PCRFast®
Realtime (Sonde)

Allgemeine Informationen /
General information

Allergene / allergens
Tierarten / animal identification
GMO

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PCRFast® Realtime (Probe)

Brief information

Easy - to - use molecular biological test (PCR) for the detection of specific DNA fragments using realtime (probe with Fam reporter and non fluorescence quencher) in food, animal feed and pharmaceutical product. The test kit contains 96 reaction vials. All reaction vials contain a specific primer pair and an internal amplification control (probe with HEX reporter and non fluorescence quencher) for investigating inhibitory effects.

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

Sample preparation

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

For some matrices other extraction or purification methods may give better results.

Time requirement:	extraction...............................	1 hr
(10 samples)	PCR - setup................................	15 min
	PCR......................................	1.5 hrs

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1. Test principle

PCRFast® is an easy - to - use molecular biology test for the detection of specific DNA fragments in food, animal feed and pharmaceutical products. The test system is in accordance with international PCR standards (ISO).

All reaction vials contain the for the PCR reaction necessary specific primers and probe in an optimum amount and an internal amplification control for investigating inhibitory effects.

The 88 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 8 red - coloured reaction vials** contain homologous, specific control DNA. These reaction vials are used for the **positive control PTC** (check on functionality of MasterMix).

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. The target sequence is then amplified in a PCR thermocycler. Is the target present the probe hybridises on the built up DNA fragments. Thereby the reporter (Fam/HEX) and the quencher (non fluorescence) of the probe will be seperated and the reporter radiates a fluorescence signal (Fam: 520 nm, HEX: 553 nm). The intensity of the signal increases with the amount of synthesised DNA which could be measured and displayed. If the target sequence is not present no increase in the fluorescence is detectable.

2. Reagents provided

Each kit consists of:

11 x **strips (colourless),** each with 8 x 0.2 ml reaction vials (88 reaction vials), coated with specific primers and probe (for the detection of DNA in the sample, negative control NTC and extraction control ETC) and an internal amplification control.

1 x **strip (red - coloured)** with 8 x 0.2 ml reaction vials each (8 reaction vials), coated with specific primers, probe and specific control DNA (for positive control PTC) and an internal amplification control.

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)
- centrifuge, > 2,500 x g (for 15 ml and 50 ml centrifuge vials)
- **PCR thermocycler**,
  e.g. Applied Biosystems 7500, STRATAGENE Mx 3005 P
- filter tips for micropipettes: 2 - 20 µl, 20 - 200 µl and 100 - 1000 µl, e.g. Gilson Pipetman P, each with filter tips
- 50 ml graduated centrifuge vials with screwing caps
- 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
- glycogen
- 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

PCR

MasterMix double concentrated (hotstart recommended),
e.g. AmpliTaq Gold® PCR MasterMix, Applied Biosystems No. 4318739; Brilliant® II QPCR MasterMix, Stratagene No. 600804; QuantiTect Probe PCR Kit, Qiagen No. 204343

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

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

The following optimized **CTAB protocol** gives good results with different matrices. Depending on the matrix, other extraction methods may give better results.

For food matrices, such as vegetable and animal fats or lecithins, a hexane extraction protocol should be used.

6.1. DNA extraction

Per analysis series one PCR setup for the extraction control ETC should be carried only with reagents, but without sample material.

Prepare two weighted units per sample for a double determination.

(1) weigh exactly **2 g of homogenized sample** in a 50 ml centrifuge vial, add **10 ml CTAB buffer** and **30 µl proteinase K solution (20 mg / ml)**;

thereafter shake vigorously (to ensure uniform distribution / moistening of sample material)

note: for highly swelling samples, e.g. starches, weigh exactly **2 g of the sample** in a 50 ml centrifuge vial, add **20 ml CTAB buffer** and **30 µl proteinase K solution (20 mg / ml)**; thereafter shake vigorously (to ensure uniform distribution / moistening of sample material)

For species identification of homogenous samples (e.g. filets,....) weigh **200 mg of sample** in a 2 ml reaction vial, add **1 ml of CTAB buffer** and **30 µl of proteinase K solution (20 mg / ml)**; shake vigorously.

