



مؤتمر عُمان الدولي السادس
للسلامة وجودة الغذاء
Oman 6th International Conference
on Food Safety and Quality

qPCR-Based Methodologies Applied to Halal Testing

Dr. Martin Mehl • 12.06.2023 • Oman



Member of NA 057-08-02 AK (DIN CEN ISO) molecular species analytics

- ISO 20813:2019

Molecular biomarker analysis — Methods of analysis for the detection and identification of animal species in foods and food products (nucleic acid-based methods) – General requirements and definitions

- DIN EN ISO 20813:2013

Further standards, guidelines as e.g.

Molecular biomarker analysis - General guidelines for single-laboratory validation of qualitative real-time PCR methods
in process; ISO 11781:202X(E)





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INTERNATIONAL
STANDARD

ISO
20813

First edition
2019-05

**Molecular biomarker analysis —
Methods of analysis for the detection
and identification of animal species
in foods and food products (nucleic
acid-based methods) — General
requirements and definitions**

*Analyse moléculaire de biomarqueurs — Méthodes d'analyse pour la
détection et l'identification des espèces animales dans les aliments et
les produits alimentaires (méthodes basées sur l'utilisation des acides
nucléiques) — Exigences générales et définitions*



NA 057-08-02 AK (DIN CEN ISO) molecular species analytics real-time PCR (qPCR) quantitative Polymerase Chain Reaction (PCR)

- Specific amplification and detection in real-time, additional fluorescent probe
ISO/TS 20224 part 1-11: ISO/TS 20224 -3 porc (**sus scrofa**)

prENxxx horse quantitative, deer... —

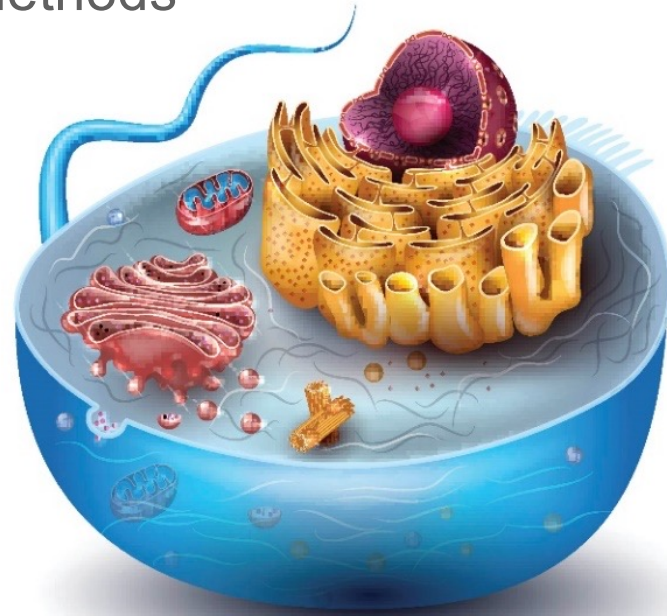
- **Barcoding:** (sequencing): Fish, muscles, meat, birds...

- **Meta-barcoding** (NGS sequencing)...

- Future projects dPCR e.g. based on § 64 projects, German food law

Species detection

- Immunological methods



- Mass spec

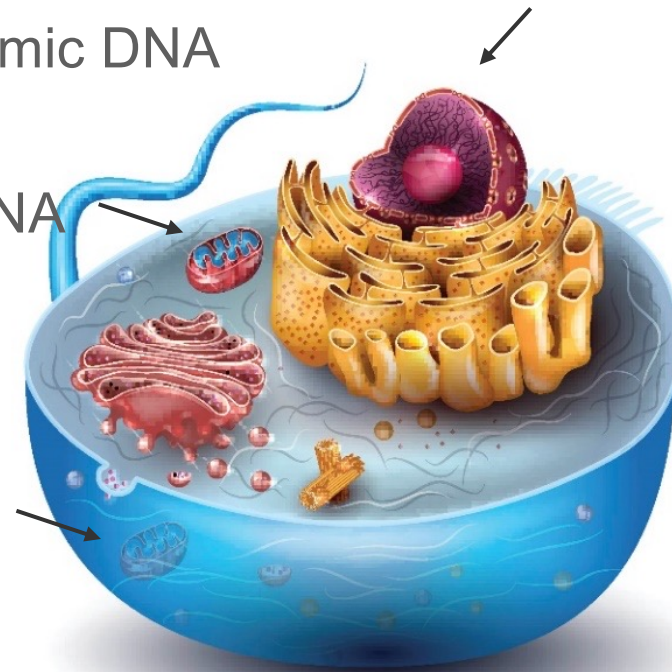
ELISA, Lateral flow
low amount for equipment
reduced sensitivity 0.1% - 1%

digestion, separation,
fingerprint peak detection
standardized reagents,
expensive equipment

- Molecular DNA (nucleotide) detection, “gold standard” specific and sensitive
- Lab equipment needed. DNA amplification:
be aware about cross contamination

Nucleus: single copy genomic DNA

Mitochondria: multicopy DNA



quantification (e.g. myostatin)

highly conserved for species

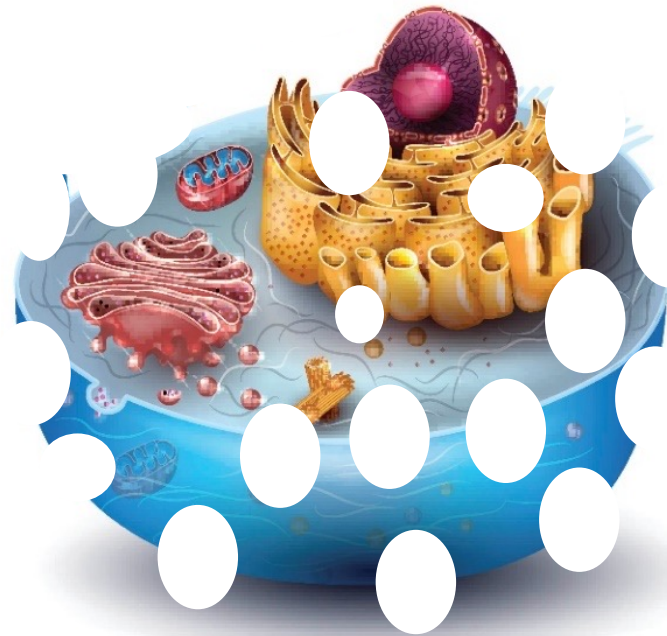
undefined, high numbers of DNA

commonly Cytochrome b (*Cytb*)
and Cyclooxygenase (COX)
used as targets

mitochondrial assays useful for sensitive assays but not for quantification

qPCR general procedure

1. Sampling
2. Homogenisation
3. Lysis/DNA Prep
4. qPCR
5. Data extraction



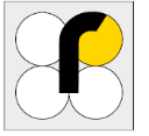


→ 100~50 μ l → 5 μ l
DNA
RNA



- surface ~ 5 x 5 cm
- Food ~ (kg) – gram (0.05 - 2 gram subsample)
- Beverages, liquids ~ ml

Representative sampling and homogenisation most crucial for all detection methods



sampling

DNA prep

set-up

qPCR run

data



representative (!)
sampling and
homogenisation

subsample e.g. 10 g

50 mg – 2 g

final sample !



- manually (spin-filter)

- automation (magnetic beads)

