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Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions

Microbiologie des aliments — Réaction de polymérisation en chaîne (PCR) en temps réel pour la détection des micro-organismes pathogènes dans les aliments — Exigences générales et définitions

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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.

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ISO 22119 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

The polymerase chain reaction (PCR) has been shown to be a fast, sensitive, and specific method for detection of food-borne pathogens. Further developments of the technology allow the detection of specific PCR products generated by the amplification process. The principle relies on the excitation of fluorescent markers during the PCR process.

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

ISO/TS 20836, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction* (*PCR*) *for the detection of food-borne pathogens — Performance testing for thermal cyclers*

ISO 20837, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction* (*PCR*) *for the detection of food-borne pathogens — Requirements for sample preparation for qualitative detection*

ISO 20838, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction* (*PCR*) *for the detection of food-borne pathogens — Requirements for amplification and detection for qualitative methods*

ISO 22118, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction* (*PCR*) *for the detection and quantification of food-borne pathogens — Performance characteristics*

ISO 22119, *Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction* (*PCR*) *for the detection of food-borne pathogens — General requirements and definitions*

ISO 22174, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction* (*PCR*) *for the detection of food-borne pathogens — General requirements and definitions*

The following Technical Specification is in preparation:

ISO/TS 13136, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Shiga toxin-producing* Escherichia coli (*STEC*) *belonging to O157, O111, O26, O103 and O145 serogroups — Qualitative real-time polymerase chain reaction (PCR)-based method*

Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions

1 Scope

This International Standard defines terms for the detection of food-borne pathogens in foodstuffs, and isolates obtained from them, using the polymerase chain reaction (PCR). This International Standard also specifies requirements for the amplification and detection of nucleic acid sequences (DNA or RNA after reverse transcription) by real-time PCR.

The minimum requirements laid down in this International Standard provide the basis for comparable and reproducible results within individual and between different laboratories.

This International Standard is also applicable, for example, to the detection of food-borne pathogens in environmental samples and in animal feeding stuffs.

NOTE Because of the rapid progress in this field, the examples given are those most frequently in use at the time of development of this International Standard.

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 20838, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for amplification and detection for qualitative methods*

ISO 22174:2005, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

3 Terms and definitions

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

3.1
real-time polymerase chain reaction
real-time PCR
enzymatic procedure which combines the *in vitro* amplification of specific DNA segments by a process of denaturation, annealing of specific primers, and synthesis of DNA with the detection of specific PCR products during the amplification process

NOTE 1 Generally, the amplification reaction mixture contains one or more specific DNA probes coupled with one or more fluorescent dyes. Using this technology, the signal is generated after specific hybridization of the probes to the target nucleic acid sequence and excitation with light of a definite wavelength.

NOTE 2 The use of non-specific DNA-binding fluorescent dyes can be applied if positive results are verified in accordance with ISO 20838.

3.2
PCR product
DNA amplified by PCR

[ISO 22174:2005, 3.4.5]

3.3
fluorescence resonance energy transfer
FRET
⟨food-borne pathogen detection by PCR⟩ distance-dependent energy transfer from a donor molecule to an acceptor molecule resulting in enhanced fluorescence of the acceptor molecule after excitation with electromagnetic radiation of a definite wavelength

NOTE Taken from Reference [2].

3.4
reporter
⟨food-borne pathogen detection by PCR⟩ fluorescent molecule used to detect the hybridization of specific probes by excitation with electromagnetic radiation of an appropriate wavelength

3.5
quencher
⟨food-borne pathogen detection by PCR⟩ fluorescent molecule serving as an energy acceptor and thus quenching the fluorescence signal of the reporter (donor)

3.6
dark quencher
molecule serving as an acceptor, which does not emit energy in a spectral range detected by the optical detection system of the real-time PCR instrument

3.7
5′-3′-exonuclease activity
ability of an enzyme, e.g. a nucleic acid polymerase, to cleave a hybridized nucleic acid molecule in the 5′-3′-direction

