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Better Training for Safer Food

Initiative

Detection of PAPs by light microscopy and PCR

Pascal Veys and Olivier Fumière

Consumers, Health And Food Executive Agency

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Methods of PAPs detection : back in time

Commission Directive 88/1998 establishing guidelines for the microscopic identification and estimation of constituents of animal origin for the official control of feedingstuffs

Commission Directive n° 126/2003 on the analytical method for the determination of constituents of animal origin for the official control of feedingstuffs

Stratfeed

Commission Regulation No 152/2009 laying down the methods of sampling and analysis for the official control of feed.

Commission Regulation No 51/2013 amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed

EURL Animal Proteins

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Commission Regulation No 152/2009

laying down the methods of sampling and analysis for the official control of feed

Annex VI : Methods of analysis for the determination of constituents of animal origin for the official control of feed

Conditions for the microscopic detection, identification or estimation of constituents of animal origin in feed

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Commission Regulation No 51/2013

amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed

- PCR = official method besides light microscopy
- Reference to Standard Operating Protocols (SOP) established by the EURL-AP and published on its website
- New and more harmonized extended light microscopy protocol
- PCR : principle and general guidelines

Better flexibility thanks to the SOPs

↳ *to add targets to the ruminant one (e.g. pig, poultry)*

↳ *to adapt the protocol if necessary (e.g. new recommended reagents)*

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Analytical methods of PAPs detection

1. Light microscopy

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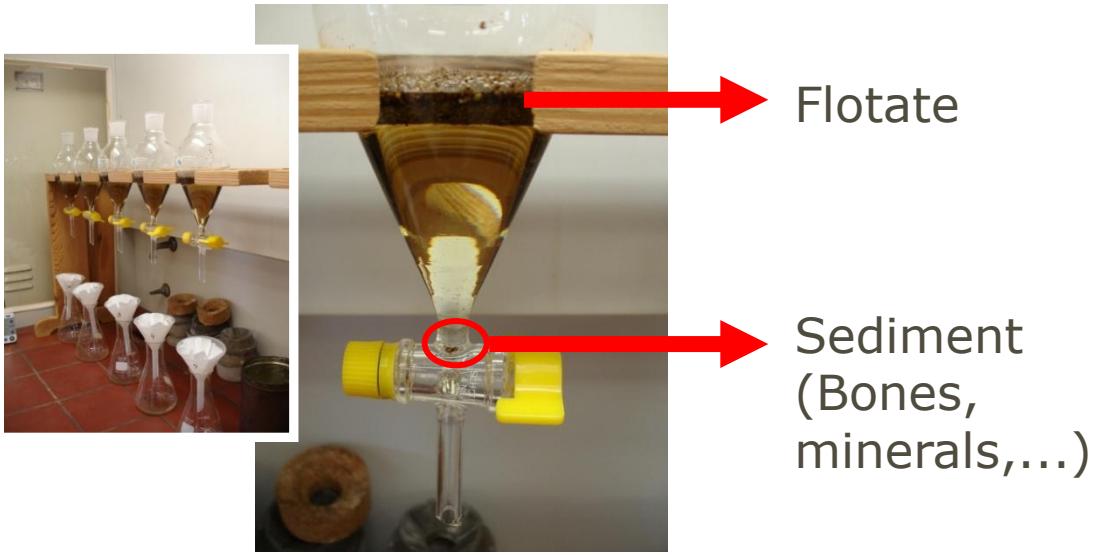
Principle

Observation of identifiable structures on sediment (TCE) and flotate or raw material



Principle

Observation of identifiable structures on sediment (TCE) and flotate or raw material

Staining can be used (optional SOP):

