



PCRFast[®] Realtime (SYBR[®] Green) und Gel

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General information

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PCRFast[®]

Realtime (SYBR[®]Green) and gel detection

Brief information

Easy - to - use molecular biological test (PCR) for the detection of specific DNA fragments using realtime (SYBR[®]Green) and / or gel detection in fish tissue. The test kit contains 96 reaction vials. All reaction vials contain a specific primer pair.

48 reaction vials (red - coloured) contain additional homologous, specific control DNA for the PCR positive control (PTC) and inhibition control (ITC).

This product was developed by our partner **Applied Food Technologies (AFT), US** (www.appliedfoodtechnologies.com).

Sample preparation

We recommend the sample extraction using a modified CTAB method (ISO 21571, Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Methods for nucleic acid extraction).

| | | |
|-------------------|--------------------------|-------------|
| Time requirement: | extraction..... | 40 min |
| (10 samples) | PCR - setup..... | 15 min |
| | PCR..... | 2 hrs |
| | gel electrophoresis..... | 15 - 30 min |

PCRFast[®]

ist ein eingetragenes Warenzeichen der ifp Institut für Produktqualität GmbH. ifp führt auch Auftragsanalytik durch.

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1. Test principle

PCRFast[®] is an easy - to - use molecular biology test for the detection of specific DNA fragments in fish tissue. The test system is in accordance with international PCR standards (ISO).

All reaction vials contain specific primers in an optimum amount.

The colourless reaction vials are used for the detection of **specific DNA extracted from the sample**, for the **negative control NTC** (check on MasterMix for contamination) and for the **extraction control ETC** (check on extraction for contamination).

In addition to the primers, **the red - coloured reaction vials** contain homologous, specific control DNA. These reaction vials are used for the **positive control PTC** (check on functionality of MasterMix), for the **inhibition control ITC** (check on isolated DNA for inhibitors) and for the verification of the result using the melting curve analysis.

12.5 µl of double concentrated MasterMix and, subsequently, **12.5 µl of the extracted DNA sample** is added into the reaction vial. The MasterMix contains an optimum concentration of polymerase, nucleotides and magnesium chloride, in realtime additionally with SYBR[®]Green. Afterwards, the target sequence is amplified in a PCR thermocycler. The amplified DNA is made visible by realtime (SYBR[®]Green) and gel electrophoresis with ethidiumbromide. Using realtime (SYBR[®]Green) positive results have to be verified by melting curve analysis and if possible by gel electrophoresis with ethidiumbromide. Therefore the melting curve of the sample should be compared with the melting curve of the inhibition control ITC.

2. Reagents provided

Each kit consists of:

- 6 x **strips (colourless)**, each with 8 x 0.2 ml reaction vials (48 reaction vials), coated with specific primers (for the detection of DNA in the sample, negative control NTC and extraction control ETC)
- 6 x **strips (red - coloured)** with 8 x 0.2 ml reaction vials each (48 reaction vials), coated with specific primers and specific control DNA (for positive control PTC and inhibition control ITC)

3. Required reagents and instruments, not provided

Instruments and lab materials

- vortexer
- shaking incubator 60 - 65 °C (140 °F - 149 °F)
- microcentrifuge, > 14,000 x g (for 1.5 ml and 2.0 ml reaction vials)
- PCR thermocycler:**
 - Gel e.g. ABI 7500, Eppendorf MasterCycler, with heating block for 0.2 ml reaction vials
 - Realtime e.g. Applied Biosystems 7500, STRATAGENE Mx 3005 P, each with melting curve analysis software
- gel electrophoresis equipment and gel documentation system
- filter tips for micropipettes: 2 - 20 µl, 20 - 200 µl and 100 - 1000 µl, e.g. Gilson Pipetman P, each with filter tips
- 1.5 ml or 2.0 ml reaction vials

Reagents

DNA extraction

- CTAB buffer (CTAB 2 %, NaCl 1.4 mol / l, Tris 0.1 mol / l, EDTA 0.02 mol / l, pH = 8.0)
- chloroform (alternatively: Ready Red, Q-BIOgene; Fa. Q-Biogene / MP Biomedicals Europe, Cat. RYRED001)
- proteinase K
- isopropanol absolute
- ethanol 70% (denatured)
- 0.1 x TE buffer (Tris 1 mmol / l, EDTA 0.1 mmol / l)
- DNA purification kit with silica gel column, e.g. PCRFast DNA Purification
- water, deionized
- DNA extraction kit e.g. PCRFast[®] CTAB Tissue Extraction

