

*Last PCR developments for species specific  
detection of MBM in feed : transferability  
and single particle analysis*

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# Overview

- Context
- Transferability of a PCR method
  - Transferability - Why?
  - The rationale behind the transferability protocol
  - How to test the transferability protocol ?
  - The inter-laboratory trial
- Single particle analysis
  - Aim of this approach
  - Design of an adapted DNA extraction protocol
  - Further refinements for the extraction step

# Context




- Bovine spongiform encephalopathy
  - ➔ Total ban of Processed Animal Proteins (PAPs) in feed
- Reference method : classical optical microscopy
- + Alternative methods : e.g. Real Time PCR
- Pro and cons of the PCR :
  - species specific
  - sensitive
  - problems with authorised ingredients

## Transferability of a PCR method

### Why do we need to check if a PCR designed for PAP detection is transferable?

# Available PCR methods

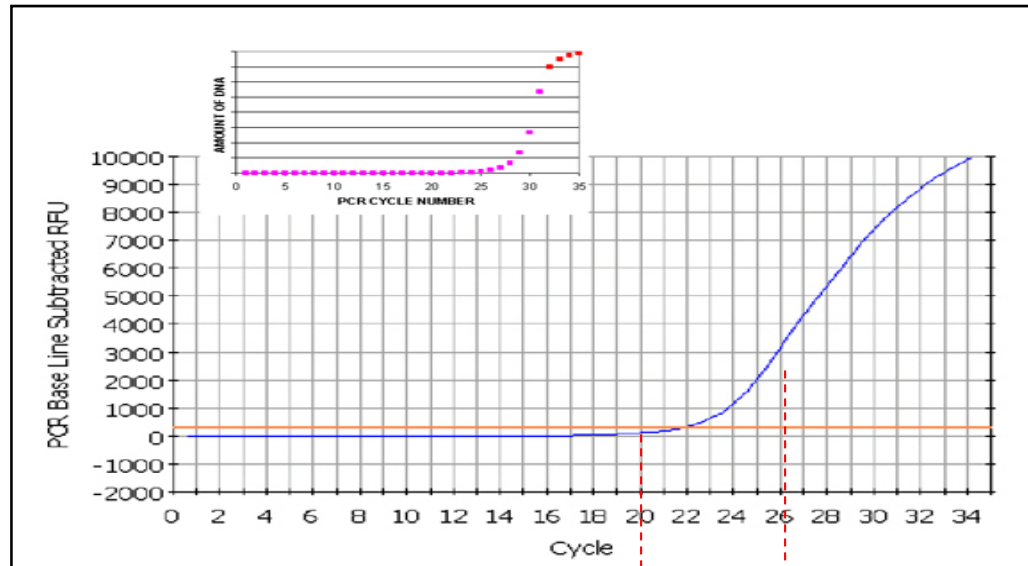
Prado *et al.*, Journal of Analytical and Food Chemistry, 2007

		CRA-W 	TNO 	VLA 
DNA extraction	Test portion size	100 mg ( duplicate/ ground)	1 g (ground)	40 g (unground)
	Extraction method	Magnetic beads	Guanidin HCl + Magnetic beads	Chelex resin
PCR	DNA target	Mitochondrial DNA	Highly repetitive genomic sequences	Mitochondrial DNA
	Amplicon size	68 bp (cattle)	83 bp (ruminants) 142 bp (cattle)	108 bp (cattle)
	Cut-off limit	<b>40 cycles</b>	<b>35 (ruminants)</b> <b>40 cycles (cattle)</b>	<b>35 cycles</b>
Publication status		Published (article 2006)	Unpublished	Published (article 2009)