(2) incubate for 90 min (or overnight) at 60 °C (140 °F) while shaking constantly; chill down to room temperature (< 30 °C (86 °F))

(3) centrifuge for 5 min not less than 2,500 x g

(4) pipette **500 µl chloroform (alternatively ReadyRed)** into a chloroform resistant 2.0 ml reaction vial, add **700 µl of the supernatant** from (3) and vortex vigorously for 15 sec

(5) centrifuge for 15 min at not less than 14,000 x g (if the supernatant is not clear, centrifuge again for 5 min)

(6) pipette **500 µl isopropanol** absolute into a 2.0 ml reaction vial, add **500 µl supernatant** from (5); for low - DNA samples: pipette **2 µl glycogen solution (20 mg / ml)** additionally inside the lid of the reaction vial, close the vial and shake

(7) incubate at room temperature for 30 min

(8) centrifuge for 15 min at not less than 14,000 x g

(9) carefully take up the supernatant by means of a pipette (**note:** with low DNA samples the pellet may not be visible, continue the cleaning procedure anyway), add **500 µl ethanol 70 %** to the pellet and shake

(10) centrifuge for 5 min at no less than 14,000 x g

(11) carefully take up the supernatant by means of a pipette and centrifuge again for a short time (15 sec at not less than 14,000 x g); take up the rest of the ethanol by means of a pipette and dry the pellet for 15 min at 50 °C (122 °F), (or for 1 hour at room temperature) so that the remainder of the ethanol can evaporate

(12) re - suspend the pellet in **100 µl 0.1 x TE buffer** by vortexing; for pellets which are difficult to re - suspend use an ultrasonic bath or leave overnight at 4 °C and then vortex

(**note**: if the re - suspended solution ought to be turbid, then proceed in the manner indicated in section 6.2; take the whole amount of 100 µl for the columns)

In special cases a cleaning step using silica columns could be necessary, such as with soy products (soy flour or soy beans), chocolate or foodstuffs containing cocoa. The entire 100 µl of DNA solution from (12) should be used for the columns.

6.2. DNA cleaning using silica columns, e.g. PCRFast® DNA Purification

For handling the purification procedure, see the manufacturer's enclosed extraction protocol. It is recommended to elute the DNA in the final purification step with 100 µl elution buffer.

Note:
An ethanol residue inhibits the PCR; let the columns dry, e.g. for 10 min at 37 °C (98.6 °F) before the elution step (the final step of the DNA purification).

6.3. Evaluation of the DNA concentration

More than 100 ng of whole-DNA should not be used for the PCR setup (examination of UV measurement at 260 nm, or estimation by means of agarose gel).
Consequently, in the case of samples with a high DNA content (soya flour, maize flour, sausage samples, …), DNA extracts usually have to be diluted with 0.1 x TE buffer.

DNA extracts with inhibiting effects (foodstuffs containing cocoa, chocolate, …) should likewise be diluted with 0.1 x TE buffer.
For example, with 100 µl volume in step 6.1 (12) and / or 100 µl elution volume in step 6.2, the following dilution ratios have proved satisfactory:

sausage sample	1 : 40 to 1 : 80	maize flour	1 : 20
soya flour	1 : 20	lecithin	pure
starch	pure	chocolate	1 : 10 and 1 : 20
animal feed	1 : 10 to 1 : 20		

7. PCR setup

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	-	-
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

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

Instrument settings

Reporter: Fam and HEX
Quencher: non fluorescence
Reference dye: ROX (depending on used master mix)

for the cycler profile see QS data sheet

Note:
The validation was done with Brilliant II QPCR MasterMix, Stratagene and the thermocyclers specified in section 3. The specified cycler profile may need to be adjusted to the respective instrument and the MasterMix.

7.3. Detection

- the evaluation of the reaction with realtime (Sonde) is done by using the amplification plot
- in principle the evaluation should be done using the software of the realtime thermocycler. Are the results not satisfactory using the instrument presettings, the threshold and the baseline settings could be changed.

8. Evaluation

8.1. Evaluation matrix realtime

The following table shows the evaluation. Positive reactions should give a final fluorescence value which is clearly higher than the threshold.