PCR

only for gel detection

double concentrated MasterMix (Hotstart recommended)

e.g. AmpliTaq Gold[®] PCR MasterMix, Applied Biosystems No. 4318739;
Qiagen HotStarTaq MasterMix Kit Qiagen No. 203443; Brilliant II QPCR
MasterMix, Stratagene No. 600804

for gel detection and / or Realtime (SYBR[®]Green)

double concentrated MasterMix (Hotstart recommended)

e.g. Power SYBR[®] Green PCR MasterMix, Applied Biosystems No.
4367659; QuantiTect SYBR[®]Green PCR Kit Qiagen No. 204143;
Brilliant II SYBR[®] Green MasterMix Stratagene No. 600548

Detection

- agarose
- ethidiumbromide
- buffer for gel preparation, to cover the agarose gel and as electrophoreses running buffer:
 - 1 x TAE buffer (Tris 40 mmol / l, acetic acid 20 mmol / l, EDTA 1 mmol / l)
- gel loading buffer, e.g. 5x (sucrose 5 %, orange G 0.25 %)
- DNA length standard, 50 - 1000 bp

4. Precautions

- all laboratory and PCR work should be carried out with the usual safety equipment in accordance with CEN/ISO recommendations
- to reduce the risk of contamination, all working operations should be performed wearing gloves and using filter tips
- the sample extraction, PCR setup and detection should be carried out in separate rooms
- ethidiumbromide is a hazardous DNA substance and should be used with care
- UV light can damage DNA: be careful when handling UV radiation from the transilluminator

5. Storage instructions

The test kit or reaction vials should be stored at 2 - 8 °C (35.6 - 46.4 °F).

6. Sample extraction (DNA isolation)

For the PCR analysis a **double extraction** for each sample and an **extraction control ETC** for each analysis run is recommended (ISO 21571, Foodstuffs - methods of analysis for the detection of genetically modified organisms and derived products - methods for nucleic acid extraction).

6.1. DNA extraction

- 1) weigh **200 mg tissue** into a 2 ml reaction vial, add **1 ml CTAB buffer** and **25 µl proteinase K solution (20 mg/ml)**; then shake vigorously
- 2) place the reaction vial from into the shaker incubator for 90 min at 60 °C (140 °F) (or over night); then cool to below 30 °C (86 °F)
- 3) centrifuge at no less than 14,000 x g for 10 min
- 4) place **600 µl chloroform** (or ReadyRed) into a 2.0 ml reaction vial, add **900 µl supernatant from (3)** and vortex vigorously for 15 sec
- 5) centrifuge at no less than 14,000 x g for 10 min (should the supernatant not have turned clear, centrifuge for another 5 min)
- 6) **place 500 µl Isopropanol, absolute** into a 2.0 ml reaction vial and add **625 µl of the upper phase from (5)**; close reaction vial, turn upside down, mixing well (eventually the genomic DNA can be seen as a fibre in the solution), then vortex vigorously for 5 sec
- 7) centrifuge at no less than 14,000 x g for 10 min
- 8) carefully remove supernatant with a pipette; add **500 µl ethanol (70 %)** to the pellet and shake
- 9) centrifuge at no less than 14,000 x g for 10 min
- 10) carefully remove supernatant with a pipette, centrifuge briefly once more (15 sec at no less than 14,000 x g), remove remaining ethanol with a pipette and leave pellet to dry in the open reaction vial for 10 min at 37 °C (98.6 °F) in desiccator cabinet (or for 1 h at room temperature) to remove the ethanol
- 11) resuspend pellet in **100 µl 0.1 x TE buffer** by vortexing; resuspend sparingly soluble pellets in an ultrasonic bath; or leave over night at 4 °C (39.2 °F) and then dissolve the pellet by vortexing vigorously
- 12) use **12.5 µl from (13)** directly or corresponding dilutions for analysis

Notes:

- not more than 100 ng total DNA should be used in the assay (check with UV measurement at 260 nm or estimate using agarose gel)
- experience has shown that DNA extracts in tissue samples must therefore be diluted with 0.1 x TE buffer