- Alizarin Red → bones, scales
- Cystine reagent → hairs, feathers

Distinction of PAPs from **terrestrial, fish** ...and avian origin

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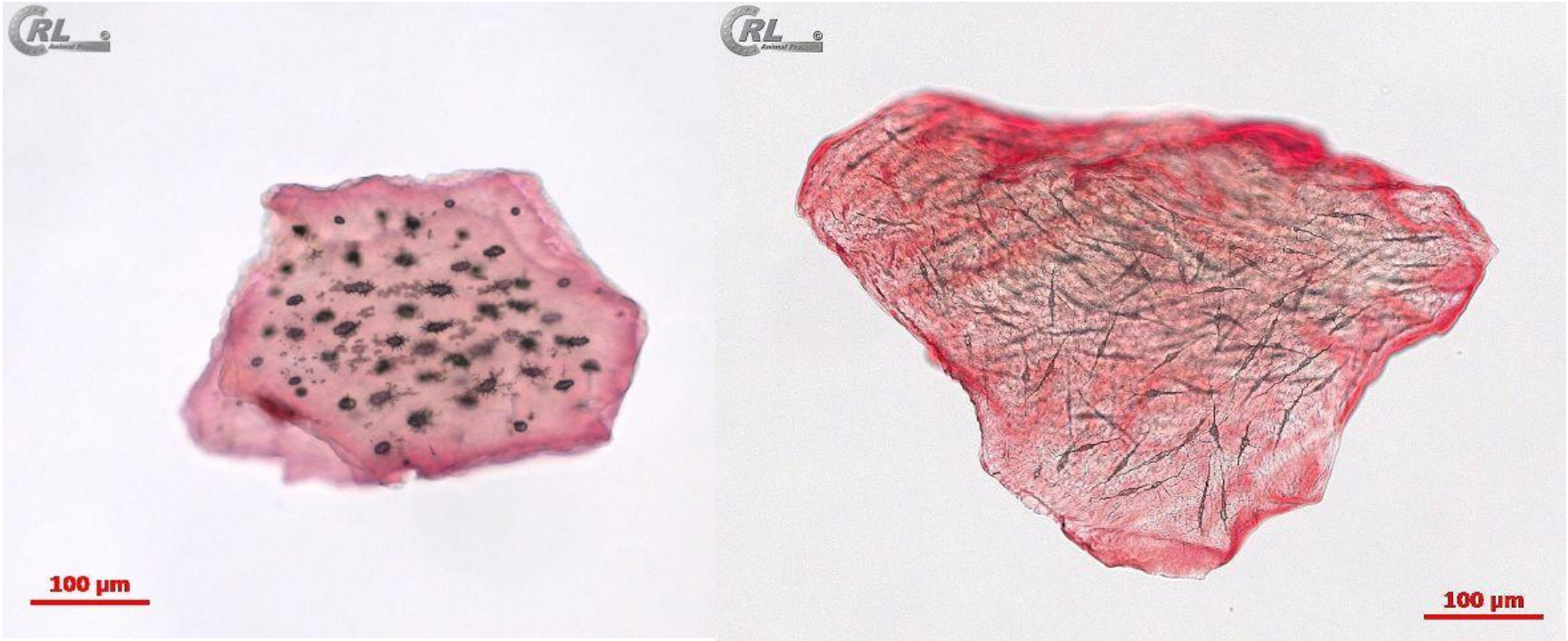
Identification : Terrestrial <> Fish