Sample	Internal amplification control (ITC)	Negative control NTC	Positive control PTC	Result
+	+			sample positive
no amplification	+			sample negative
no amplification	no amplification			inhibition*
		no amplification		MasterMix not contaminated
			+	MasterMix functional

* dilute the extracted sample DNA and amplify again
+ = amplification signal
Table 3: Evaluation PCRFast® Realtime (probe)

8.2.2. Examples for the evaluation of the amplification plots

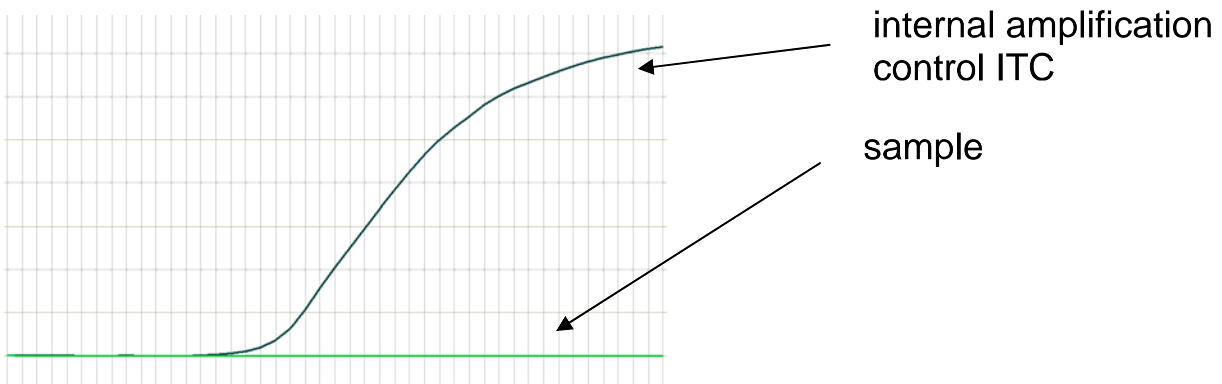

Fig. 1: Sample is negative (no amplification of the sample)

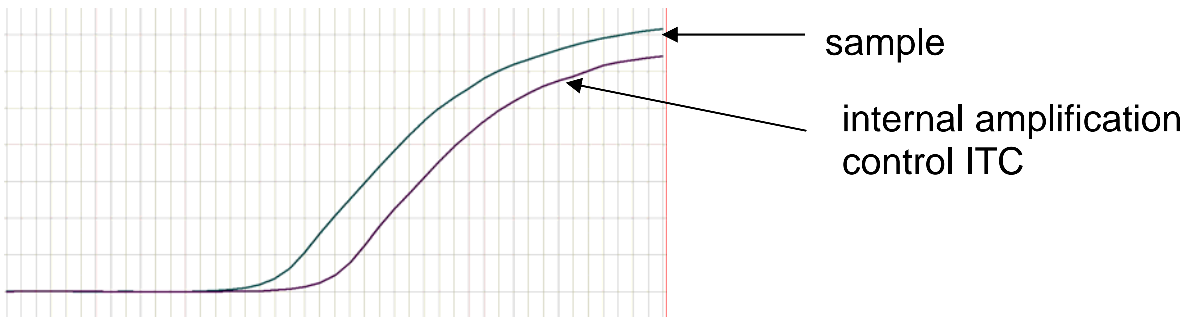

Fig. 2: Sample is positive

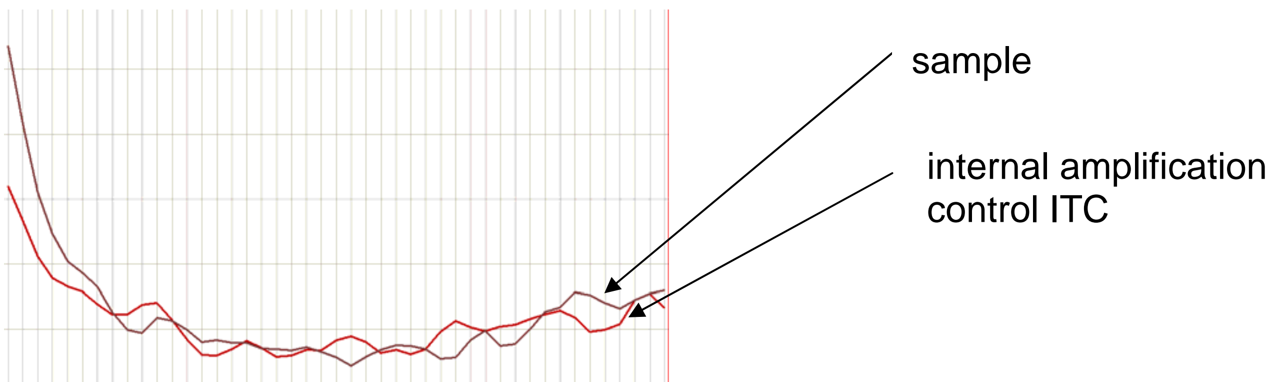

Fig. 3: Inhibition of the reaction, dilute the extracted DNA and repeat amplification

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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