**All 3 labs are able to detect ~ 0.1 % of MBM alone or in mixture with different materials such as fishmeal**

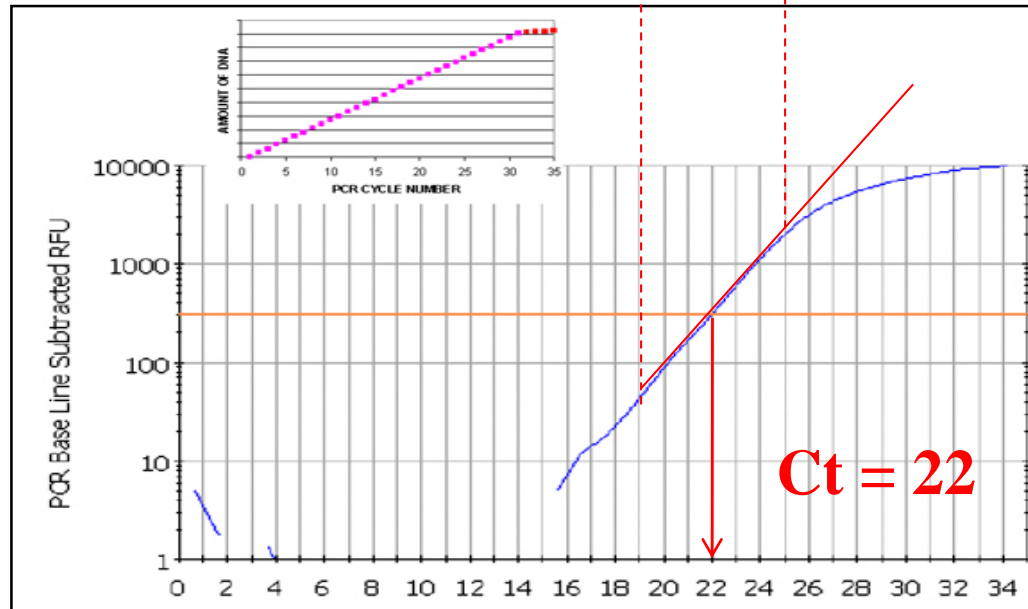
# The concept of threshold cycle

Linear scale



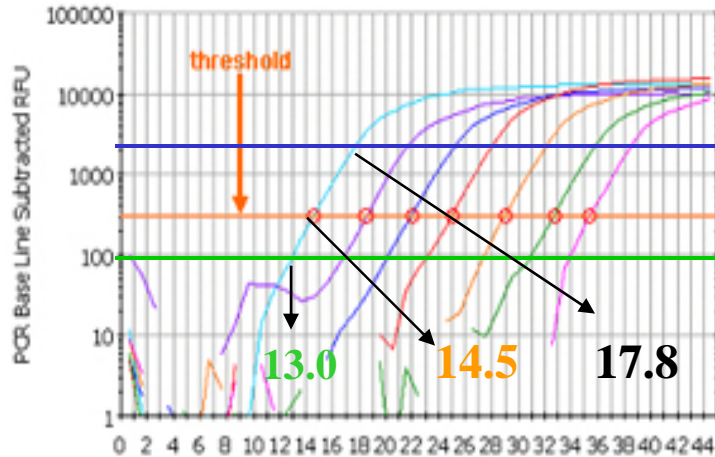
Logarithmic scale

*Exponential  
amplification phase*



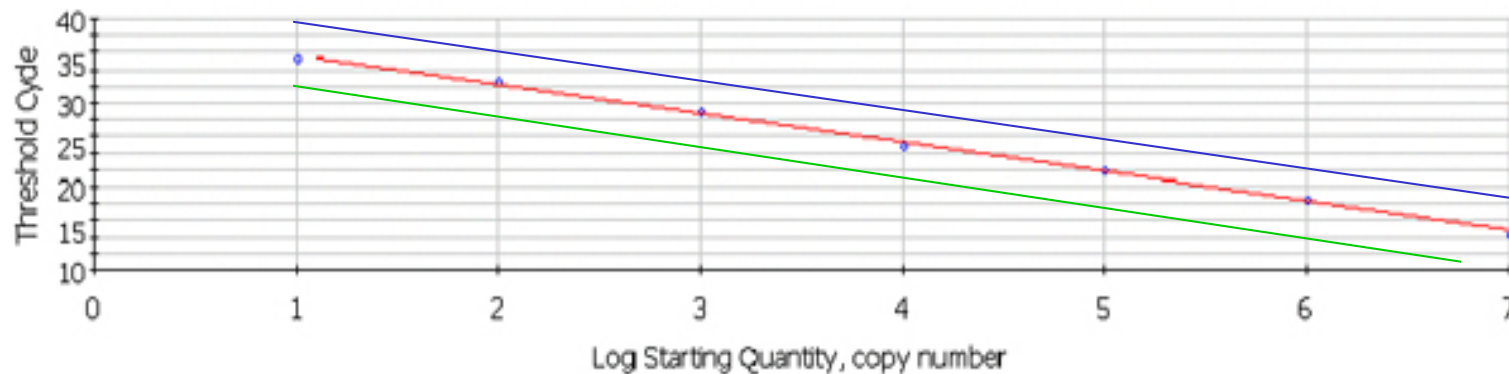
# The concept of threshold cycle

## Relativity of the Ct concept



Correlation Coefficient: 0.999 Slope: -3.488 Intercept: 39.204  $Y = -3.488 X + 39.204$