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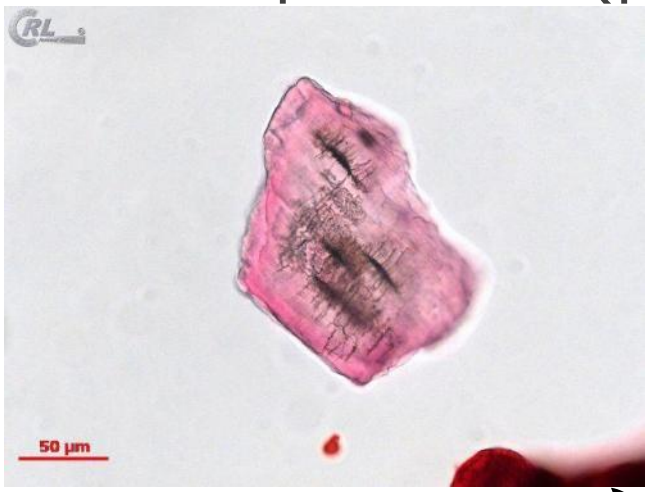
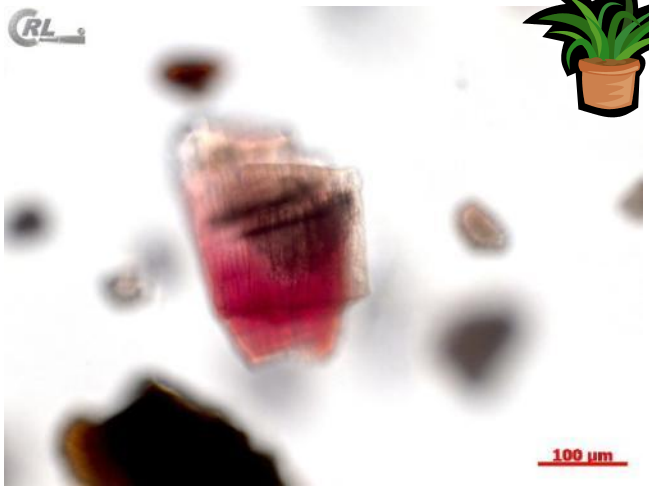
Expertise... (plant or animal ?)

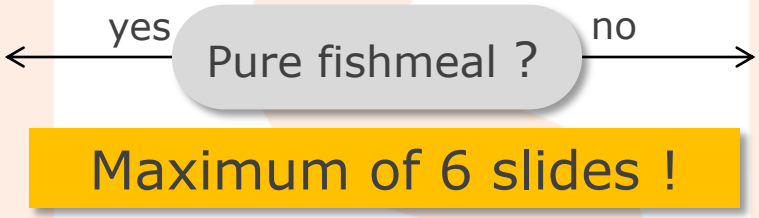




Microscopic examination (I)

1. Slide preparation in accordance with **SOPs**
   - coverslips
   - …

2. Number of slides



3. Sequence of observations of slides ?
   - cfs. diagrams
   - use of stereomicroscope = optional
   - **strictly** respect diagrams !