~ 1-2 hrs



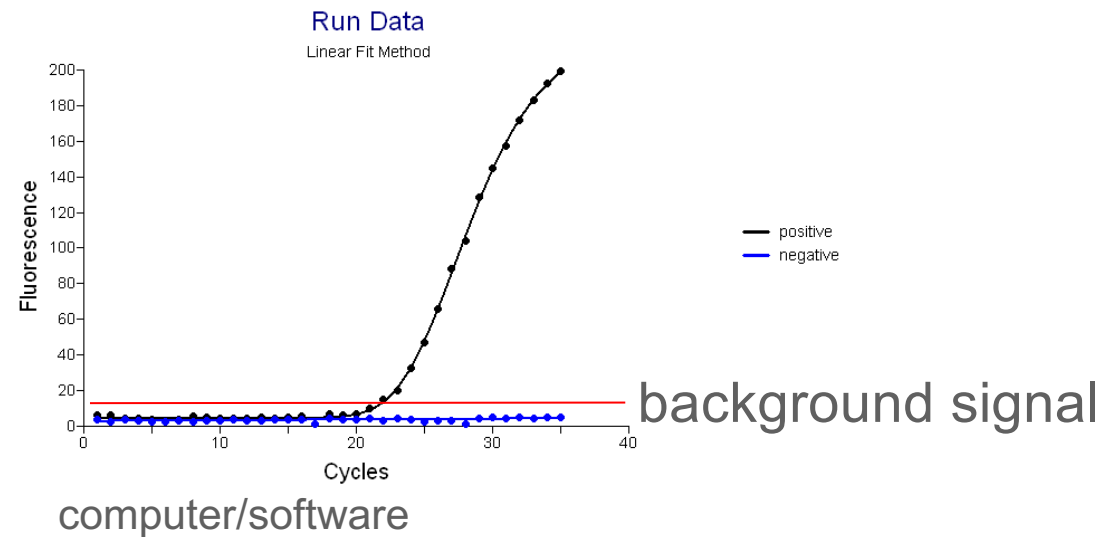
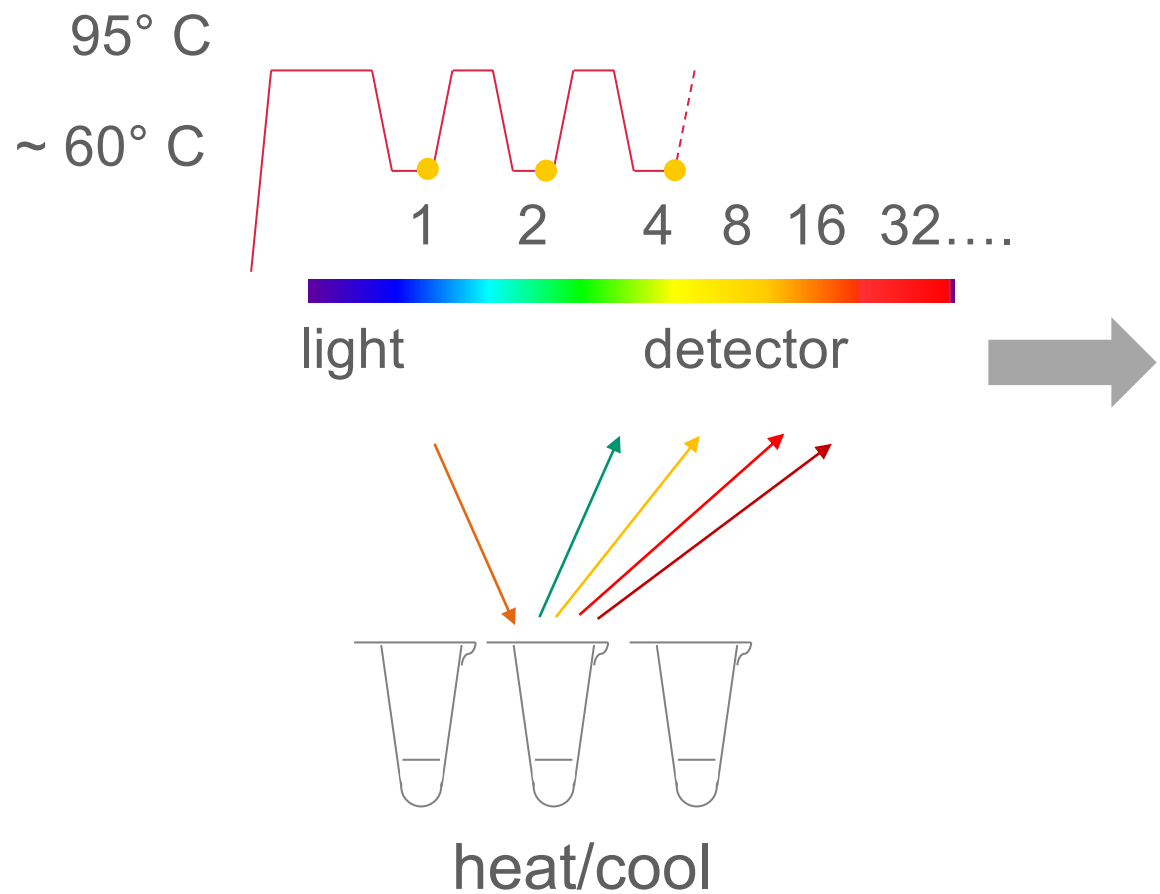
qPCR setup:
mastermix +
DNA (controls)



qPCR run and data extraction

data handling

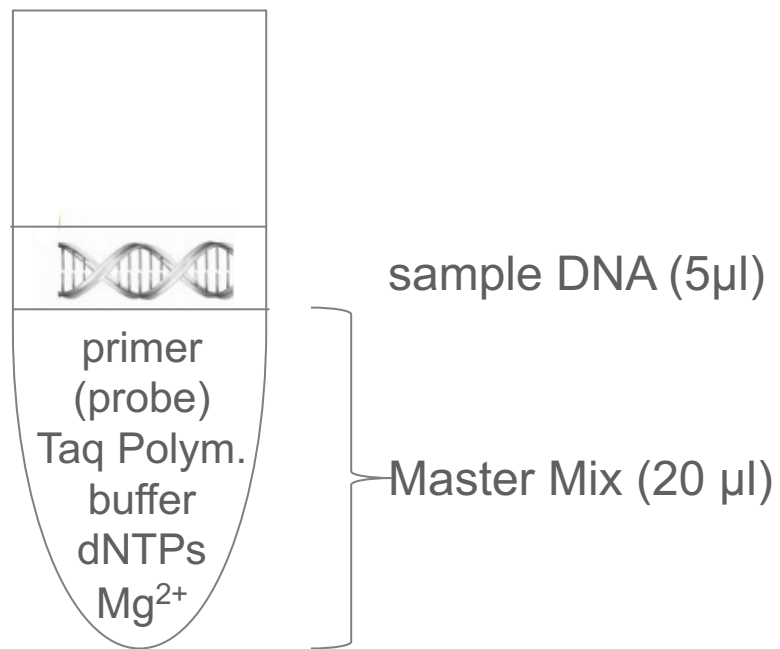
qPCR



-  FAM
-  HEX
-  ROX
-  Cy5



procedure of DNA amplification and detection:



nucleotides

Guanine

Adenine

Cytosine

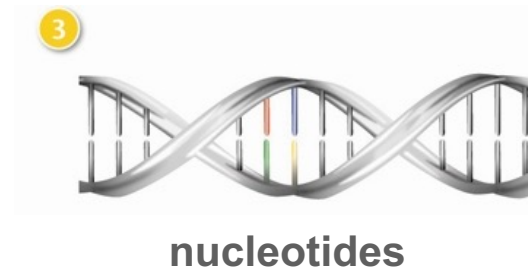
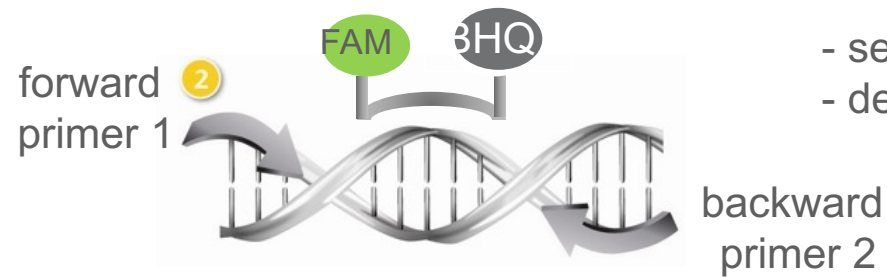
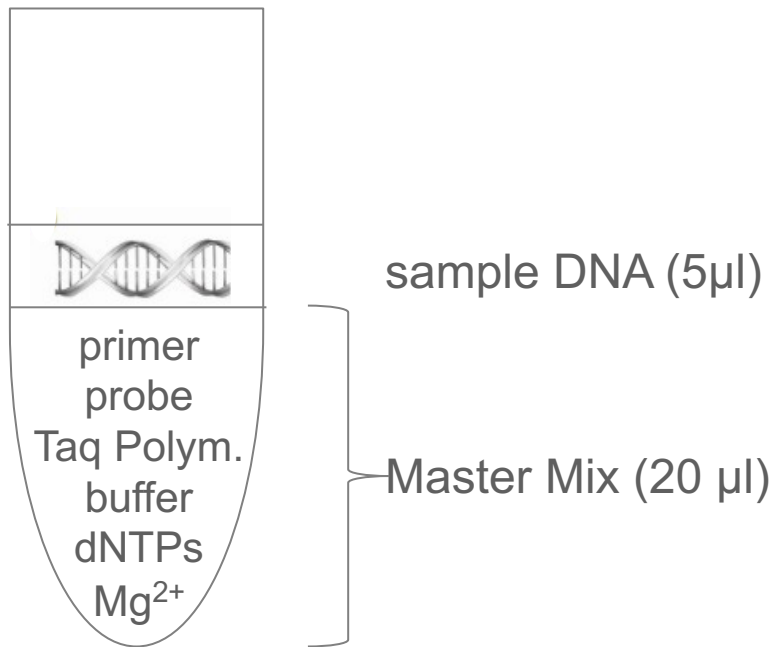
Thymine



procedure of DNA amplification and detection:

probe: specific sequence, labelled with fluorescent dye and quencher

- second sequence: higher security
- detection in real-time



Guanine
Adenine
Cytosine
Thymine

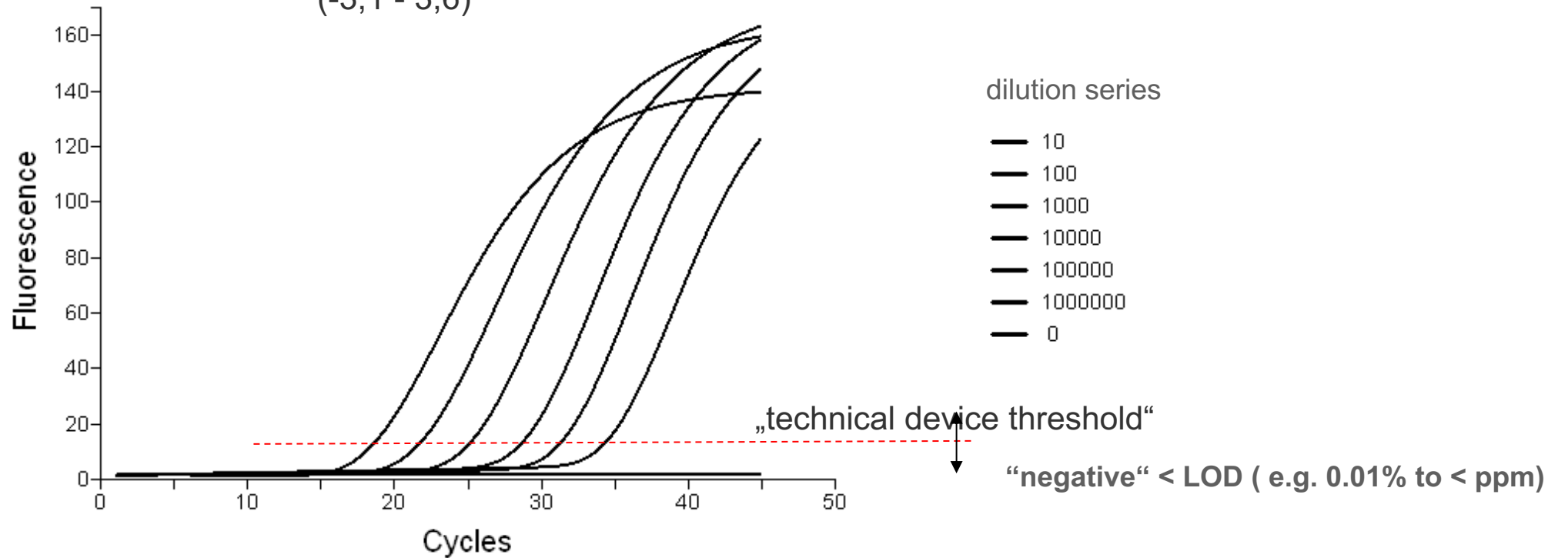


Ct values

high DNA amount

low DNA amount

3,322 at $\Delta 10 \times$
(-3,1 - 3,6)

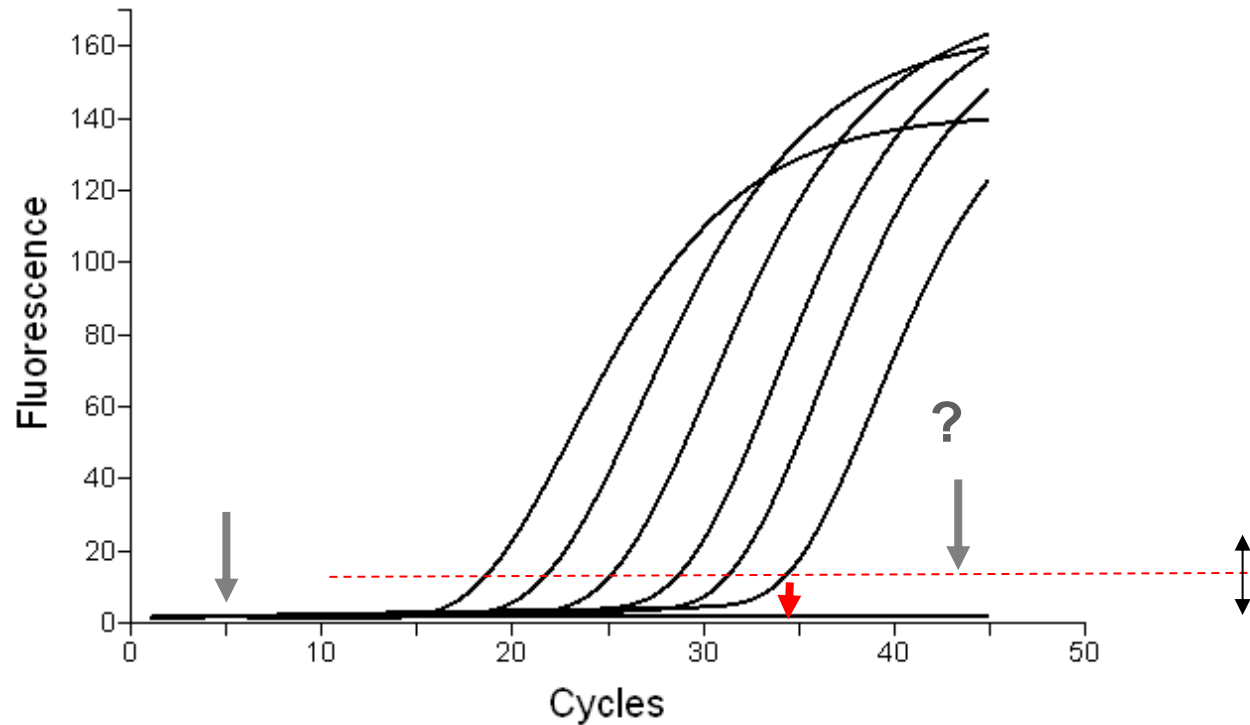


qPCR – qualitative, or relative quantification

Ct values

high DNA amount

low DNA amount



Algorithms as e.g.

LinRegPCR

Ct cycle threshold

Cp crossing point

Cq cycle quantitative

value

< LOD (e.g. 0.01% to < ppm)



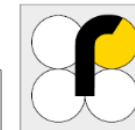
Amount	dilution	percentage	ppm
1	10^0	100	%
			1.000.000 ppm



Amount	dilution		percentage		ppm
1	10^0	100	%	1.000.000	ppm
0.1	10^{-1}	10	%	100.000	ppm



Amount	dilution		percentage		ppm
1	10^0	100	%	1.000.000	ppm
0.1	10^{-1}	10	%	100.000	ppm
0.01	10^{-2}	1	%	10.000	ppm



Amount	dilution	percentage	ppm
1	10^0	100 %	1.000.000 ppm
0.1	10^{-1}	10 %	100.000 ppm
0.01	10^{-2}	1 %	10.000 ppm
0.001	10^{-3}	0.1 %	1.000 ppm

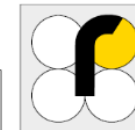
e.g. GMO
EU: 0.9%
legal treshhold

e.g. horse meat scandal 2013: informal, non – legal thresholds in EU:

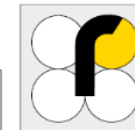
> 1 % ingredient or severe incompetence

0.1 - 1 % internal controls in factory recommended

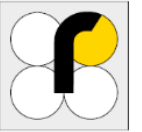
< 0.1 % contamination, “technical unavoidable” contamination? trace



Amount	dilution	percentage	ppm		
1	10^0	100	%	1.000.000	ppm
0.1	10^{-1}	10	%	100.000	ppm
0.01	10^{-2}	1	%	10.000	ppm
0.001	10^{-3}	0.1	%	1.000	ppm
0.0001	10^{-4}	0.01	%	100	ppm



Amount	dilution	percentage	ppm		
1	10^0	100	%	1.000.000	ppm
0.1	10^{-1}	10	%	100.000	ppm
0.01	10^{-2}	1	%	10.000	ppm
0.001	10^{-3}	0.1	%	1.000	ppm
0.0001	10^{-4}	0.01	%	100	ppm
0.00001	10^{-5}	0.001	%	10	ppm



Amount	dilution	percentage	ppm
1	10 ⁰	100 %	1.000.000 ppm
0.1	10 ⁻¹	10 %	100.000 ppm
0.01	10 ⁻²	1 %	10.000 ppm
<hr/>			
0.001	10 ⁻³	0.1 %	1.000 ppm
0.0001	10 ⁻⁴	0.01 %	100 ppm
0.00001	10 ⁻⁵	0.001 %	10 ppm
0.000001	10 ⁻⁶	0.0001 %	1 ppm
<hr/>			
0.0000001	10 ⁻⁷	0.00001 %	0.1 ppm
0.0000000... (not zero)			

“ingredient”



Sensitivity of qPCR in muscle meat ≤ 1 ppm ...contamination in production?
 difficult interpretation (y/n)...