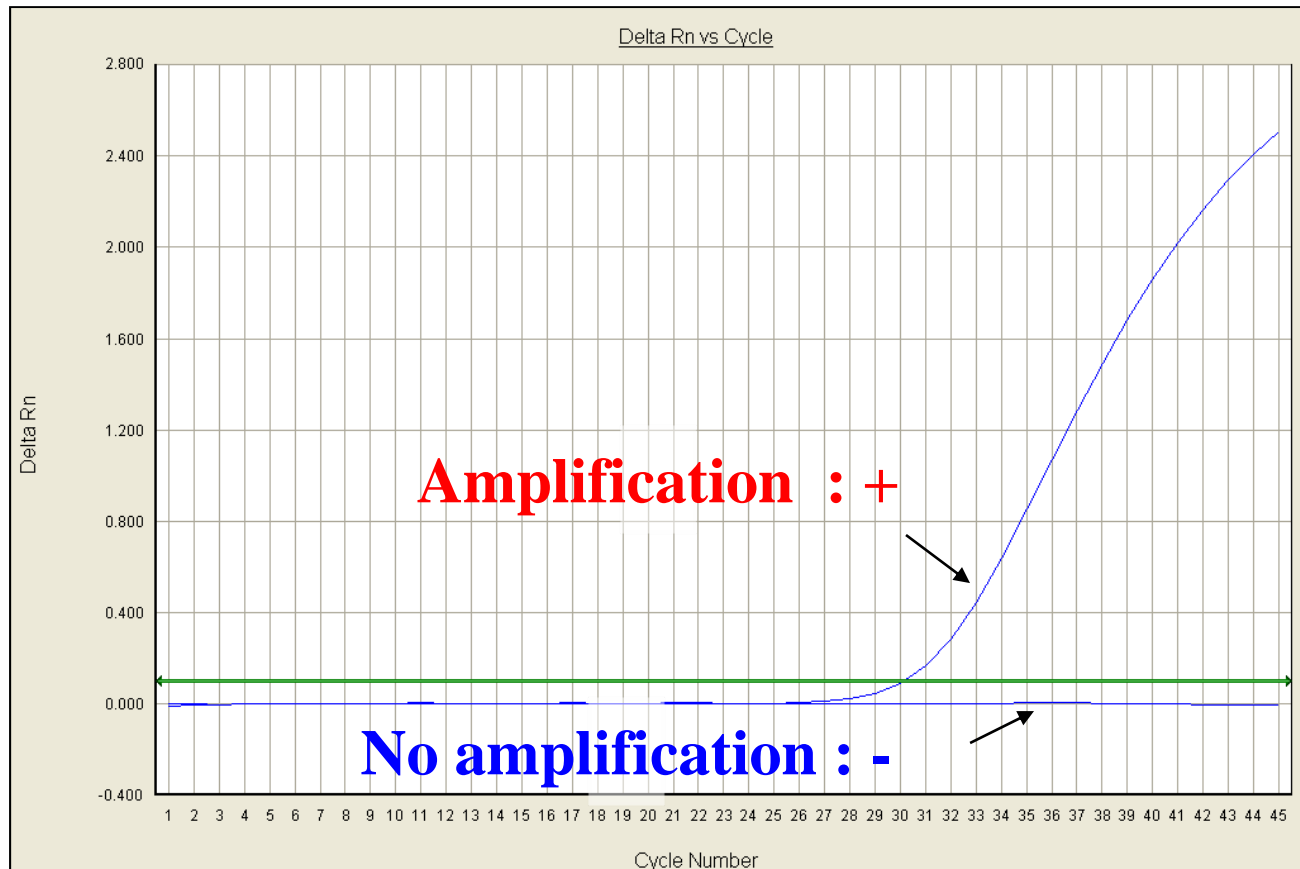
□ Unknowns  
● Standards



PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

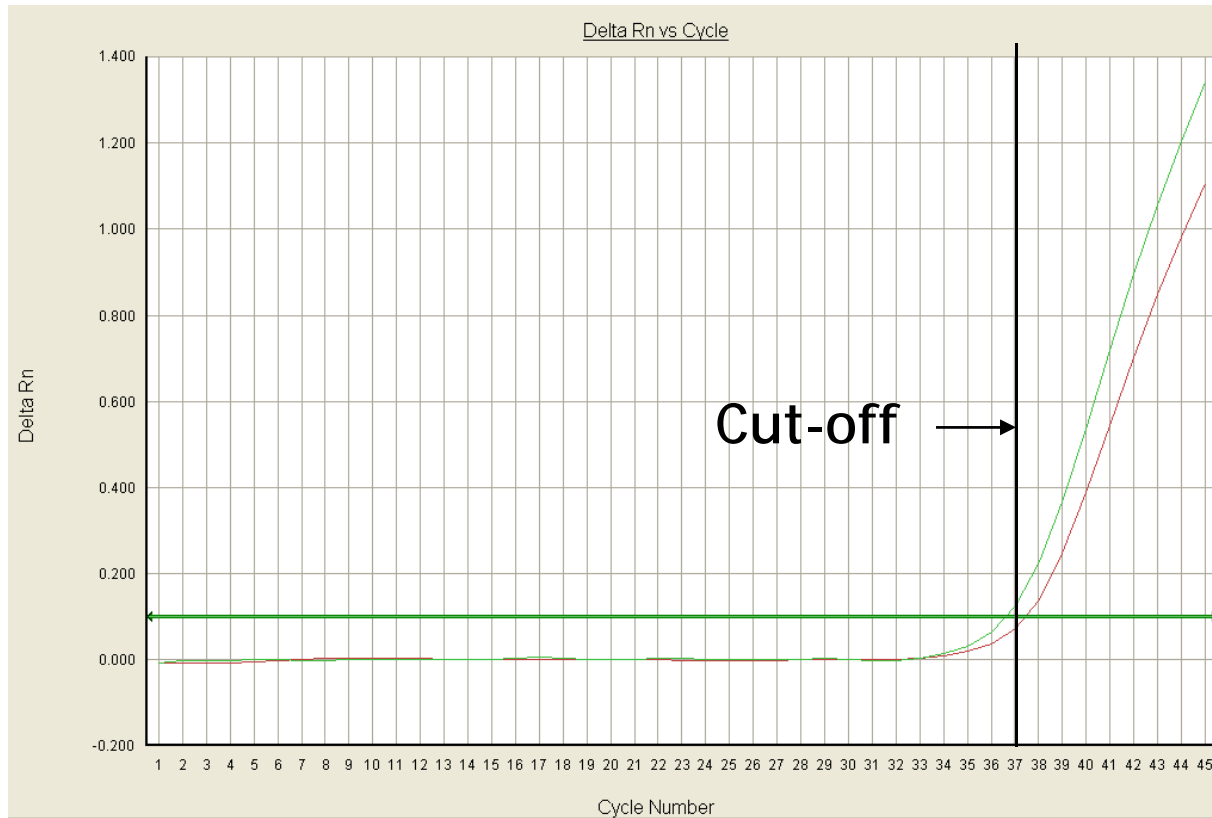
# The concept of cut-off value

## Clear-cut signals



# The concept of cut-off value

## Late signals



# *Request of DG-Sanco (2008)*

## ✓ Request

- Organisation of an interlaboratory study proving that PCR is working for the purpose of MBM detection in a **network of laboratories**

## ✓ Consequence

- The method must be **transferable**

## ✓ Problem

- Each of the three methods is using a **cut-off value** to define what is a positive and what is a negative value.
- **BUT** : the cut-off value is defined in terms of Ct (which is a **RELATIVE** value not an **ABSOLUTE** one), so, in essence, the methods are not directly transferable!