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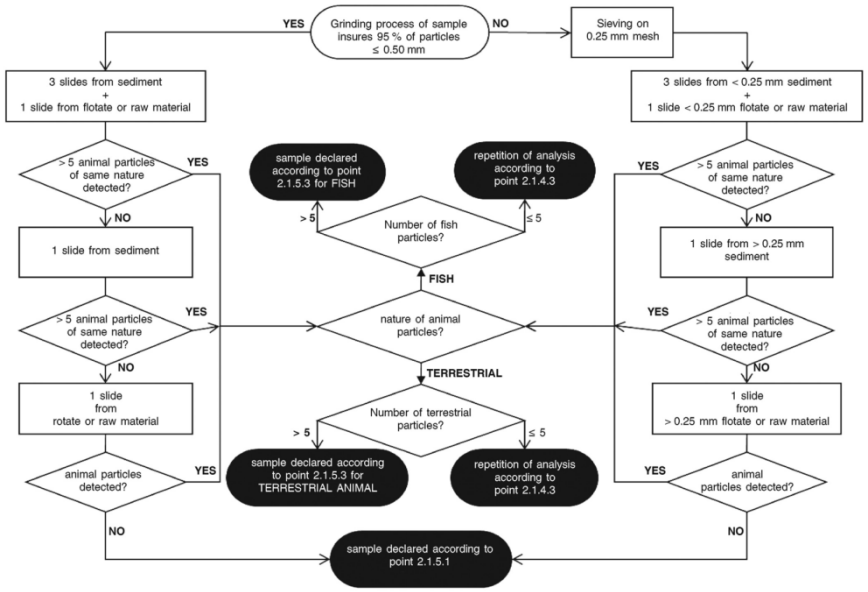
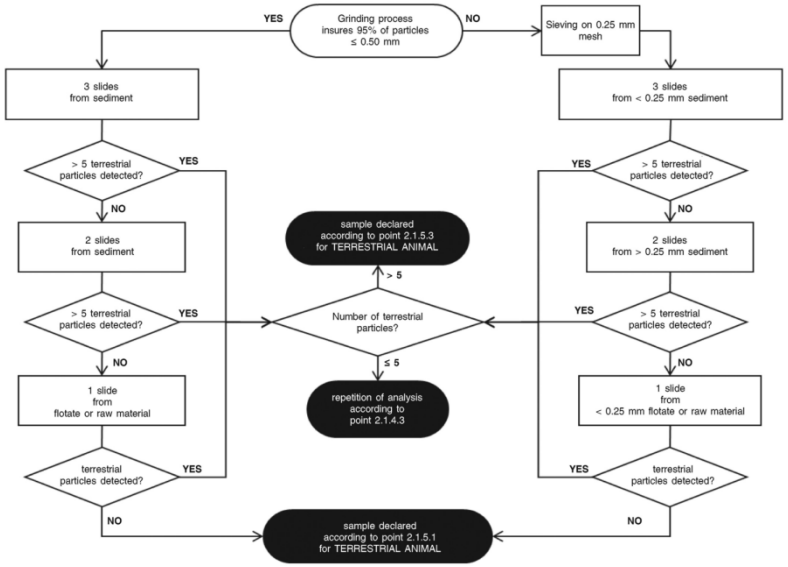
Microscopic examination (II)

Compound feed & feed materials



Fishmeals



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Results expression (I)

for each nature ! (FISH + TERRESTRIAL)

no particle detected :

« as far as was discernible using…., no particle from… was detected in the submitted sample »

1-5 particles detected on average

« as far as was discernible using…., no more than 5 particles from… were detected on average per determination in the submitted sample. The particles were identified as [bone, cartilage, muscle…]. This low level is below the LOD… risk of false positive result »

>5 particles detected on average

« as far as was discernible using….,more than 5 particles from… were detected on average per determination in the submitted sample. The particles were identified as [bone, cartilage, muscle…]»

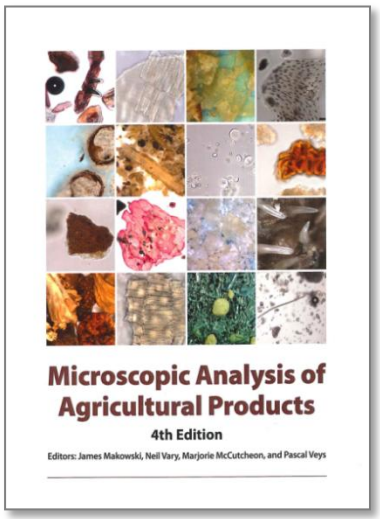
Results expression (II)

report shall mention:

1. Type of material
   - sediment,
   - flotate or raw material
2. Number of determinations
3. If presieving : in which fractions (sieved fraction, pelleted fraction or kernels) particles have been detected.

LM advantages and drawbacks

- Ease of use
- Cheap
- Very sensitive (<0.01%)
- Disclosure of adulteration
- References



- Skilled people, *real* microscopists
- Continuous training
  - new feed compounds and by products
  - Keeping skills at the top
- No species identification
- Based on particle detection only, some ingredients are not always visible
- Only qualitative…!