Sensitivity	Limit of detection (LOD)	all target DNA to be detected
	absolute LOD_{abs} usually 95%	dilution series in relevant range
	relative LOD LOD_{rel}	dilution in relevant material (A)
	assymetric LOD	multiplex qPCR
	Limit of Quantification (LOQ)	
Specificity detected	1. <i>in silico</i> , 2. in practice	only target DNA has to be
	inclusivity, excludivity	no “false positives“
Robustness	othogonal variation	same results, even by variation

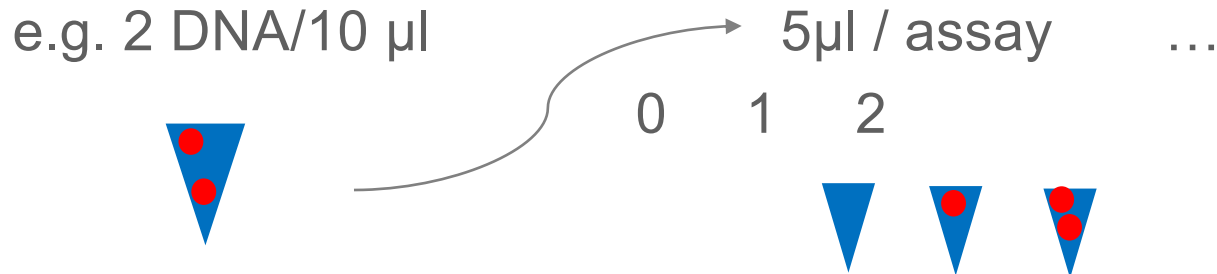
Sensitivity Limit of detection (LOD) **all target DNA** to be detected

absolute LOD_{abs} usually 95% dilution series in relevant range

relative LOD LOD_{rel} dilution in relevant material (A)

Dilution series, e.g. 19 of 20 samples positive: LOD_{95}

Low amount of DNA: statistical aspects: **Poisson Distribution**



Orthogonal robustness study, e.g. according to **TS 17329-1: 2021**

multifactorial test with 3 x LOD₉₅ DNA
Cq values should not have high variation

Factor	1	0
PCR equipment	A	B
PCR mastermix	X	Y
Primer concentration	protocol	-30%
Probe concentration	protocol	-30%
Volume mastermix	19 µl (-5%)	21 µl (+5%)
Annealing temperature	+ 1 °C	- 1 °C

Interlaboratory study (ring trials) and proficiency tests as internal, DRRR, FAPAS



ISO 20224-3 2019E

Molecular biomarker analysis — Detection of animal derived materials in foodstuffs and feedstuffs by real-time PCR — Part 3: Porcine DNA detection method

Table 1 — Oligonucleotides

Name	DNA sequence of the oligonucleotide	Final concentration in PCR
Porcine beta actin gene as the target sequence (GeneBank accession number: DQ452569.1) ^a		
Porcine-97bp-F	5'-CGTAGGTGCACAGTAGGTCTGAC-3'	400 nmol/l
Porcine-97bp-R	5'-GGCCAGACTGGGGACATG-3'	400 nmol/l
Porcine-97bp-P	5'-[FAM]-CCAGGTCGGGGAGTC-[NFQ-MGB ^b]-3'	200 nmol/l

a	PCR Product = 335 - cgtagg tgcacagtag gtctgaectg actcccgcac ctggggtccc cagcacactt agccgtgttc cttgcactct ctgcatgtcc ccagtctggc c - 431 - DQ452569.1
b	FAM: 6-Carboxyfluorescein, MGB: Minor Groove Binder (non-fluorescent chromophore)

10 min 95 °C initial denaturation
 15 s 95 °C denaturation
 60 s 60 °C annealing/elongation
 45 cycles

The absolute limit of detection ($LOD_{95\%}$) for the method is ≤ 5 DNA copies. The collaborative trial of the ovine detection method was carried out at the same time as collaborative trials for the bovine, porcine and chicken detection methods. Bovine, ovine, porcine and chicken target DNA sequences were synthesized and cloned into the pUC57 vector (2710 bp in length, GenBank/EMBL accession number Y14837). This constructed plasmid pUC57-bopc (3127 bp in length) was sequenced to ensure that only one copy of the ovine, bovine, porcine and chicken target DNA sequence was inserted (Fig. 1). No deletion or insertion mutations were found in the inserted sequences (Fig. 2). The target sequences of corresponding PCR methods are indicated in bold capital letters.

GSO 2649:2021

Gulf Standard ● ACTIVE

Detection Of Porcine DNA- Test Method- Food And Food Products

Scope

This standard describes the adapted and modified methods for the qualitative detection of porcine deoxyribonucleic acid (DNA) in food and food products. It is applicable to raw materials, processed and highly processed food and food product. The Polymerase Chain Reaction (PCR) technique is used for the identification and confirmation of porcine DNA presence.

The method described has been optimized for different food matrices (homogenous or heterogeneous). However, due to the type of food analyzed, some of the processes may have to be modified in the laboratory. In such cases, the laboratory may proceed with the modifications, provided validation studies had been carried out.

Type	Prepare
Edition	1
Approved on	01 July 2021
Sector	Food And Agriculture Sector
ICS - 67.050	general methods of tests and analysis for food products

مواصفة قياسية خليجية ● الإصدار الحالي

الطبعة: 1 اعتمدت بتاريخ 01 يوليو 2021

الكشف عن الحمض النووي الديوكسي ريبوزي للخنزير -

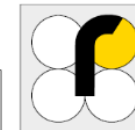
قطاع الغذاء والزراعة

القطاع

الطرق العامة لاختبار المنتجات الغذائية وتحليلها

ICS - 67.050

* ميكروبيولوجيا الأغذية، انظر 07.100.30 * التحليل الحسي للأغذية، انظر 67.240



GSO 2649:2021 adapted from MS 2627:2017

CTAB, Phenol extraction; DNA

classical PCR with post-PCR gel electrophoresis detection or qPCR

primers e.g. 5'- GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA-3'
5'- GCT GAT AGT AGA TTT GTG ATG ACC GTA-3'

3-step PCR 94°C, 30s / 54°C, 30s / 72°C, 40s, 2 min extinction
35 cycles

positive results should be in in the range of Ct 32-34

LOD: 0.01% (w/w)

Commercial kits as Pork Sens Plus

No “halal test” !

sensitive test for detection of pork (sus scrofa),

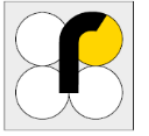
highly specific (as other sus scrofa tests)

very sensitive: ~ 0.4 ppm in muscle meat

validated, suitable for food, not validated for other products as cosmetics, environmental samples etc.



Example: Pork sens detection



DNA preparation using PREP Basic/Advanced (simple spin filter system) or automation

Qualitative real-time PCR with internal amplification control (IAC), commercial Pork sens assay

5 min	95 °C	initial denaturation
45 cycles:		
15 s	95 °C	denaturation
30 s	60 °C	annealing / elongation

Detection **FAM**: *sus scrofa*

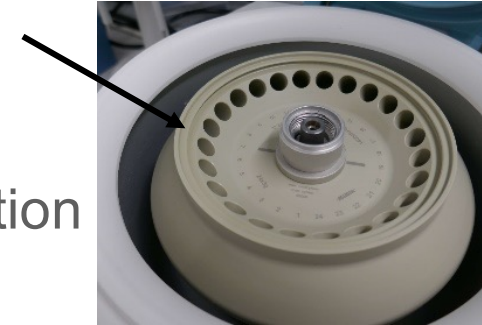
HEX: IAC

inclusivity/exclusivity: *sus scrofa (domestica)*

Example: Pork sens detection

Highly sensitive

Do not change settings (thermoprofile), be aware of contamination



Validated for food and ingredients,
but not for cosmetics, pharmaceuticals, environmental material

If result is positive, it may have an impact, careful handling of data interpretation mandatory

Laboratory staff procedure, controls, contamination?

Lab management controls, replicates, samples, contamination, sampling out of
laboratory?