7. PCR**7.1. Preparation**

- take the strips from the foil bag and separate the required number of reaction vials
- return unused strips / reaction vials together with the desiccant to the foil bag and seal it well, store at 2 - 8 °C (35.6 - 46.4 °F)
- prepare the necessary amount of MasterMix (12.5 µl for each reaction)

7.2. Setup

- pipette the following volumes into the reaction vials:

| | reaction vial | Master-Mix | extract sample | extract extraction control* | water deionized |
|-----------------------------------|---------------|------------|----------------|-----------------------------|-----------------|
| for each sample extraction | | | | | |
| sample | colourless | 12.5 µl | 12.5 µl | - | - |
| inhibition control ITC* | red | 12.5 µl | 12.5 µl | - | - |
| per each analysis run | | | | | |
| positive control PTC | red | 12.5 µl | - | - | 12.5 µl |
| negative control NTC | colourless | 12.5 µl | - | - | 12.5 µl |
| extraction control ETC | colourless | 12.5 µl | - | 12.5 µl | - |

Table 1: PCRFast® pipetting steps

* recommended

–close the reaction vials (centrifugation is recommended) and place in the PCR thermocycler; the amplification is effected according to the given thermocycler temperature profile:

–**for the cycler profile see QS data sheet**

Note:

The validation is done with AmpliTaqGold[®] PCR MasterMix, Applied Biosystems, Brilliant[®] II QPCR MasterMix Stratagene, Power SYBR[®] Green MasterMix, Applied Biosystems, Brilliant[®] II SYBR[®] Green QPCR MasterMix Stratagene, the thermocyclers ABI 7500, Stratagene Mx3000P and Mx3005P, TECHNE Personal - Cycler TC - 3000 and Eppendorf Mastercycler; for other cyclers and MasterMixes an adaption of the cycler profile could be necessary.

7.3. Detection

7.3.1. Agarose gel detection

- electrophoresis is recommended in 2.0 - 2.5 % agarose and colouring with ethidiumbromide
- carry out the gel electrophoresis using manufacturer's recommendations

For example:

- prepare 2.0 - 2.5 % agarose gel (1.0 - 1.25 g agarose and 2 - 4 µl ethidiumbromide solution (10 mg / ml) dissolved in 50 ml 1 x TAE buffer by heating, thereafter cast the gel)
- cover agarose gel with 1 x TAE buffer
- add the gel loading buffer to the PCR amplificate, e.g. add 6 µl with 5 x loading buffer to the PCR reaction vial (25 µl), load 10 µl of the mixture onto the agarose gel
- load DNA length marker
- run the gel electrophoresis for 15 min to 30 min at 3 - 6 V / cm (depending on the electrophoresis system)
- make the gel visible on a transilluminator and document it

7.3.2. Realtime (SYBR[®]Green)

- the evaluation of the reaction with realtime (SYBR[®]Green) is done by using the amplification plot and the melting curve analysis

- if the amplification blot shows an increase in the fluorescence, the amplificate has to be verified by melting curve analysis
- therefore the melting curve of the amplificate has to be compared with the melting curve of the inhibition control ITC
- for positive samples the melting curve of the samples should be consistent with the melting curve of the inhibition control ITC (deviation of temperature ± 0.5 °C)
- for an additional verification of a positive sample it is recommended to visualize the amplificate by agarose gel electrophoresis with ethidiumbromide

8. Evaluation

8.1. Agarose gel detection

The following table summarizes the evaluation. When a positive test is carried out, the band of the sample and the positive control band are at the same level.

8.1.1. Evaluation matrix gel detection

| sample | inhibition control ITC | positive control PTC | negative control NTC | extraction control ETC | result |
|---------|------------------------|----------------------|----------------------|------------------------|---|
| ■ | ■ | ■ | no band | no band | sample positive |
| no band | ■ | ■ | no band | no band | sample negative |
| no band | no band | ■ | no band | no band | inhibition* |
| | | | ■ | | MasterMix or water contaminated |
| | | | | ■ | contamination in the sample preparation |
| | | no band | | | MasterMix not active |

* dilute the extracted DNA sample once again and re-amplify

■ = band

Table 2: PCRFast® evaluation

8.1.2.. Example for a typical Agarose gel pattern

A typical agarose gel pattern is depicted in the following:

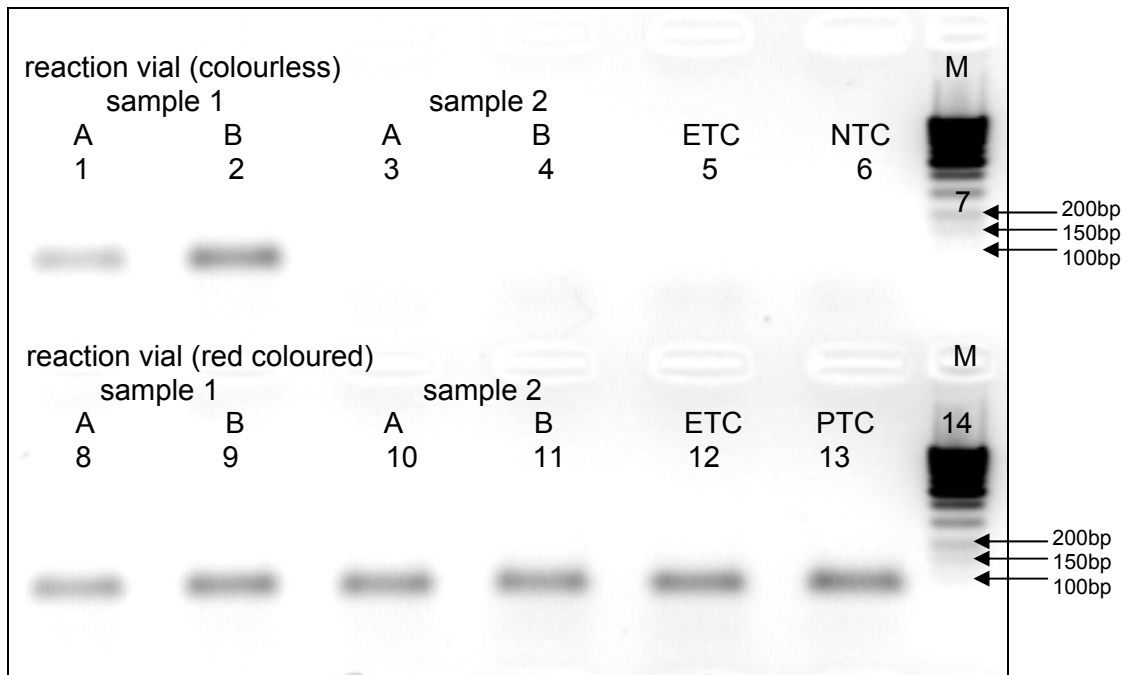


Fig. 1: Example for an agarose gel pattern PCRFast® Hazelnut upper tracks 1 to 7 (colourless reaction vials, with specific primers)

track 1: sample 1 extraction A: positive
 track 2: sample 1 extraction B: positive
 track 3: sample 2 extraction A: negative
 track 4: sample 2 extraction B: negative
 track 5: extraction control ETC: negative
 track 6: negative control NTC: negative
 track 7: DNA length marker

lower tracks 8 to 14
 (red - coloured reaction vials, with specific primers and hazelnut DNA)

track 8: sample 1 extraction A: positive
 track 9: sample 1 extraction B: positive
 track 10: sample 2 extraction A: positive
 track 11: sample 2 extraction B: positive
 track 12: extraction control ETC: positive
 track 13: positive control PTC: positive
 track 14: DNA length marker

8.2. Realtime (SYBR[®]Green)

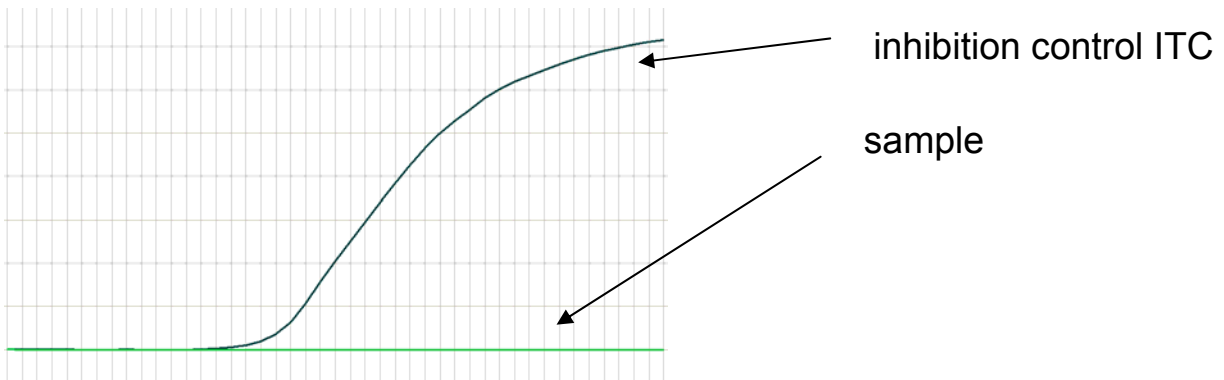
8.2.1. Evaluation matrix realtime (SYBR[®]Green)