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Analytical methods of PAPs detection

2. The PCR (Polymerase Chain Reaction)

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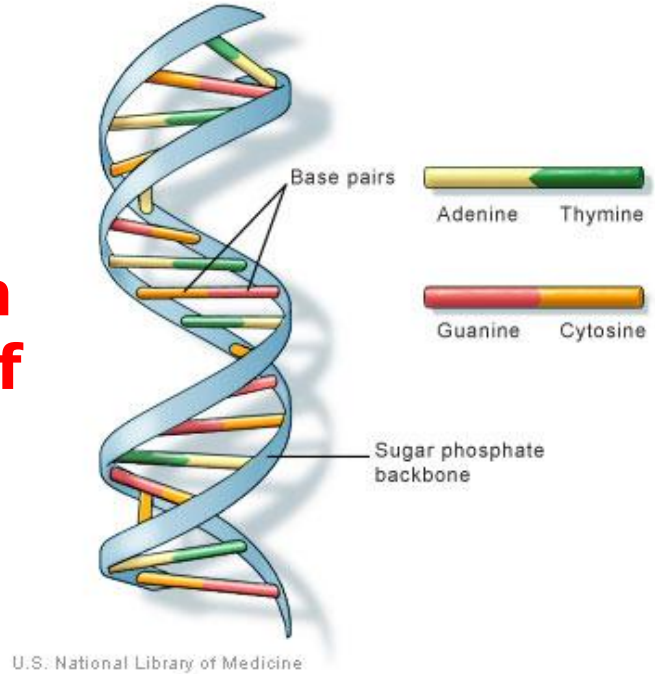
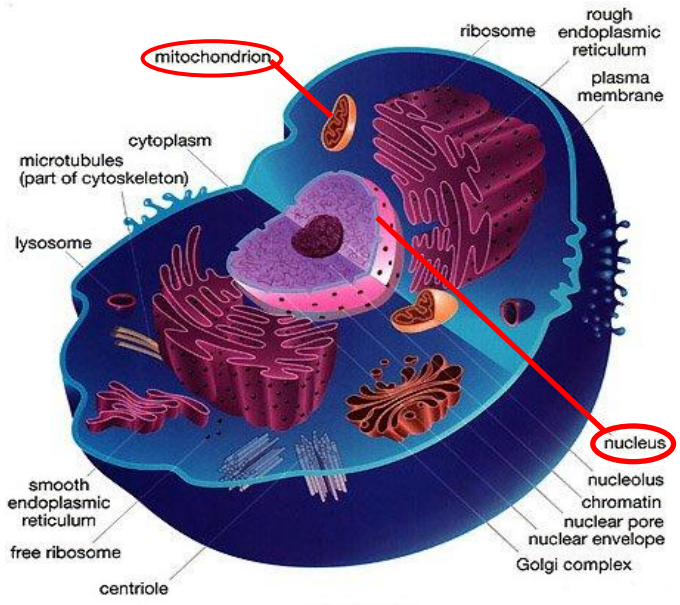
The target of the PCR : the DNA