Management how to classify and handle this (decision)

Communication how to communicate

Example: Pork sens detection

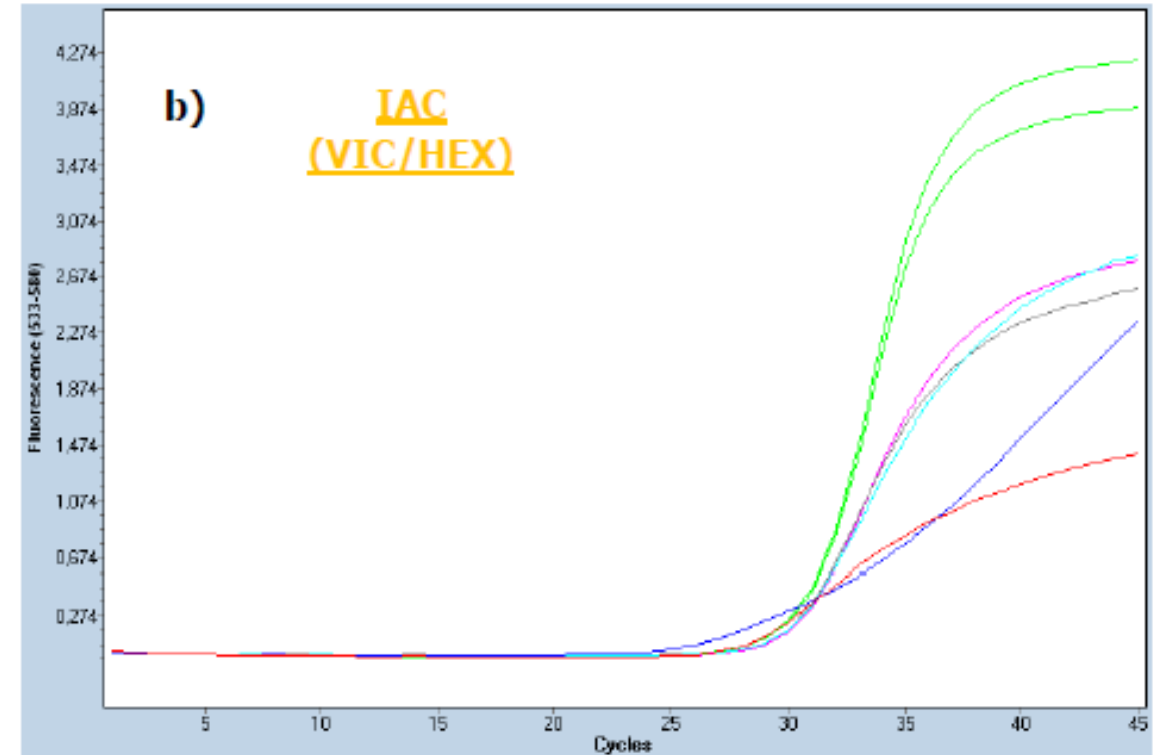
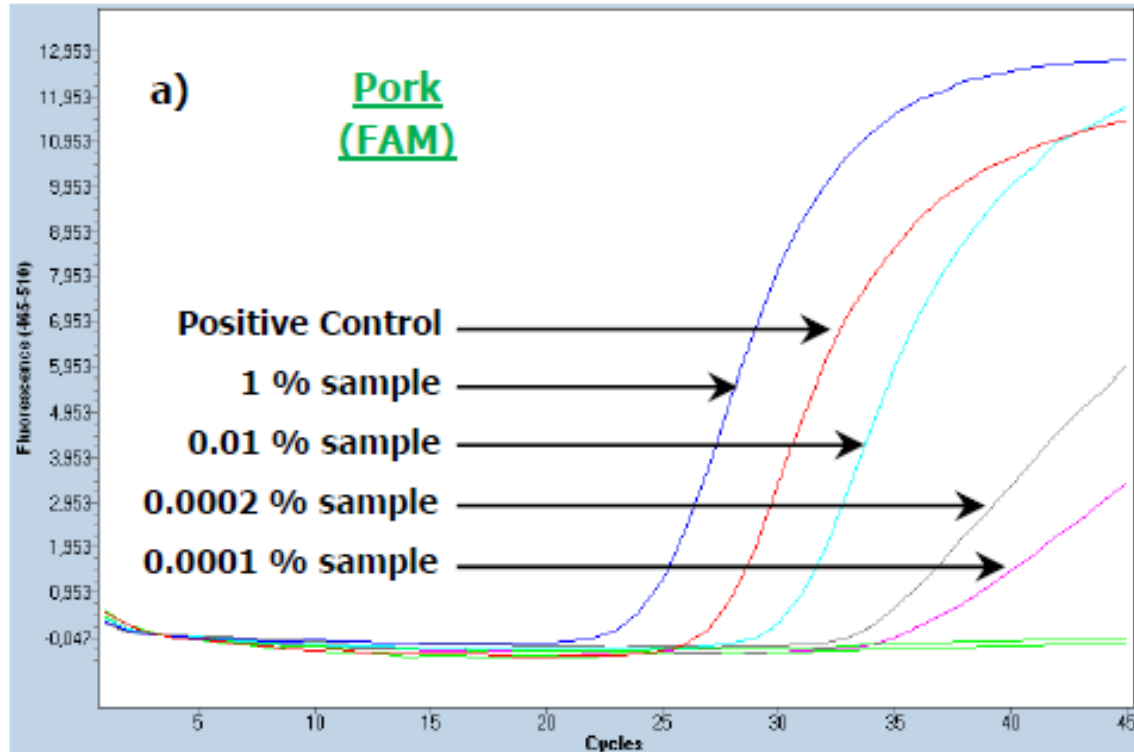
Management how to classify and handle this (decision)

Communication how to communicate

Guidelines e.g.



Example: Pork sens detection: results



Example: Pork sens detection

Can be used for gelatin and products (with reduced sensitivity)

Proficiency tests: FAPAS (lyophilized meat)

DRRR:

Gelatine	pork
Sweets (gummi drops)	pork
meat	different species
milk	different species

REPORT



DRRR-Proficiency Testing

RVEP 222167

porcine and beef DNA in gelatine

molecular biological analytic

Dr. Sonja Schötterl
Inspector*

Regina Wörz
PT-Assistent



date: 24.05.2023

* responsible for planning, organization and evaluation of the present proficiency testing scheme

Proficiency testing scheme accredited by A2LA according to ISO/IEC 17043:2010.



Deutsches Referenzbüro für Ringversuche und Referenzmaterialien GmbH
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Thorsten Heibig M.Eng.

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4. test items



- sample 1: fish gelatine; RM CP L TA SG 4
- Manufacturing process: Industrially manufactured goods
 - Dimension: plastic bag & 1,0 g
 - Material: typical fish gelatine
- sample 2: beef gelatine; RM CP L TA SG 5
- Manufacturing process: Industrially manufactured goods
 - Dimension: plastic bag & 1,0 g
 - Material: typical beef gelatine
- sample 3: porcine gelatine; RM CP L TA SG 6
- Manufacturing process: Industrially manufactured goods
 - Dimension: plastic bag & 1,0 g
 - Material: typical porcine gelatine

results (qualitative) Identification of the animal species pork					 Deutsches Referenzbüro für Ringversuche und Referenzmaterialien	
customer data					performance	
lab code no.	method description	sample set			number of correct findings	performance
		sample 1 <i>negative</i>	sample 2 <i>negative</i>	sample 3 <i>positive</i>		
1	CTAB TaqMan	negativ	negativ	positiv	3	successful
2	SureFood® PREP Advanced TaqMan, Kim et. al.	negativ	negativ	positiv	3	successful
3	CTAB+magnetic Beads Maxwell Promega Taqman	negativ	negativ	positiv	3	successful
4	DNeasy® mericon® Food as manufacture description with some modification and prep basic extraction (r-biopharm) protocol 1 mericon Pig KI (qaigen) and SureFood® ANIMAL ID Pork SENS (R-biopharm)	negativ	negativ	positiv	3	successful
5	Magnetic Beads RT PCR	negativ	negativ	positiv	3	successful



2023 PT with grinded meat samples

REPORT

DRRR-Proficiency Testing

RVEP 222166

beef, pork, horse

molecular biological and immunological analytic

Dr. Sonja Schötter Inspector' Yeliz Elüstü PT-Assistant

date: 28.04.2023

* responsible for planning, organization and evaluation of the present proficiency testing scheme

Deutsches Referenzbüro für Ringversuche und Referenzmaterialien

Proficiency testing scheme accredited by A2LA according to ISO/IEC 17043:2010.

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Dr. rer. nat. Ulrich Leist
Thorsten Heilig, M.Eng.