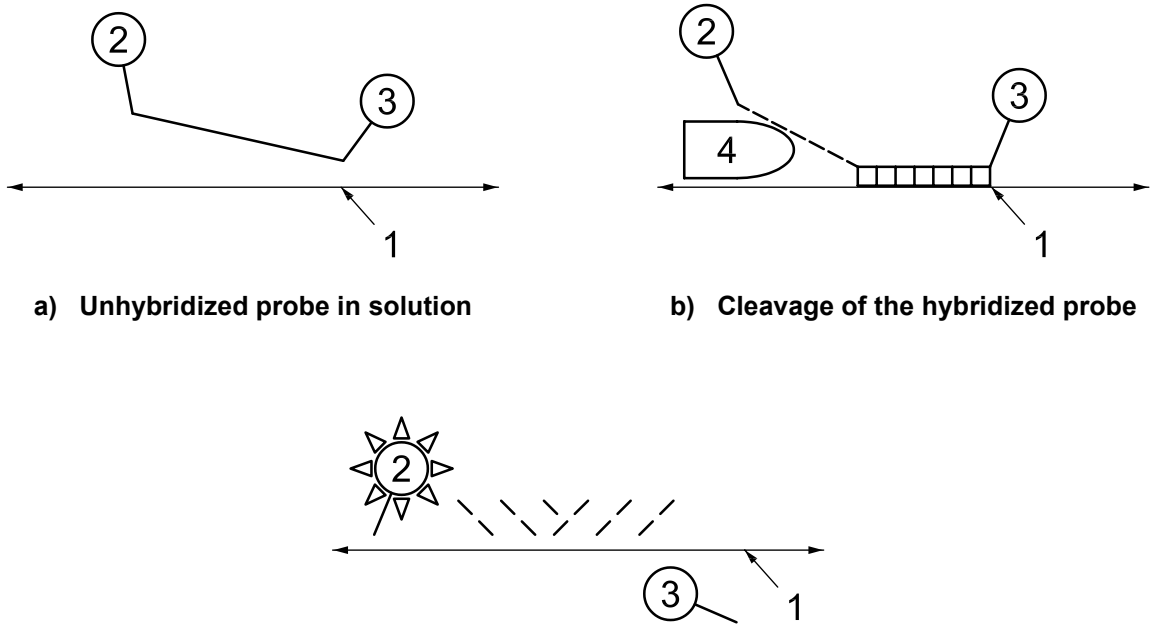
NOTE The activity of 5′-3′-exonuclease is double stranded DNA specific. It is dependent on the type of enzyme and can be present, for example, in Taq-, Tth- and Tfl-polymerase.

3.8
fluorescent probe
oligonucleotide or oligonucleotide analogon of defined sequence coupled with one or more fluorescent molecules

NOTE Any system emitting a fluorescence signal after specific hybridization to the target nucleic acid sequence which can be detected by the specific equipment can be used as a fluorescent probe.

3.9
hydrolysis probe
fluorescent probe coupled with two fluorescent molecules which are sterically separated by the 5′-3′-exonuclease activity of the enzyme during the amplification process

NOTE The principle of a hydrolysis probe is illustrated in Figure 1.



a) Unhybridized probe in solution

b) Cleavage of the hybridized probe

c) Cleaved probe resulting in reporter fluorescence after excitation

Key

1 DNA substrate
2 fluorescent molecule (reporter)
3 quenching molecule
4 enzyme

Figure 1 — Principle of a hydrolysis probe

3.10
hybridization probe
system of two fluorescent probes coupled with one fluorescent molecule each, where one molecule serves as donor and the other serves as acceptor

NOTE The principle of a hybridization probe is illustrated in Figure 2.

a) Unhybridized probes in solution

b) Hybridized probes resulting in acceptor fluorescence

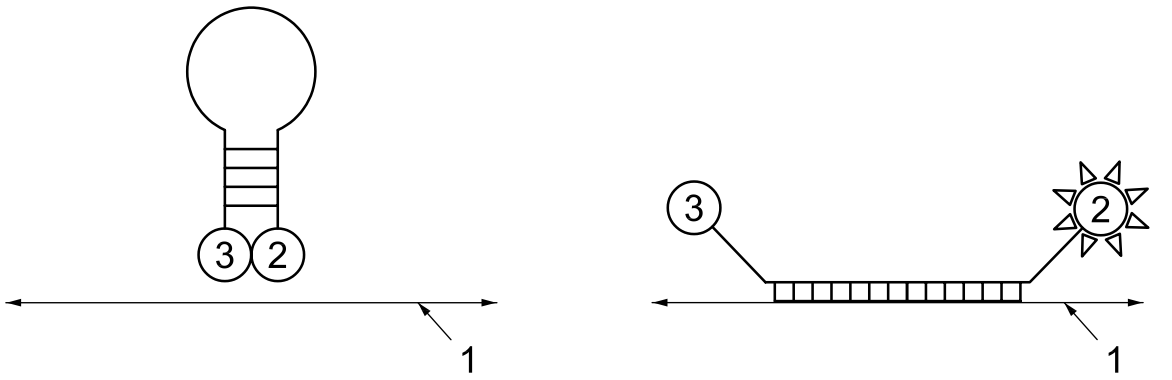
Key

1 DNA substrate
2 acceptor molecule
3 donor molecule

Figure 2 — Principle of a hybridization probe

3.11
molecular beacon
fluorescent probe consisting of three different parts: a central part complementary to the target nucleic acid sequence, plus a 5′-part and a 3′-part which are complementary; the reporter is attached to one arm of the molecule, while the end of the other carries a quencher