# Transferability of a PCR method

## The rationale behind the designed transferability protocol

# *Used rationale for the transferability protocol*

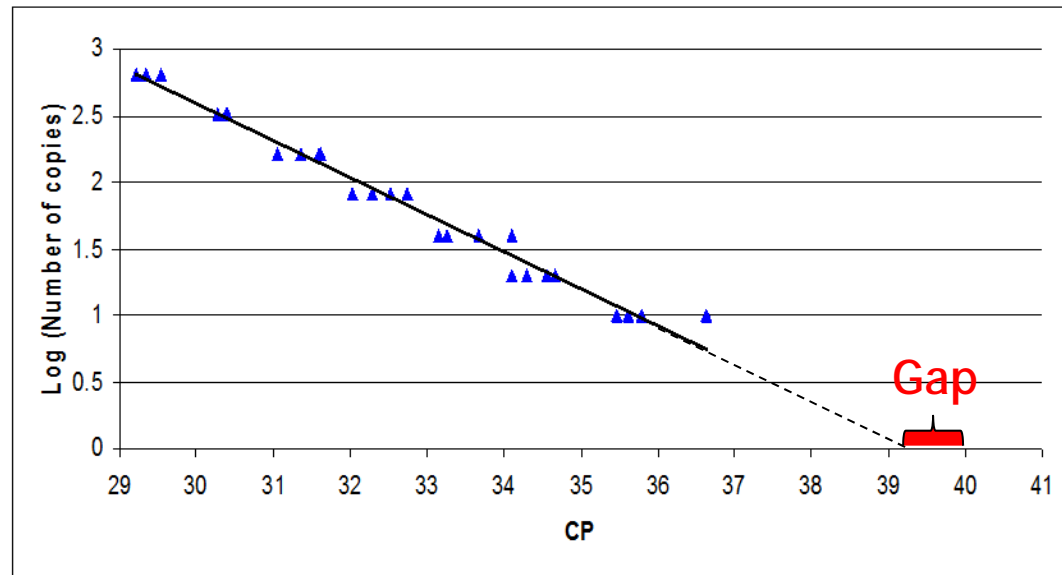


- Empirically in the normal conditions used at CRA-W on the first real-time thermocycler on which the test was designed it appears that a cut-off value of 40 is fit for purpose (based on analysis of a lot of negative samples)
- This relative Ct value could be translated in terms of a more absolute values like the number of the copies of the target that correspond to it
- This would mean that a calibration curve could be built and then be used to define based on a determined number of targets corresponding to the cut-off what is the corresponding Ct

*But...*

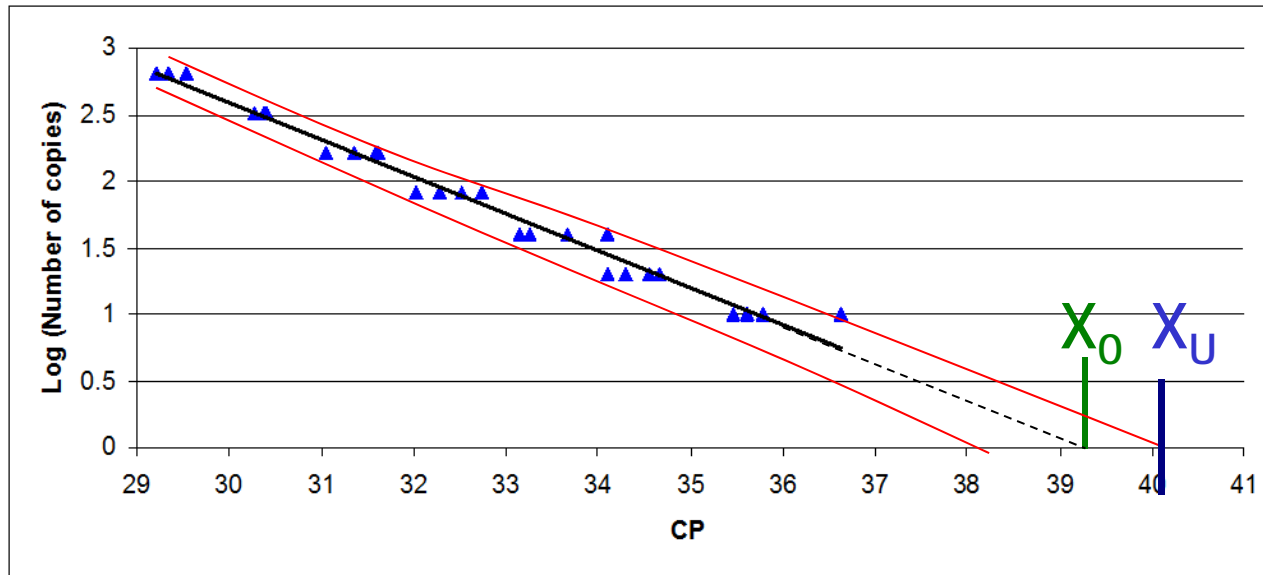
✓ Calibration with :  
7 points and 4 replicates/point

Well	Number of copies /5µl	CP
B8	640	29.34
C8	640	29.34
D8	640	29.53
E8	640	29.22
B7	320	30.27
C7	320	30.38
D7	320	30.41
E7	320	30.38
B6	160	31.35
C6	160	31.62
D6	160	31.60
E6	160	31.05
B5	80	32.28
C5	80	32.03
D5	80	32.53
E5	80	32.75
B4	40	33.67
C4	40	34.10
D4	40	33.25
E4	40	33.16
B3	20	34.11
C3	20	34.29
D3	20	34.66
E3	20	34.55
B2	10	35.47
C2	10	35.62
D2	10	36.63
E2	10	35.80



✓ A cut-off of 40 on our platform corresponds to a starting material of less than one target !