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results (qualitative)					Deutsches Referenzbüro für Ringversuche und Referenzmaterialien	
Identification of species, sample 1						
customer data			performance			
lab code no.	method description	sample 1			number of correct findings	performance
		beef positive	pork positive	horse positive		
1	ISO 20	positiv	positiv	n/a	2	
2,1	§ 64 LFGB, L 08.00-62, 2019-03	positiv	positiv	positiv	3	successful
2,2	16S rDNA-Metabarcoding (NGS) nach Dobrovoiny et al. (2019) Food Chemistry, 272, 354-361.	positiv	positiv	positiv	3	successful
3	Extraktion: NukleoSpin Food mit Einwaage 200mg, qPCR LFGB L08.00-62 50 Zyklen	positiv	positiv	positiv	3	successful
4	PCR-RFLP (Wolf et al., 1999), real-time PCR (Laube et al., 2007 and Köpkel et al., 2009), EURL	positiv	positiv	positiv	3	successful
5,1	In-house method based on ISO 20224-3 ; 2020 combination with International Journal of Food Science and Technology 38; 2003: 1111-8, Real-Time PCR	positiv	positiv	n/a	2	
5,2	In-house method based on ISO 20224-3 ; 2020 combination with International Journal of Food Science and Technology 38; 2003: 1111-8, Real-Time PCR	positiv	positiv	n/a	2	
5,3	In-house method based on ISO 20224-3 ; 2020 combination with International Journal of Food Science and Technology 38; 2003: 1111-8, Real-Time PCR	positiv	positiv	n/a	2	
6	GEN-IAL GmbH GEN-IAL® First-Beef PCR Kit, GEN-IAL GmbH GEN-IAL® First-Pig PCR Kit, GEN-IAL GmbH GEN-IAL® First-Horse PCR Kit	positiv	negativ	positiv	2	
7	RT-PCR	positiv	negativ	positiv	2	

results (qualitative)					Deutsches Referenzbüro für Ringversuche und Referenzmaterialien	
Identification of species, sample 1						
customer data			performance			
lab code no.	method description	sample 1			number of correct findings	performance
		beef positive	pork positive	horse positive		
8	relativ quantitative real-time PCR	positiv	positiv	n/a	2	
9	real-time PCR LOD 0,01%	n/a	positiv	positiv	2	
10	Real-time PCR	positiv	n/a	n/a	1	
11	CTAB-Chloroform RT-PCR 4S Zyklen	positiv	positiv	positiv	3	successful

further PTs in autumn:

- animal species
- insects in cereals / in feed

www.DRRR.de

Insects



- EU: novel food, ongoing approvals for increasing number of insects (protein)

Tenebrio molitor

Hermetia illucens

Locusta migratoria

Alphitortus diaperionus

↑ > Panorama > Ritter Sport preist Schokoladensorte "Ganze Grille" an - Kunden reagieren extrem sauer

Besondere Edition

Ritter Sport preist Schokolade der Sorte "Ganze Grille" an - Kunden reagieren extrem sauer

Von **Eva Orthenburger** ▾

17.2.2023, 09:17 Uhr



Mit diesem Bild sorgte Ritter Sport auf Instagram für einen Scherz - doch dieser ging nach hinten los.

© Ritter Sport



marketing joke

mostly Burgers,

powder

so far not
commercial
successful in
Europe

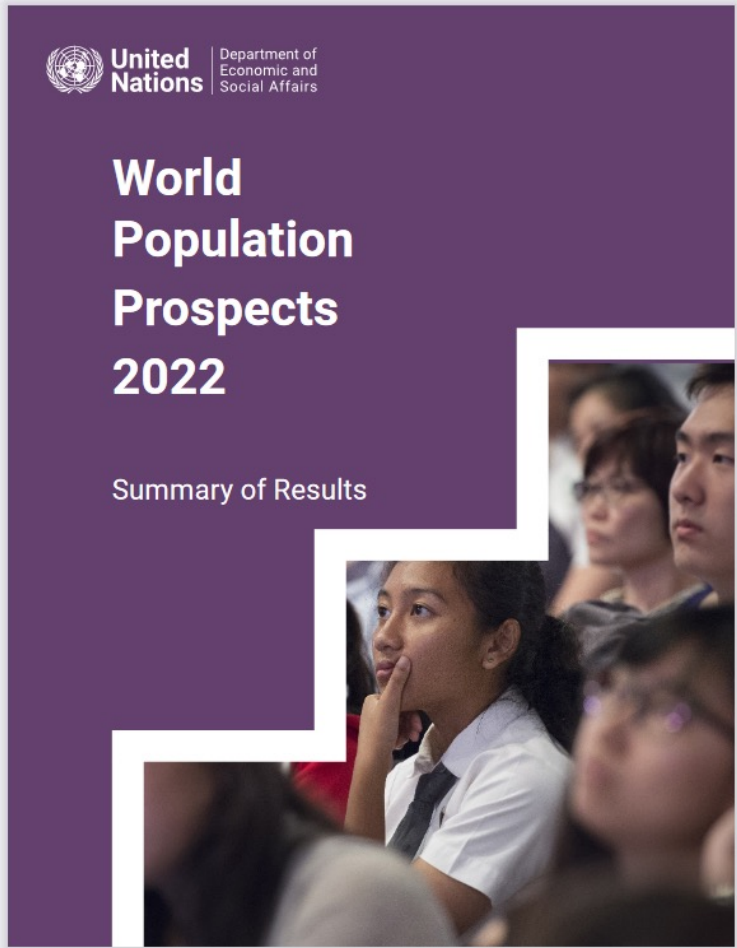
must be labelled

...diesem Bild sorgte Ritter Sport auf Instagram für einen Scherz - doch dieser ging nach hinten los. © Ritter Sport



Allergen
 Risk
 “crustaceans
 mites”

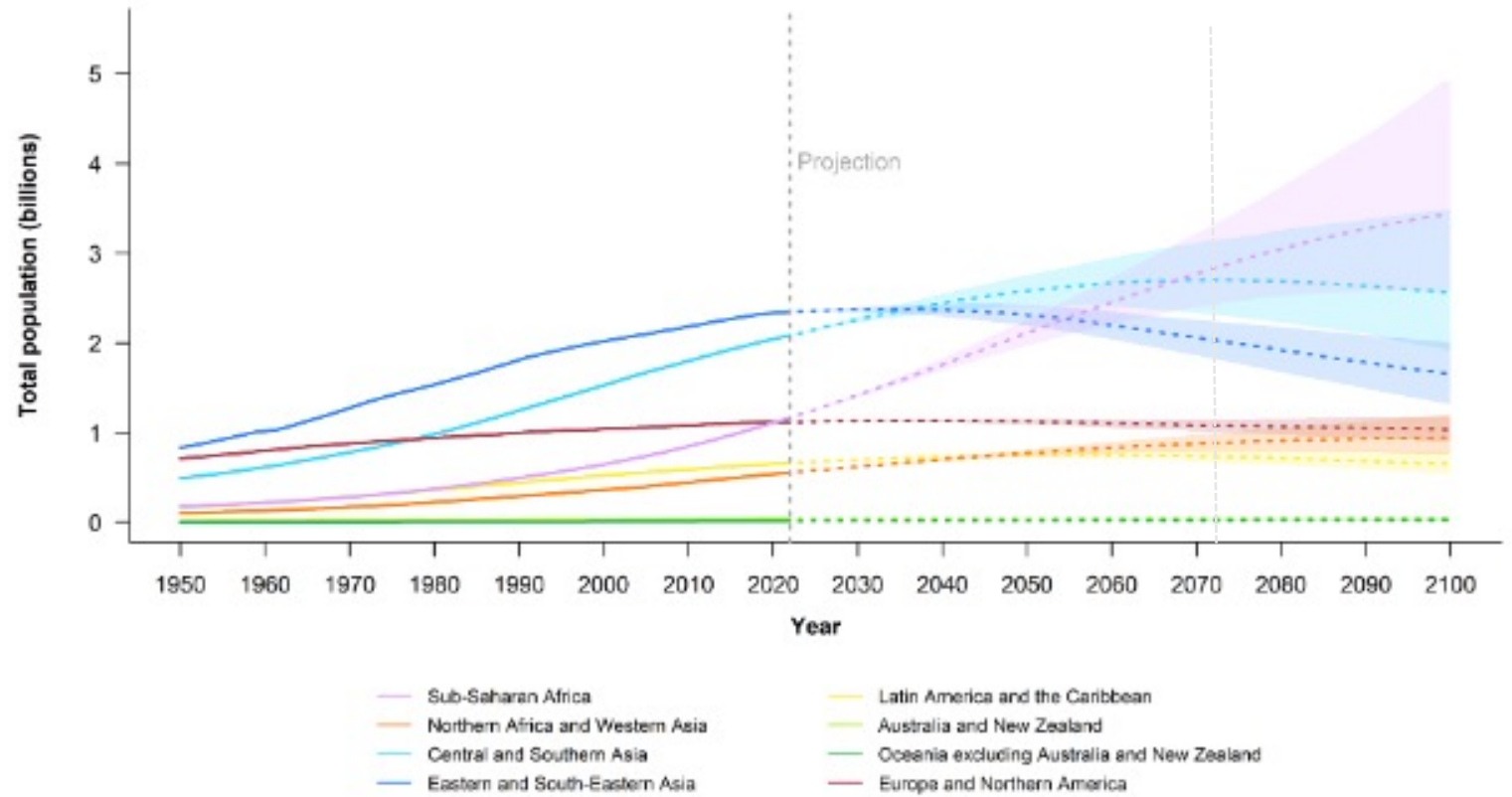
50 years from now...