NOTE The principle of a molecular beacon is illustrated in Figure 3.



a) Unhybridized molecular beacon in solution

b) Hybridized molecular beacon resulting in reporter fluorescence

Key
1 DNA substrate
2 fluorescent molecule (reporter)
3 quenching molecule

Figure 3 — Principle of a molecular beacon

3.12
probe for detection of a specific pathogen DNA sequence
probe with a sequence complementary to the DNA of a pathogen with a reporter emitting a signal of a definite wavelength which can be detected by the optical detection system

3.13
probe for detection of an internal control nucleic acid sequence
probe with a reporter designed to confirm amplification performance

NOTE 1 The probe emits a signal clearly distinguishable from the signal of the probe designed for the detection of the specific pathogen.

NOTE 2 The application of internal controls requires the use of an instrument able to detect signals of different wavelength.

3.14
passive reference
fluorescent molecules present in the reaction mix used to normalize the signal

NOTE These may be coupled nucleic acid sequences or other molecules not taking part in the reaction.

3.15
baseline fluorescence detection level
“baseline”
point at which a reaction reaches a fluorescence intensity above the background

3.16
background fluorescence
"background"
intrinsic level of fluorescence resulting from the reagents and consumables used

3.17
threshold cycle crossing point
point of the amplification curve at which the fluorescence signal rises above the baseline or crosses a predefined threshold setting

4 Principle

4.1 General

Real-time PCR analysis generally consists of:

a) amplification of specific target sequences by PCR in the presence of fluorescent probes;

b) binding of fluorescent probes during each amplification cycle;

c) generation of a fluorescent signal by excitation during each cycle;

d) monitoring of the fluorescence signals by the optical detection system;

e) data analysis.

NOTE For screening purposes, fluorescence signals from DNA double strand binding dyes can also be used.

4.2 Probes for real-time PCR

4.2.1 Hydrolysis probes

The hydrolysis probe is a specific oligonucleotide present in the PCR assay together with the PCR primers. One end of the probe bears a fluorescent reporter molecule with an emission spectrum which is quenched by a second molecule located at the other end.

The probe hybridizes to the target nucleic acid sequence. During the extension step, the 5′-3′-exonuclease activity of the DNA polymerase cleaves the hybridized probe. After cleavage, the reporter is separated from the quencher, resulting in an increase in the fluorescence intensity of the reporter. The resulting fluorescence signal is proportional to the production of specific PCR product.

The 3′-end of the probe should be blocked to prevent its extension during the PCR.

4.2.2 Hybridization probes

Two hybridization probes are present in the PCR assay as specific oligonucleotides in addition to the PCR primers. These probes, each containing a fluorescent molecule, one of which serves as a donor, the other as an acceptor, hybridize to the target nucleic acid sequences. After hybridization, both dyes are in a close proximity so that fluorescence resonance energy transfer occurs on excitation and the acceptor molecule generates a detectable signal. The resulting fluorescence signal is proportional to the production of a specific PCR product.

The 3′-end of the probes should be blocked to prevent its extension during the PCR.

4.2.3 Molecular beacons

The molecular beacon is a specific oligonucleotide present in the PCR assay together with the PCR primers.

When molecular beacons bind to a complementary target sequence at the hybridization temperature, they undergo a conformational transition that forces the stem apart. This results in a probe-target-hybrid that is longer and more stable than the stem. This separates the reporter and the quencher, resulting in a reporter signal (see Reference [3]). The resulting fluorescence signal is proportional to the production of specific PCR product.

5 General laboratory requirements

General laboratory requirements shall be in accordance with ISO 22174.

6 Reagents and materials

6.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and sterile distilled or demineralized water or water of equivalent purity free of nucleic acids and nucleases suitable for molecular biological analysis.

Avoid the use of any reagents or consumables, e.g. components of enrichment broths, whose fluorescence interferes with the detection system.

Special requirements apply to the reagents specified in 6.2 to 6.6.

6.2 DNA polymerase and reaction buffer

6.2.1 DNA polymerase

A thermostable polymerase (possibly with reverse transcriptase activity) is used for PCR. It should be used as directed by the manufacturer.