The DNA = desoxyribonucleic acid

The DNA is a molecule present in almost all the cells and tissues of an organism





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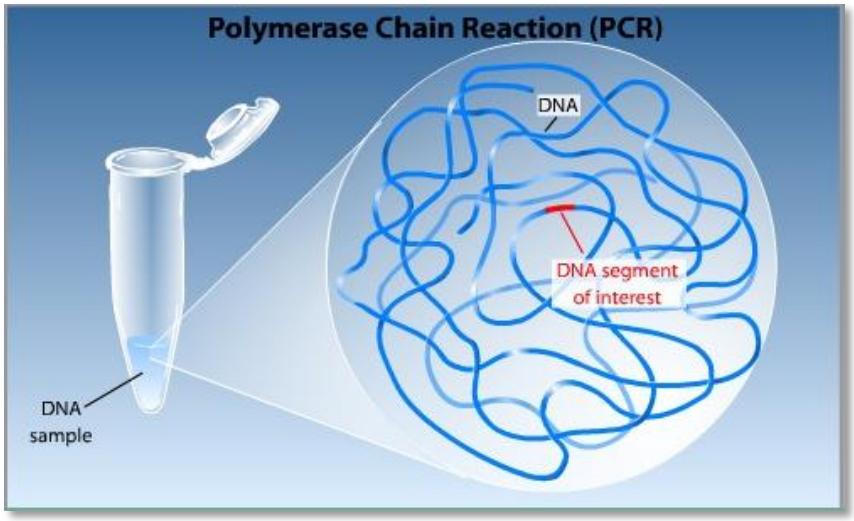
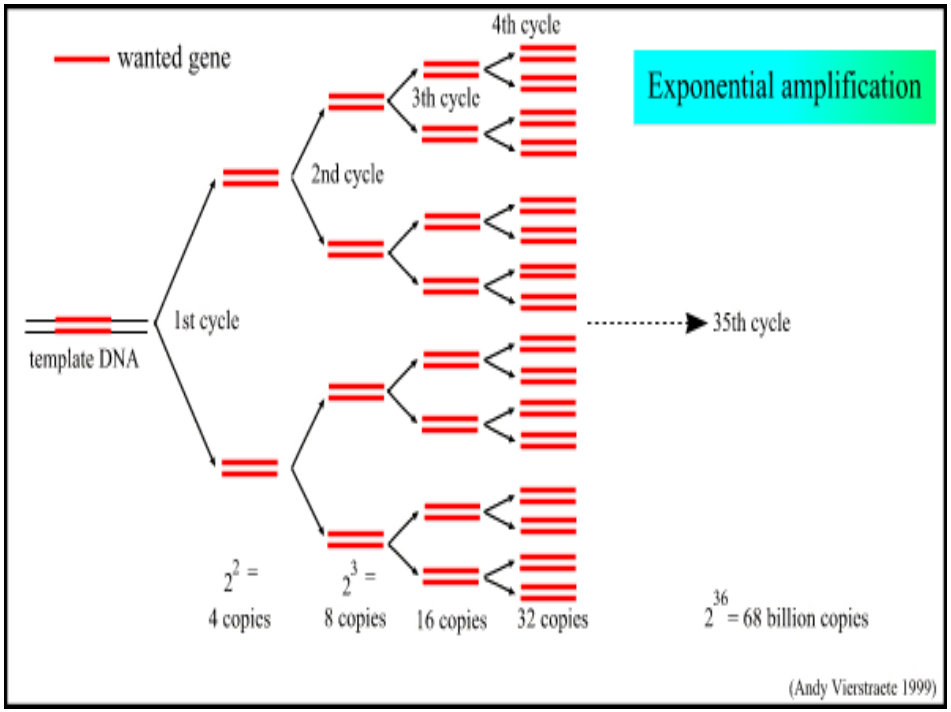
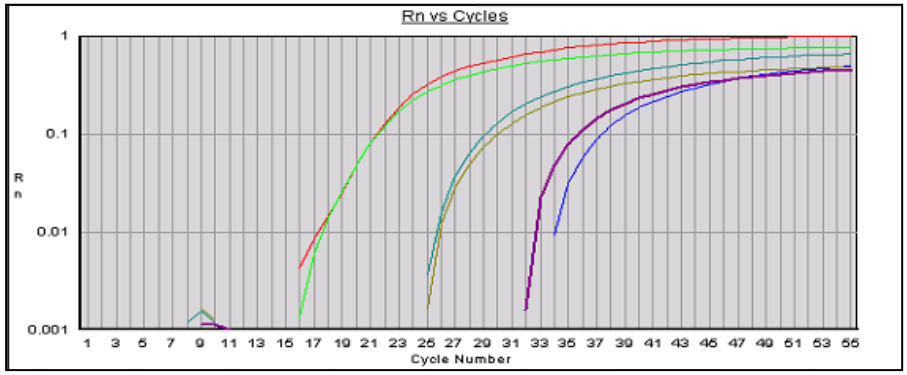
The target of the PCR : the DNA