# Statistical solution to the problem



The problem of the cut-off determination solved statistically to find  $X_u$

+

Optimisation of the calibration (number of points and replicates) : 3 points 40, 160 and 640 with three replicates for each

# Transferability of a PCR method

## How to test the transferability protocol ?

# *Aim of the study*

With the methodology developed for the transfer, check if the calculated cut-off value is appropriately set to :

- detect 1 or more copies (positive results)
- have no more than 5 % of signals after the cut-off due to the presence of 1-2 copies of the target (false negative results)

Samples (plasmids diluted in maize DNA)  
to analyse close to 1 copy of target :  
5, 2, 1, 0.6, 0.4 and 0.1 copy  
+ controls : 80 and 0 copy

# *Problems when handling tiny number of targets*

- **With very low copy numbers of targets, stochastic distribution of these copies due to pipetting has to be considered : when pipetting a volume that should contain 1 target, there is one chance on three approximately that the volume contains NO target!**
- **To circumvent this problem, samples are to be tested in triplicates and a sample is considered as positive as long as one of the triplicates is positive (i.e. a Ct value lower than the one defined by the protocol as being the cut-off value).**

# Transferability of a PCR method

## The inter-laboratory trial

# Participants

19 laboratories: 17 from the European Union  
1 from Japan  
1 from Australia

22 thermocyclers from 4 companies:

4 LC480

1 LC480 II (Roche Diagnostics)

1 ABI 7300

2 ABI 7500

1 ABI 7500 Fast

1 ABI 7700

2 ABI 7900

1 Step One Plus (Applied Biosystems)

2 iCycler

1 MyiQ

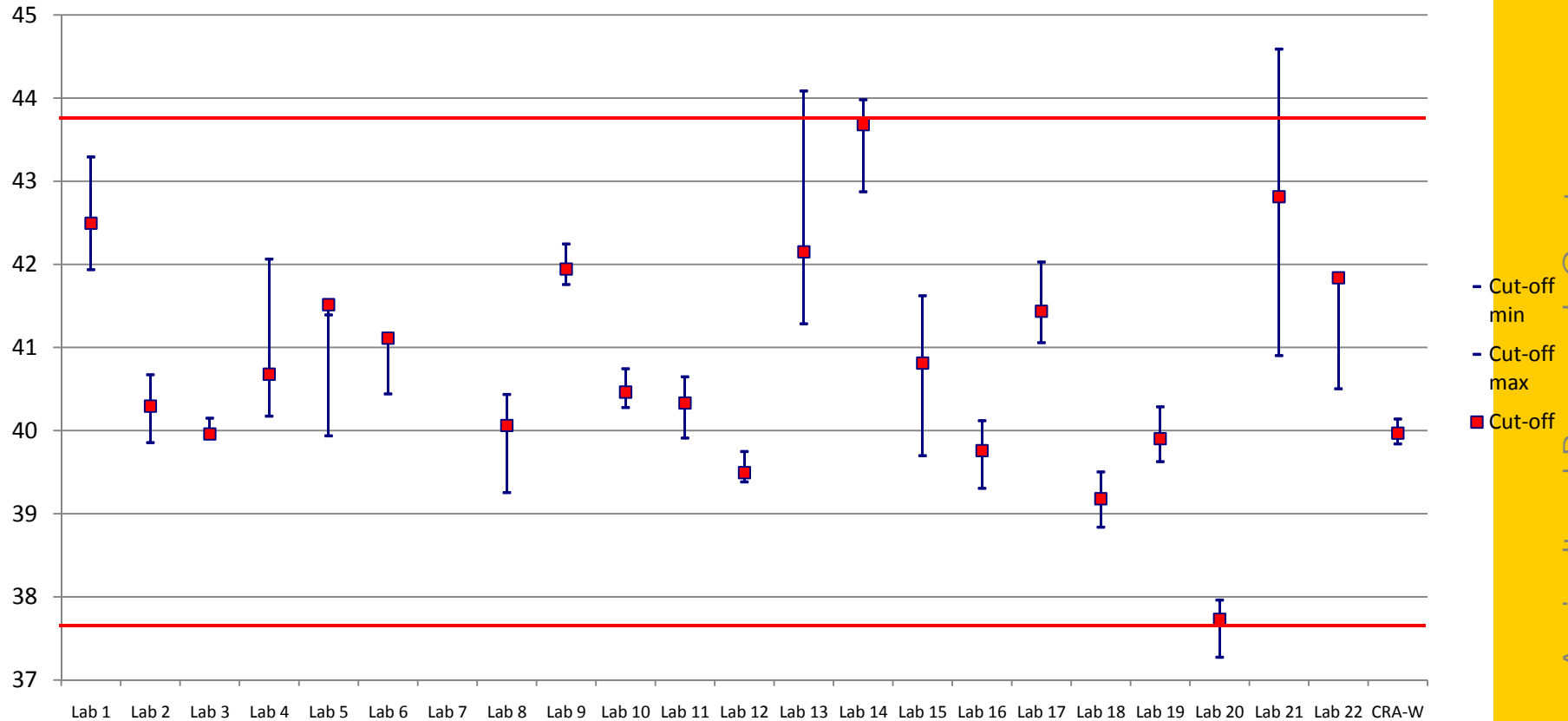
2 CFX (BioRad)