NOTE This can be a purified, native enzyme, or a purified, genetically engineered recombinant form of the enzyme.

If hydrolysis probes are to be used, the DNA-polymerase should possess 5′-3′-exonuclease activity. For RNA analysis, a mixture of reverse transcriptase and DNA polymerase enzymes or a DNA polymerase with reverse transcriptase activity is required.

Each DNA polymerase may need different experimental conditions.

6.2.2 Reaction buffer

The reaction buffer shall be in accordance with ISO 20838.

6.3 Deoxyribonucleoside triphosphates (dNTPs) for PCR

The dNTPs shall be in accordance with ISO 20838.

6.4 Primers

The primers shall be in accordance with ISO 20838.

6.5 Fluorescent probes for real-time detection

Oligonucleotides shall be of appropriate quality, and designed to detect a sequence present in the specific PCR-product. The probe sequence should be highly complementary to the target DNA sequence.

6.6 Internal amplification control

The amplification efficiency of a test sample can be verified by using a control DNA fragment added to the same reaction vessel as the DNA extract of a test sample. It is either possible to use the same primer pair for amplification of the control DNA fragment as for amplification of the target DNA (homologous internal amplification control) or another primer pair (heterologous internal amplification control). The system with target and the system with the internal amplification control should result in approximately equal amplification efficiencies. The concentration of the added control DNA fragment should be as low as possible in order to detect even small inhibitions and at the same time give a statistically reproducible positive result. Further, it should be ascertained that the internal amplification control does not affect the detection level of the target. The following reduce the risk of negative interactions on the detection level:

— a slightly lower amplification efficiency of the homologous internal amplification control than the target DNA;

— a reduced concentration of primers for the heterologous internal amplification control.

The internal amplification control may be added to the sample at an early stage of analysis and thus at the same time act as a control of the extraction procedure.

6.7 Reagents to prevent carry over

The carry-over prevention reagents shall be in accordance with ISO 20838.

7 Apparatus

7.1 General

The hardware shall be in accordance with ISO 22174.

The laboratory shall use properly maintained equipment suitable for the methods employed.

7.2 Special apparatus and equipment

In addition to usual laboratory equipment, and the equipment indicated in ISO 22174, the following apparatus shall be used.

7.2.1 Thermal cycler equipped with:

a) an energy source suitable for the excitation of fluorescent molecules;

b) an optical detection system, for detection of fluorescence signals generated during PCR.

The application of internal controls requires the use of an instrument capable of detecting signals of different wavelength.

7.2.2 Reaction vessels and **caps** or **closures** which can be repeatedly heated to 100 °C and cooled to 4 °C without damage and which do not influence the fluorescence signal generated during the amplification process.

8 Laboratory sample

Extracts containing nucleic acids from any matrix appropriate to the field of application are suitable as laboratory samples provided that there is no demonstrable PCR inhibition or fluorescence interference.

9 Procedure

9.1 Sample preparation

Nucleic acid extraction and/or purification of the test sample should be carried out in accordance with an appropriate method, e.g ISO 20837 [1].

The resulting nucleic acid solution should contain a sufficient amount of target nucleic acid of sufficient quality so that for qualitative analysis one target microorganism to 10 target microorganisms or viral genome equivalents in the test portion can be detected. An enrichment and/or concentration of the target microorganism or virus is normally required to allow qualitative analysis of low numbers of targets in the test portion.

The resulting nucleic acid solution should contain substances with neither demonstrable PCR inhibition effects nor fluorescence interference.

NOTE Fluorescence is often derived from coloured reaction vessels and some ingredients of enrichment broths.

Quantitative analysis requires a sample preparation procedure which ensures that the amount of the nucleic acid of the target microorganism or virus in the resulting nucleic acid solution is highly reproducible.

9.2 Amplification

9.2.1 General

Amplification of specific nucleic acids is performed *in vitro* through a reaction catalysed by a DNA polymerase in the presence of oligonucleotide primers, deoxynucleoside triphosphates, and fluorescent probes in a defined reaction buffer.

Additionally to conventional PCR, the set-up for the reaction mixture shall avoid the use of coloured pipette tips and reaction vessels. Care shall be taken to prevent any contamination with dust particles outside the reaction vessels.

The signal of the fluorescent probe is monitored during the amplification process.

RNA can be detected using real-time PCR if the sequence has first been transcribed into a complementary DNA sequence by reverse transcription.

9.2.2 Cycling parameters

9.2.2.1 Hydrolysis probes

In general, the amplification process uses a two-step cycling protocol with denaturation, and a combined primer and probe annealing and extension step. The fluorescence signal is monitored during the annealing and extension step.