United Nations | Department of Economic and Social Affairs

World Population Prospects 2022

Summary of Results



various scenarios: ~ 2070 -2100 population > 10 Billion

Sale of insect-based food products not allowed in Oman



OBSERVER WEB TEAM

PUBLISHED: 11:04 AM, FEB 08, 2023



The Food Safety & Quality Center (FSQC) has issued a clarification on the consumption of insect-based food products in some countries of the world.

FSQC said that such food products do not comply with the regulations and standard specifications and are not allowed to be traded in the local markets.

Consignments of all imported food products go through inspection and laboratory analysis to ensure their safety and compliance with the regulations and standard specifications.

Some of the GCC countries, including Qatar, recently announced that they will not allow the entry of insect-based food products into their markets.

The Ministry of Health in Doha said that insect products do not meet “the requirements of the technical regulations for halal food.”

“The regulations of the Gulf Cooperation Council (GCC) countries prohibit eating insects or proteins and supplements extracted from them,” it added.

The European Union Commission recently approved the use of small mealworm larvae and domestic crickets in food.

Validation report DNA / matrix DNA dilution inclusivity, exclusivity

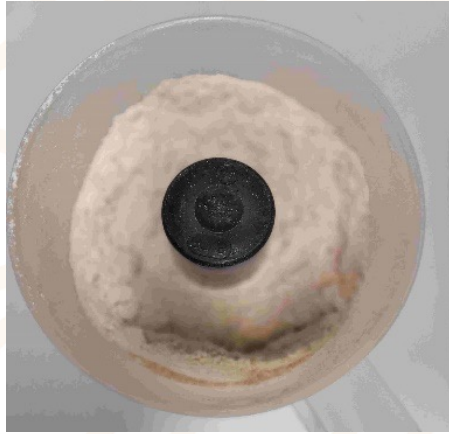
Table 1: Overview of specificity tests using SureFood® ALLERGEN Insects

sample		class / order / suborder	FAM
Achatinidae	Achatinidae	Gastropoda / Stylommatophora / Achatinia	negative
Ant	(Formicidae)	Insecta / Hymenoptera	positive
Atlantic salmon	(<i>Salmo salar</i>)	Actinopterygii / Salmoniformes	negative
Beef	(<i>Bos taurus</i>)	Mammalia / Artiodactyla / Ruminantia	negative
Beetle larva	(Pachnoda butana)	Insecta / Coleoptera / Polyphaga	positive
Black bean aphid	(Aphis fabae)	Insecta / Hemiptera / Sternorrhyncha	positive
Blue mussel	(<i>Mytilus edulis</i>)	Bivalvia / Mytilida	negative
Blue shark	(<i>Prionace glauca</i>)	Chondrichthyes / Carcharhiniformes	negative
Buffalo worm	(Alphitobius diaperinus)	Insecta / Coleoptera	positive
Bumblebee	(Bombus)	Insecta / Hymenoptera	positive
Butterfly	(Lepidoptera)	Insecta / Lepidoptera	positive
Canola	(<i>Brassica napus</i>)	Plantae / Brassicales	negative
Charlock mustard	(<i>Sinapis arvensis</i>)	Plantae / Brassicales	negative
Chicken	(<i>Gallus gallus</i>)	Aves / Galliformes	negative
Colorado potato beetle	(Leptinotarsa decemlineata)	Insecta / Coleoptera	positive
Common rough woodlouse	(<i>Porcellio scaber</i>)	Malacostraca / Isopoda / Oniscidea	negative
Common squid	(<i>Loligo spp.</i>)	Cephalopoda / Myopsida	negative
Crocodile	(<i>Crocodylia</i>)	Reptilia / Crocodylomorpha / Crocodylia	negative
Cuttlefish	(<i>Sepia officinalis</i>)	Cephalopoda / Decapodiformes / Sepiida	negative
Dragonfly	(Odonata)	Insecta / Palaeoptera	positive

depending on sample matrix,
de, DNA preparation and

was observed with DNA
rachnids with 100 %

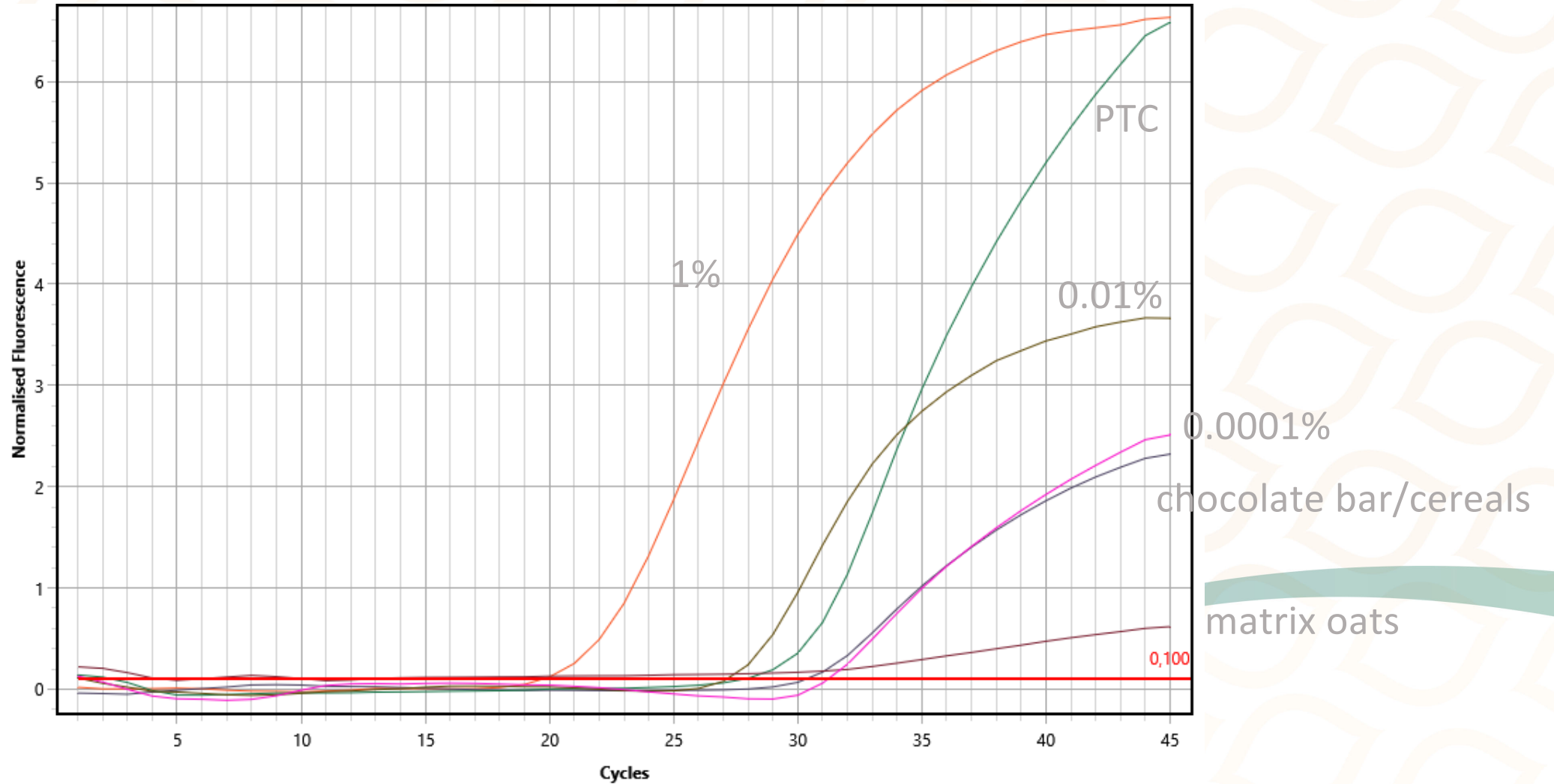
Insects, seriell 1:10 (w/w) dilution experiment: 45 gram matrix plus 5 gram sample



3 x 5 s 10.000 rpm
100% to 1 ppm
not a reference material
Laboratory material

...

