentration "a" containing 1 000 copies/µl, to the second aliquot add 25 µl of target DNA at concentration "b" containing 100 copies/µl and to the third aliquot add 25 µl of the target DNA at concentration "c" containing 10 copies/µl and mix carefully. Prepare five 50-µl reactions from each of the mixes and place them into the thermal cycler according the scheme as outlined in Figure A.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**a**									**a**		
B		**b**									**b**	
C			**c**									**c**
D					**a**	**b**	**c**	**N**				
E												
F	**a**									**a**		
G		**b**									**b**	
H			**c**									**c**

Positions labelled **a**, **b** and **c** are reaction tubes with 5 000, 500 and 50 target copies, respectively.

N = NTC (no template control)

Figure A.2 — Layout of the PCR tubes for the test in a 96-well thermal cycler

A.5.2.2 Temperature-time programme

The temperature-time programme is given in Table A.4.

Table A.4 — Temperature-time programme

Initial denaturation	2 min/95 °C
Amplification	10 s/95 °C 20 s/63 °C 20 s/72 °C
Number of cycles	40
Final extension	5 min/72 °C

A.5.3 Detection

A.5.3.1 General

PCR products are detectable by gel electrophoresis. Gel electrophoresis may be performed using either the TAE buffer system or the TBE buffer system. Use the same buffer to dissolve both the agarose and to fill the electrophoresis tank.

A.5.3.2 Agarose gel preparation, electrophoresis and staining

Weigh an appropriate amount of agarose (A.3.8) and add it to the electrophoresis buffer solution (A.3.19 or A.3.20) to reach a final concentration of ρ = 20 g/l. Gels should not be thicker than 1 cm.

Mix 5 µl of each of the PCR products with approximately 1 µl of loading buffer (A.3.21) and apply to the gel.

Apply the molecular weight marker to a separate well.

Carry out the electrophoresis at room temperature. Apply an appropriate voltage to give a maximum constant voltage of 5 V/cm between the electrodes.

After completing the electrophoresis, incubate the gel for not less than 30 min in the ethidium bromide solution (A.3.22) at room temperature, in the dark.

Any other agarose gel staining method that gives equivalent results may be applied.

Transfer the gel to a transilluminator (A.4.4). Switch on the UV light and record the DNA fluorescence by photography or video-documentation.

A.6 Interpretation of the results

The size of the PCR product is determined by comparison with the fragments of the DNA molecular weight standard (A.3.23).

If in all samples an amplification product of 116 base pairs can be detected, any overshooting of the annealing temperature of 63 °C is less than 3 °C. If no bands are visible, either there is an overshooting of the annealing temperature or insufficient denaturation. Both situations can arise from poor control of the temperatures programmed in the thermal cycler under study.

The interpretation of the results is given in Table A.5.

Table A.5 — Interpretation of results

Copy number	Acceptable performance: detectable product	Unacceptable performance: product not detectable
5 000	5 of 5	< 5 of 5
500	5 of 5	< 5 of 5
50	$\leqslant$ 5 of 5[a]	

[a] The reaction with 50 copies per reaction can produce faint bands.

In the case of a result that is interpreted as unacceptable performance (see Table A.5), the test shall be repeated. If the result is confirmed, the thermal cycler performance is considered deficient and the equipment shall be subjected to a physical performance test.

Annex B
(informative)

Physical performance test

B.1 General

This is a method to verify the temperature accuracy of thermal cyclers under cycling conditions. The test records the temperature in the liquid in a sample tube.

B.2 Principle

The temperature is measured by thermocouples or other devices that are capable of recording the temperature inside a vial containing water.

The physical test procedure is performed under PCR cycling conditions. This usually comprises a sequence of the three consecutive temperature levels used for denaturation, primer annealing and primer extension.

If the cycler has a heated lid then the measurement should be carried out with the lid operating.

B.3 Reagents

B.3.1 Water.

B.4 Apparatus

B.4.1 Thermal cycler.

B.4.2 Temperature recording unit: temperature recording probes, wires and temperature recording instrument.

Calibrate the temperature recording unit regularly for the intended temperature range.

B.5 Procedure

B.5.1 Test layout

Place the temperature recording probes at positions that are representative of the thermally controlled area. Critical positions should especially be considered. When the location of critical positions is not known, the sensors should be distributed randomly in the wells.

EXAMPLE For a 96-well cycler, divide the well area into at least 6 equally sized regions. Place at least 2 temperature recording probes in each region. This results in a minimum of 12 points where the temperature is recorded [1].

B.6 Recording temperature values

The recording rate should be set so that, as a minimum, one temperature value is recorded per position per second [1].

As a minimum, perform measurements on three consecutive runs of at least 30 cycles each.

For each of the test positions, record the mean of the highest and lowest recorded temperature at time-point t_0 (time-point when the temperature programme starts) and t_x (time-point when the temperature programme finishes) for the denaturation, annealing and extension steps [1].

B.7 Interpretation of the results

Investigate the temperature recorded at the positions showing the highest and the lowest temperatures. Both temperatures shall be either within the temperature listed in the instrument specifications or, if a statement of temperature accuracy is not given, shall not deviate more than 0,5 °C from the programmed temperature. The temperature shall stay within the programmed range for each step in the cycle and the entire run.

Thermal cyclers that do not comply with these criteria shall not be used.

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ICS 07.100.30

Price based on 13 pages