9.2.2.2 Hybridization probes

The amplification process uses a three-step cycling protocol with denaturation, primer and probe annealing and extension step. The fluorescence signal is monitored during the annealing step.

9.2.2.3 Molecular beacons

In general, the amplification process uses a three-step cycling protocol with denaturation, primer, and molecular beacon annealing and extension step. The fluorescence signal is monitored during the annealing step.

9.3 Controls

The controls outlined in ISO 22174 shall be used.

Real-time PCR allows the simultaneous detection and differentiation of the signal of the specific pathogen and of the internal amplification control. Therefore, internal amplification controls are recommended.

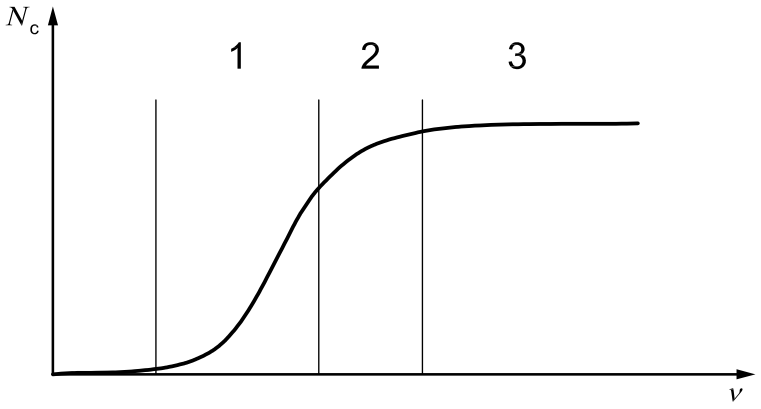
9.4 Analysis of the fluorescence data

9.4.1 Amplification plot

9.4.1.1 General

During the amplification process, the number of detectable PCR products increases. The increase of the fluorescence signal is related to the increase of the PCR products. This can be graphically displayed in an amplification curve (see Figure 4).

A typical amplification curve consists of three phases that characterize the progression of the PCR.



Key

N_c number of amplified molecules

ν number of amplified cycles

1 exponential phase
2 linear phase
3 plateau phase

Figure 4 — An amplification curve

9.4.1.2 Phase 1: Exponential phase

The exponential phase is a cycle range of high precision which is characterized by a high and constant amplification efficiency. During the exponential phase, the relationship between the PCR product to initial template can be described by Equation (1):

$$N_c = N\,(1+\eta)^{\nu} \tag{1}$$

where

N_c is the number of amplified molecules;

N is the initial number of the target molecules;

η is the efficiency of the system;

ν is the number of amplification cycles.

9.4.1.3 Phase 2: Linear phase

The linear phase is characterized by a levelling effect where the slope of the amplification curve decreases steadily. At this point, one or more components have fallen below a critical concentration and the amplification efficiency has begun to decrease. The phase is termed linear because amplification approximates an arithmetic progression rather than a geometric increase.

9.4.1.4 Phase 3: Plateau phase

At plateau, the PCR amplification stops and the signal remains relatively constant (see Reference [4]).

9.4.2 Evaluation of the fluorescence data

A positive sample generates an amplification plot with at least phase 1 of a typical amplification curve. The amplification curve of these samples crosses a defined threshold setting after a certain number of cycles. A sample with a fluorescence signal above the threshold is considered positive.

9.4.3 Quantitative analysis

9.4.3.1 General

A quantitative assay determines the fluorescence corresponding to the amount of nucleic acid target sequences generated during the amplification phase of the PCR. This can be used to determine the initial amount of target nucleic acid within the sample.

Target nucleic acids are quantified by reference to a standard curve.

Other methods can be applied if their validity has been demonstrated.

9.4.3.2 Standard curve method for quantification

Any stable and pure target RNA or DNA stock with a known concentration can be used for preparation of a standard curve by serial dilution. The amplification efficiencies of the standard curve and the target nucleic acid should be closely matched. Plasmid DNA and *in vitro* transcribed RNA are commonly used to prepare standards.

The target concentration should fall into the range of the standard curve.

An appropriate number of calibration points and replicates covering the range of quantification shall be applied [e.g. at least four calibration points with two replicates (a total of 4×2 values) or six calibration points with one measurement at each point (six values altogether)].

10 Evaluation and documentation

Evaluation is possible provided the results obtained with the controls specified in 9.3 are unambiguous.

The possible PCR results are listed in ISO 22174:2005, Table 2.

Bibliography

[1] ISO 20837, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for sample preparation for qualitative detection*

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1) Example of a product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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