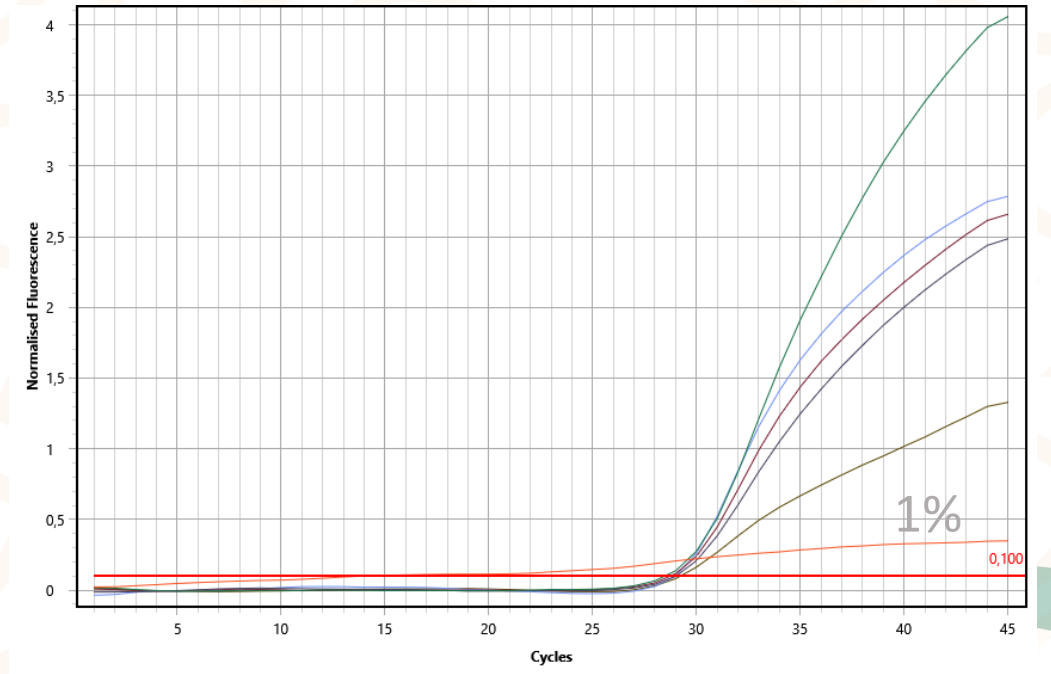
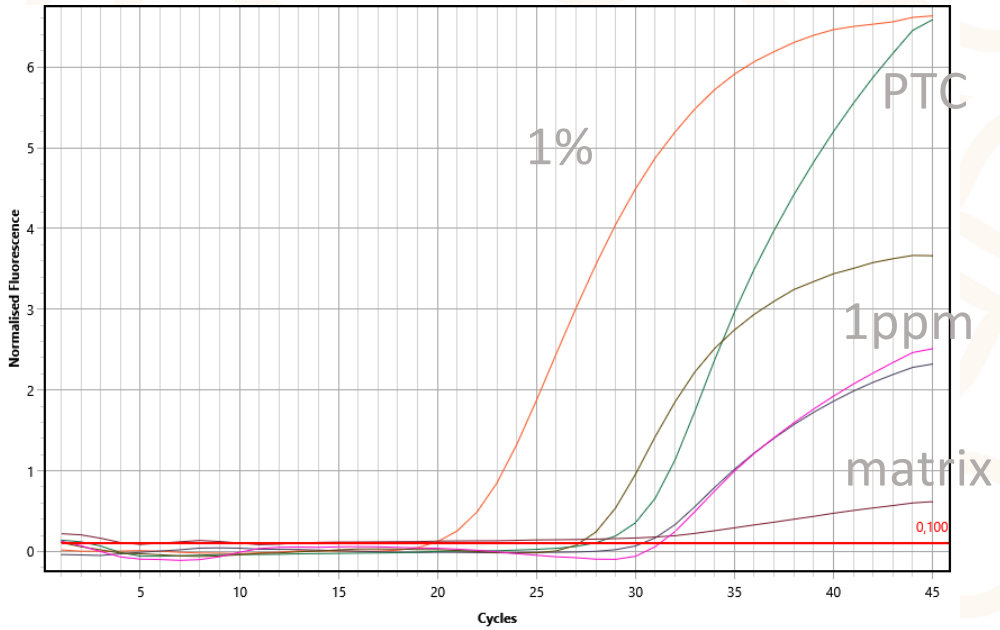
Raw data



Automatic data extraction

insects

IAC



matrix not calculated as positive, no curve, √

high protein concentration, dilute the DNA (e.g. 1:10) chocolate bar in ppm/sub-ppm range positive
 check lab conditions

Sample matrices

Example:
Gelatine

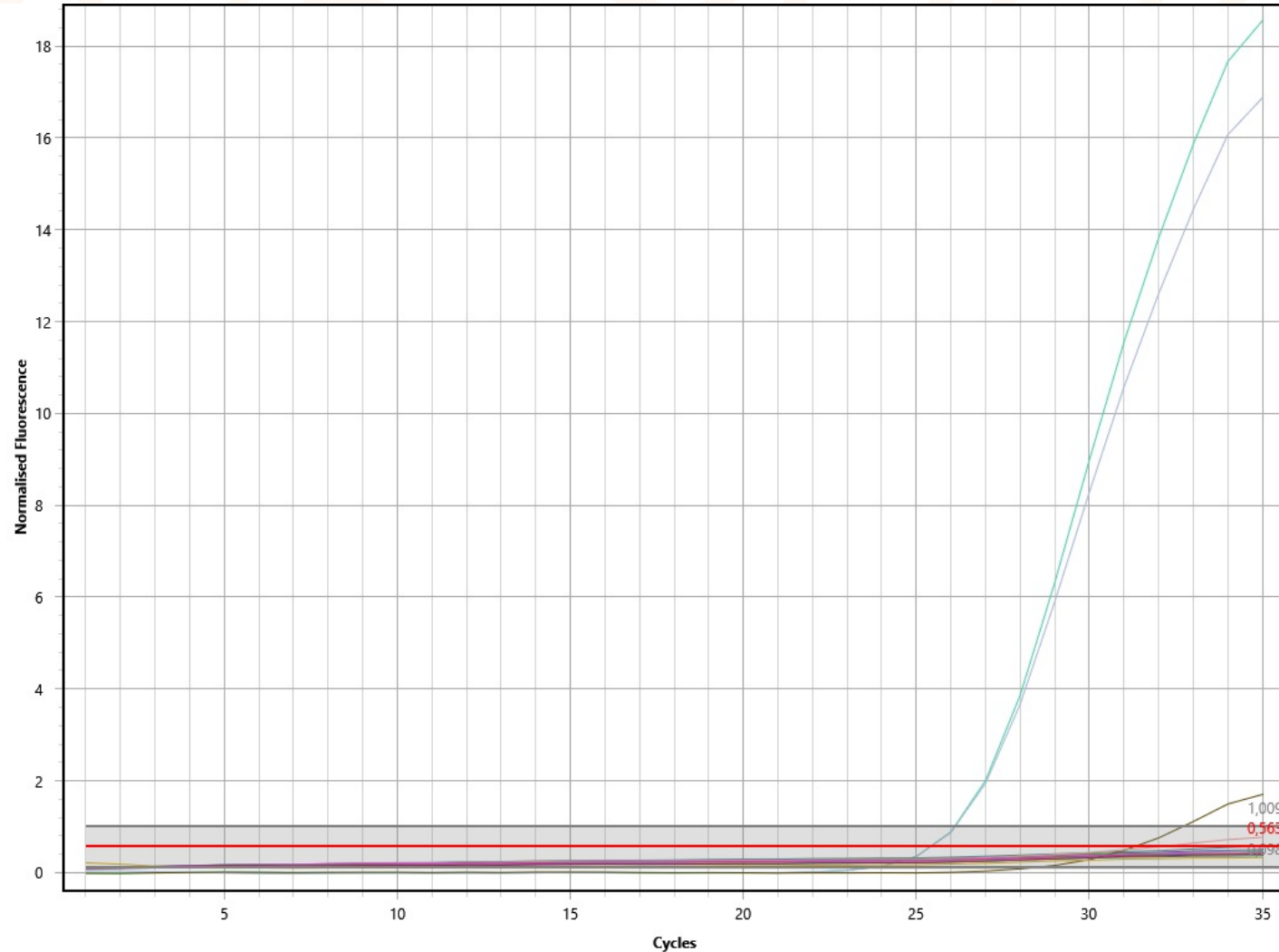
DRRR
Proficiency test

Pork sens

“problem“

1 of 2 results

(duplicate), presumptive; to be confirmed: if positive: porcine DNA detectable 2/3



Summary

modern highly processed, multi-component food can be of analytical challenge

qPCR (DNA methods) serves as sensitive technical tools - trace analytic is however difficult for correct interpretation

- Use proper controls: NTC, PTC, negative extraction (blank) controls, positive extraction controls
- Avoid contamination sources
- duplicates, confirmation
- Check not only Ct values, curves, software settings
- Standardisation, but not just SOP
- Proficiency tests, education
- Information about product, production if available

Thank you for your attention

...what about Lab (pure cell culture) meat ?

technical, regulatory treshhold for insects? locusts? bee? differentiation?





مؤتمر عُمان الدولي السادس
لسلامة وجودة الغذاء
Oman 6th International Conference
on Food Safety and Quality