2 Mx3000P

2 Mx3005P (Stratagene)

# Results

## Distribution of cut-off values

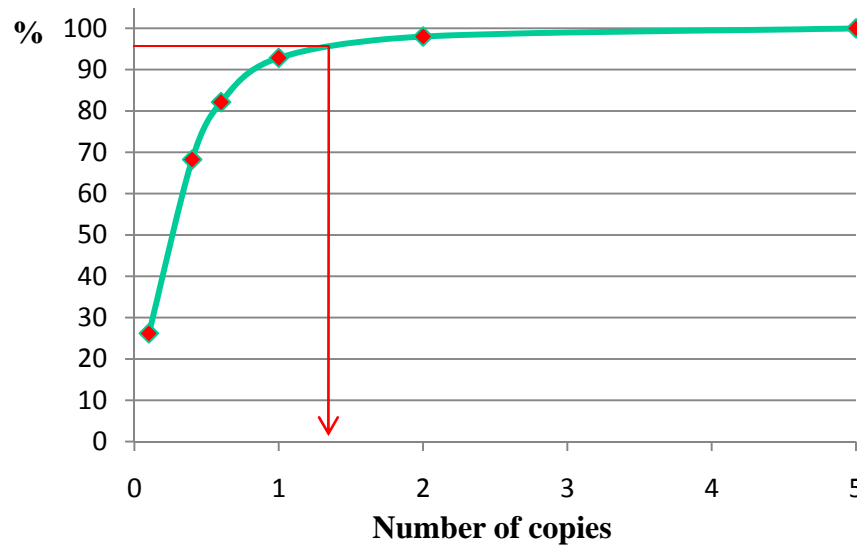


**$37.73 < \text{Cut-off} < 43.68$**

**Almost 6 units of Ct of difference !!  $2^6 = 64$**

# Results

## Percentages of blocks of triplicates detected



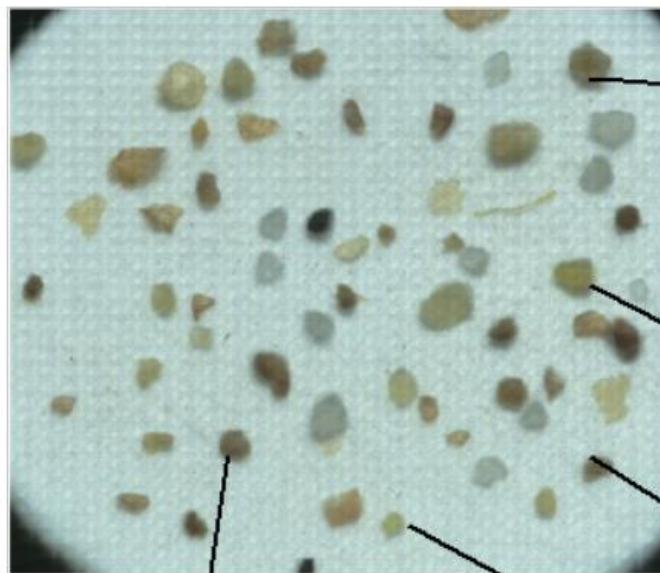
The protocol seems fit for purpose but in-depth statistical analysis is being done