| Sample | Inhibition control ITC | Result |
|--|---------------------------------------|--------------------|
| no amplification plot | amplification plot + melting curve | sample negative |
| amplification plot + melting curve, but different to ITC ($\geq 0,5$ °C) | amplification plot + melting curve | sample negative |
| amplification plot + melting curve, same to ITC ($\pm 0,5$ °C) | amplification plot + melting curve | sample positive |
| no amplification plot | no amplification plot | inhibition* |

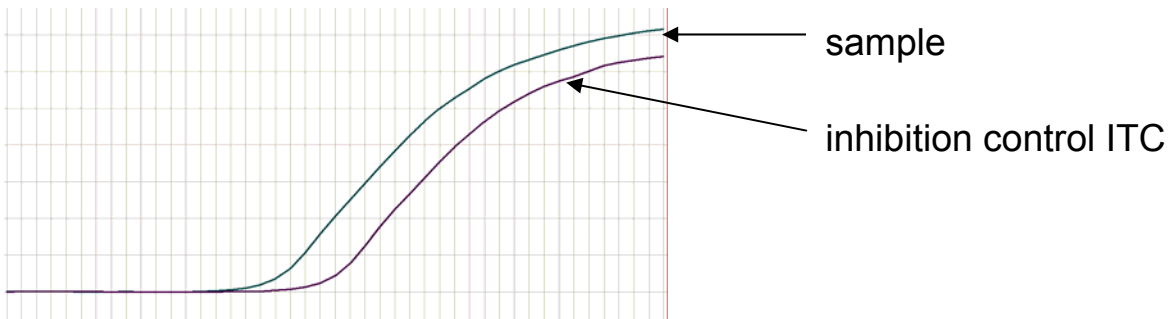
* dilute the extracted sample DNA and amplify again

Table 3: Evaluation PCRFast[®] Realtime (SYBR[®]Green)

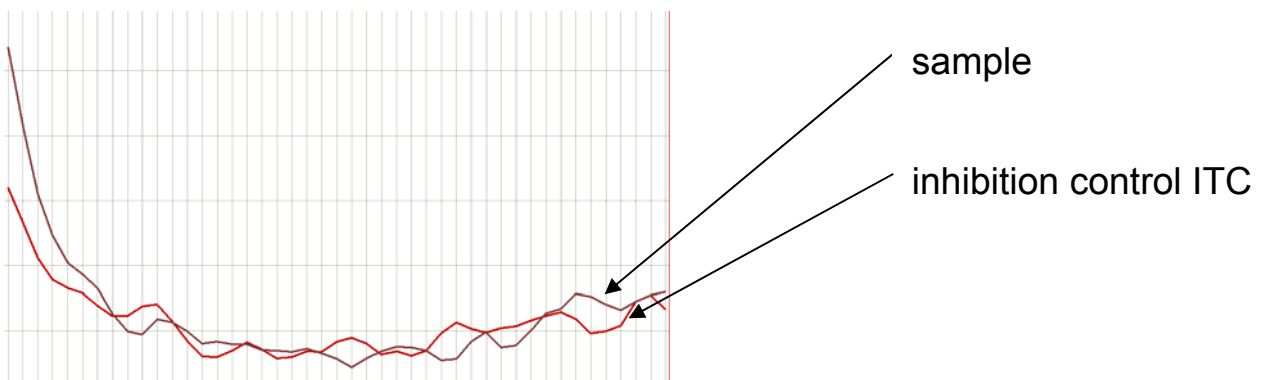
8.2.2. Examples for the evaluation of the amplification plots