It will be used in the validation of a kit developed within SAFEED-PAP

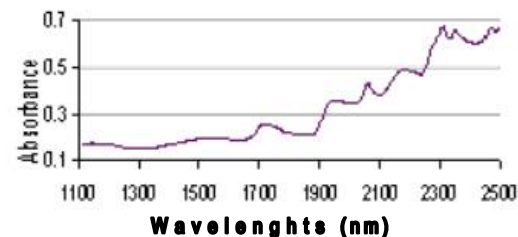
# Single particle analysis

## Aim of this approach

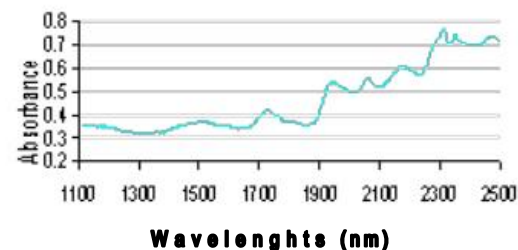
# NIR imaging based particle analysis



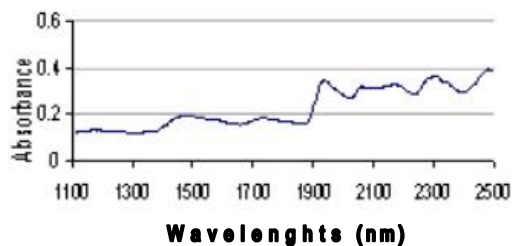
**Fish particle spectrum**



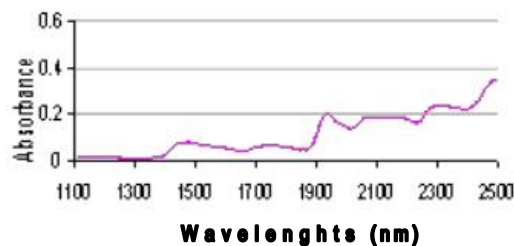
**Bone particle spectrum**



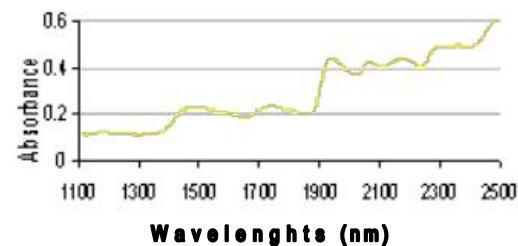
**Flax oilcake spectrum**



**Maize particle spectrum**

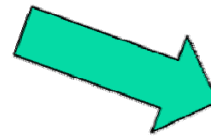


**Soybean particle spectrum**

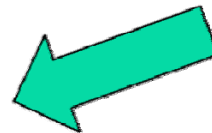




**Spreading of particles ( $> 250 \mu\text{m}$ ) on a spectralon sheet with the help of a stereomicroscope**

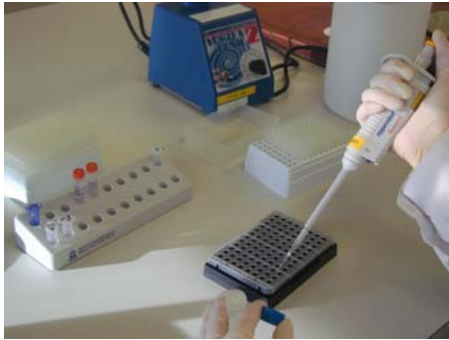


**Spectral measurements**



**Isolation of identified particles to be put in Eppendorf vials**

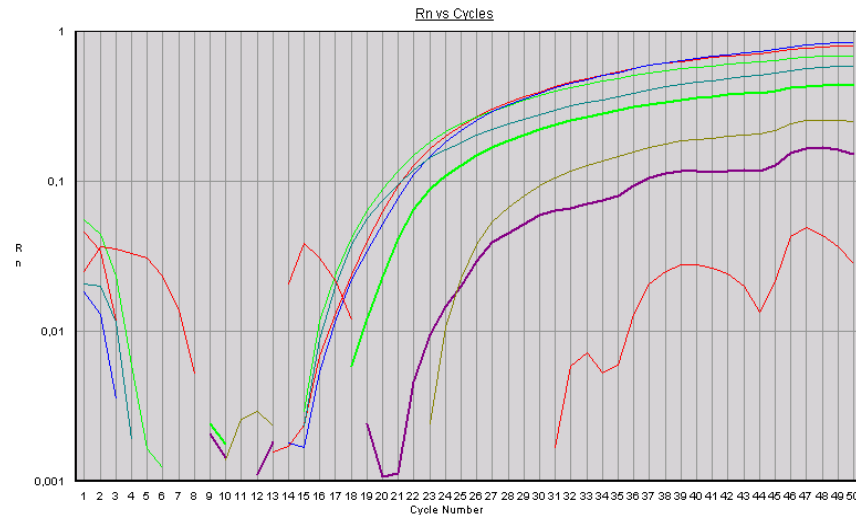




**Addition of DNA extraction reagents combined to the PCR mix**

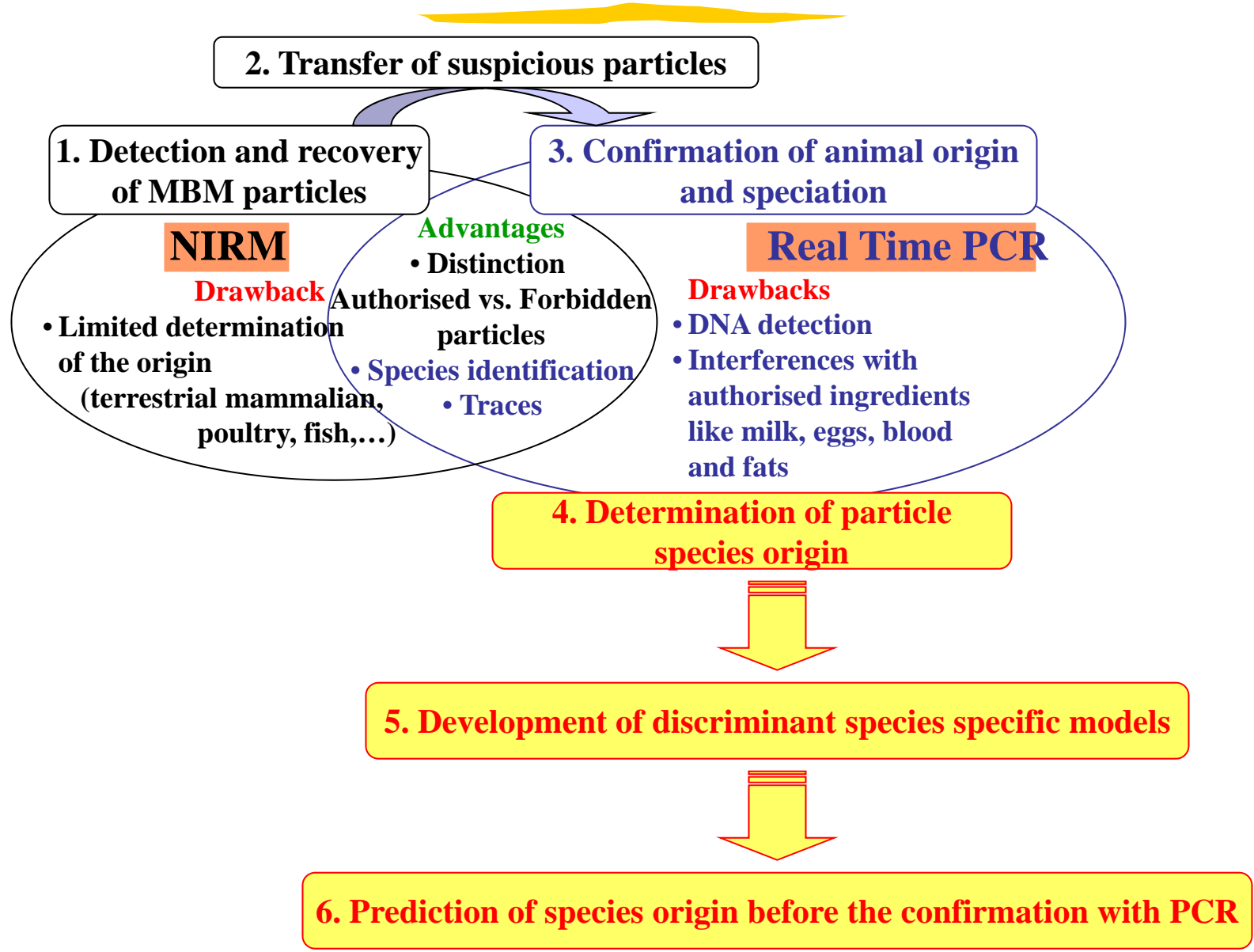


**PCR analysis of suspicious animal particles**



**Single particle PCR results of analysis**

# Development of the combination NIRM – Real Time PCR



# Single particle analysis

## Design of an adapted DNA extraction protocol

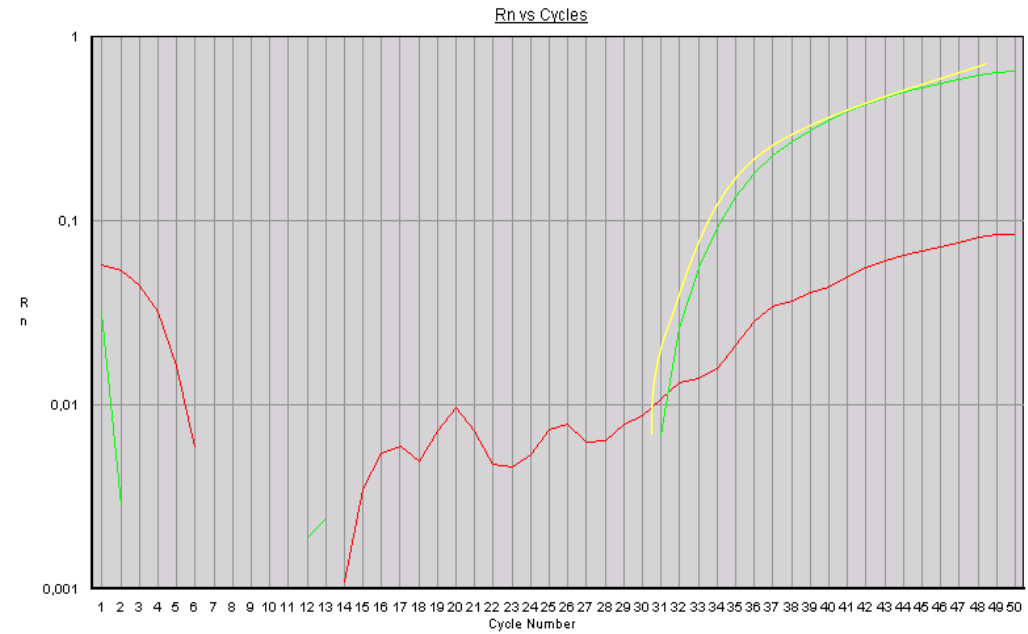
# Development of a DNA extraction protocol from a single particle

## 1. Home made protocol

The particle is put directly in the PCR mix before performing the PCR

Some improvements :

- particle in a solution of Proteinase K (1 mg/ml)
- heating step (20 min, 60 °C + 10 min , 95 °C) previous to the PCR
- particle in a greater volume of Proteinase K
- particle in a vegetal DNA + Proteinase K



### Results :

- **1 to 3 PCR / particle**
- **Aspecific signals probably due to nuclease**
- **Successful amplification rates highly dependent of the samples (40 – 100 %)**

# Development of a DNA extraction protocol from a single particle

## 3. Special extraction buffer

*Direct PCR<sup>®</sup> Lysis Reagent  
(Viagen Biotech Inc., Los Angeles, USA)*

DNA extraction buffer initially dedicated to DNA extraction from mouse tail tissue giving after lysis an extract directly usable for PCR



### Optimised protocol :

- Lysis of the particle in 7  $\mu$ l of buffer containing 0.4  $\mu$ g /  $\mu$ l of Proteinase K at 85 °C during 45 minutes
- Dilution of the lysate 5 fold in milliQ water
- 5  $\mu$ l of diluted lysate in the PCR mix

### Evaluation :

- High successful amplification rates (100 %)
- 5 PCR / particle
  - Possibility to test 5 different species or to replicate an analysis
- Clear amplification curves and no aspecific signal
- Rapid, easy to use and very flexible
- Very cheap

# Single particle analysis

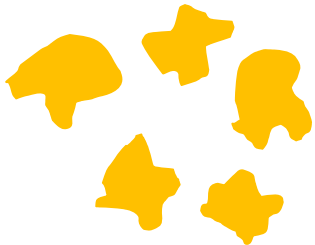
## Further refinements for the extraction step

# *Need of purification steps*

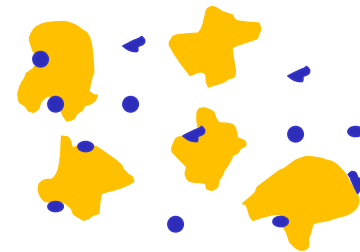
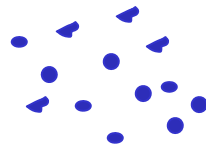
The selection of the appropriate extraction protocol was done with the help of pure meat and bone meals (originating from only one species).

When analysing a mix of material, a particle is no longer « monospecific » .

**Bone particles**



**Milk powder**



## *Need of purification steps*

**Balance between a process that is strong enough to get rid of the contaminating material (at least its DNA) and soft enough for the main particle so as to allow still sufficient extraction of DNA out of it.**

**Several procedures were tested, some based on the literature (e.g. Toyoda *et al.*, 2004 using bleach) but most are not appropriate as their results are largely dependent on the matrix. However the protocol based on use of DNA Erase seems to be the best one.**

# Acknowledgements



**Olivier Fumière, Aline Marien, Julie Hulin, Denis Roulez,  
Vincent Baeten, Isabelle Fissiaux  
Viviane Planchon & Robert Oger  
Christoph von Holst & Ana Boix  
All laboratories participating to the interlaboratory study**



**FARIMAL** : New methodology to determine the species origin of animal ingredients in feedingstuffs : combination of micro-spectroscopy techniques and Real-Time PCR



**SAFEED-PAP** : Detection of presence of species-specific processed animal proteins in animal feed

<http://safeedpap.feedsafety.org/>



**CRL-AP** : Community Reference Laboratory for the detection of animal proteins in feedingstuffs

<http://www.crl.cra.wallonie.be/>

**Many thanks for your attention**