Result: Sample is negative

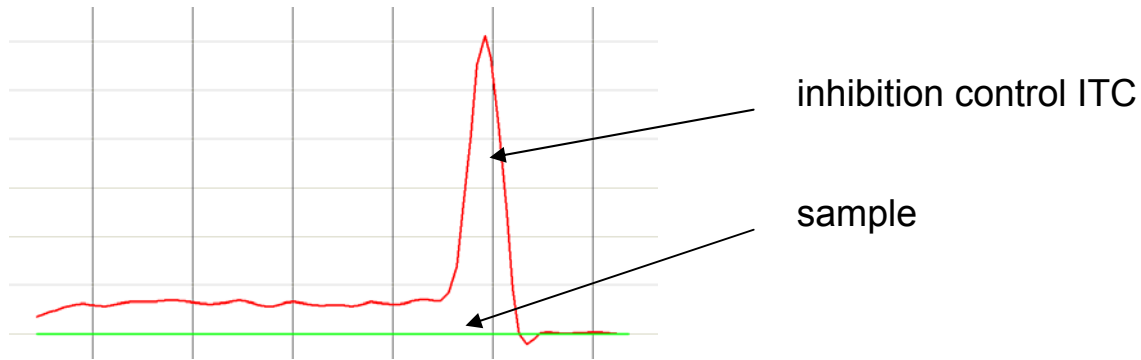


Result: Sample is positive, but verification of the positive result has to be done by means of melting curve analysis (in comparison to the melting curve of the inhibition control ITC)

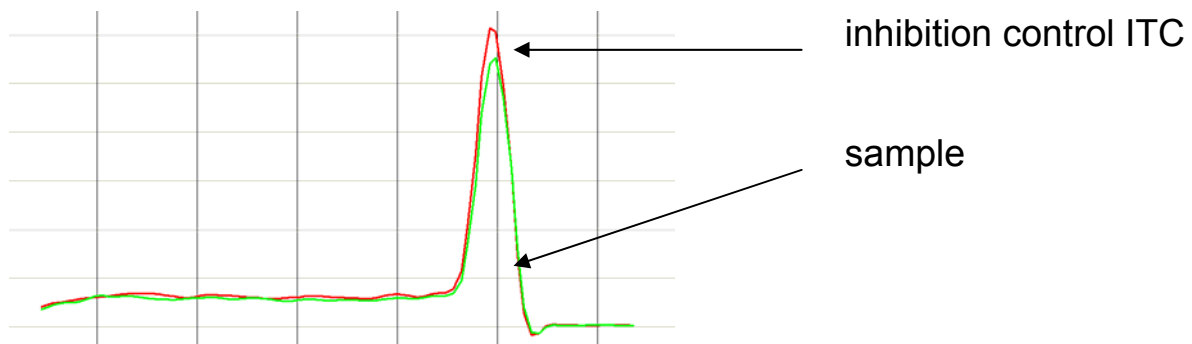


Result: Inhibition of the reaction

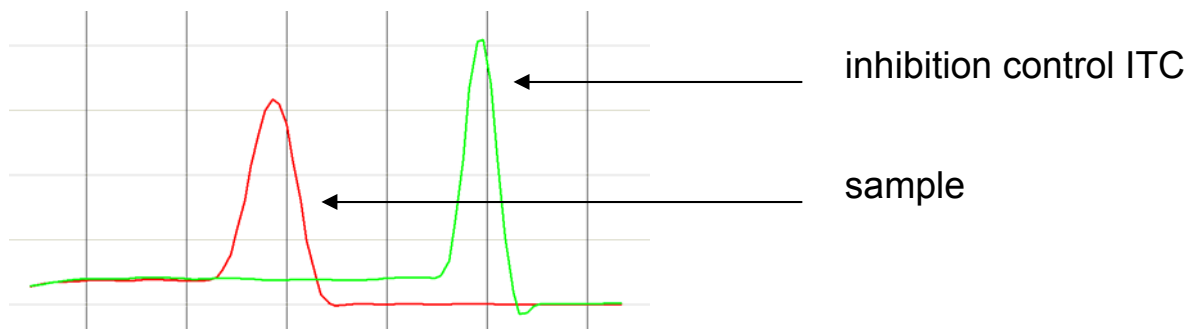
8.2.3. Examples for the evaluation of melting curves



Result: Sample is negative



Result: Sample is positive



Result: Sample is negative

9. Sensitivity

See QS data sheet.

10. Specificity

See QS data sheet.

If there are any questions concerning the test procedure or the PCR in general, please contact the Institute for Product Quality at the ifp's DNA analysis competence centre.

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Further information about the test procedure from PCRFast[®] can be found at www.produktqualitaet.com.



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11. Literature

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