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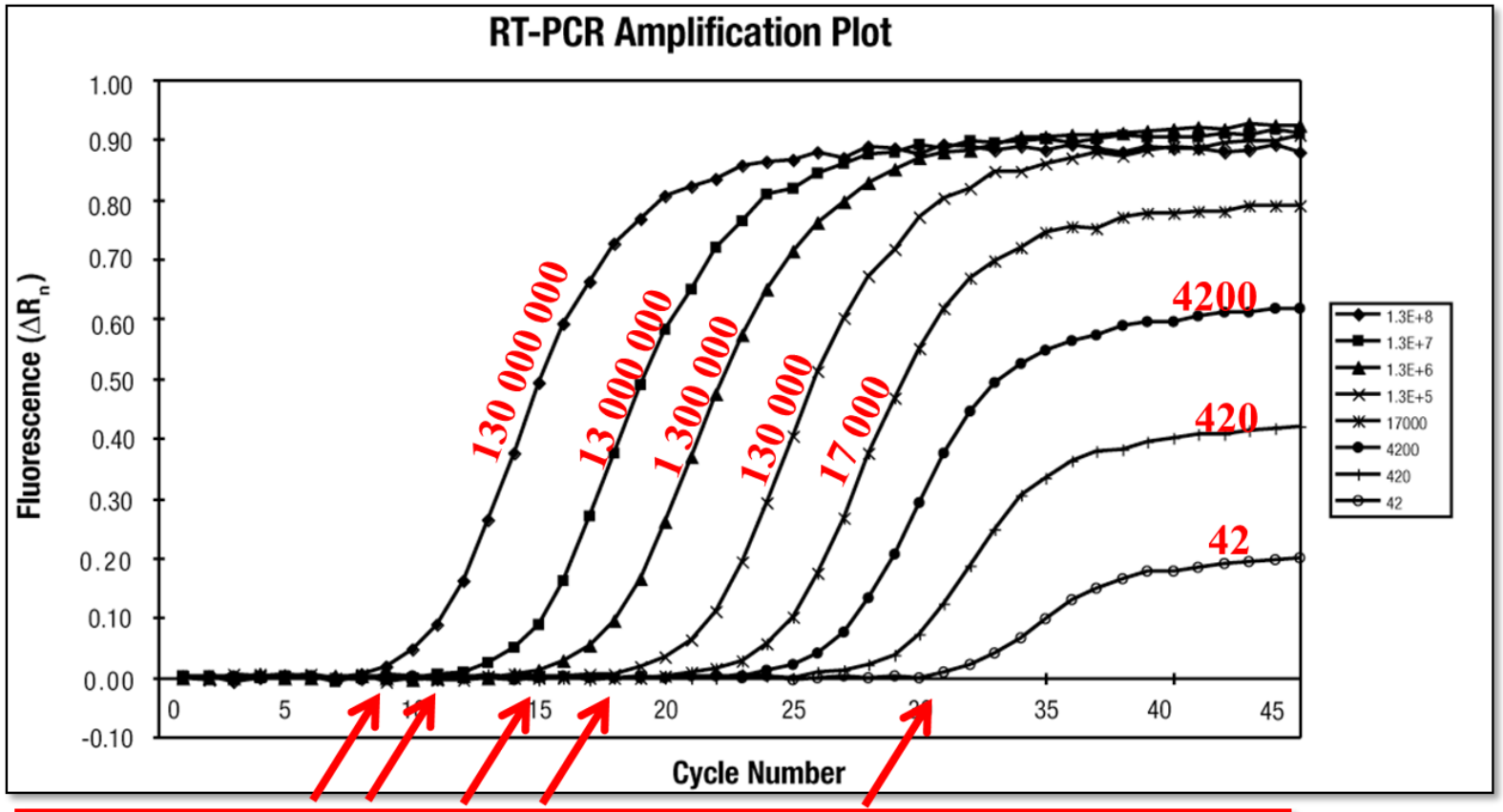
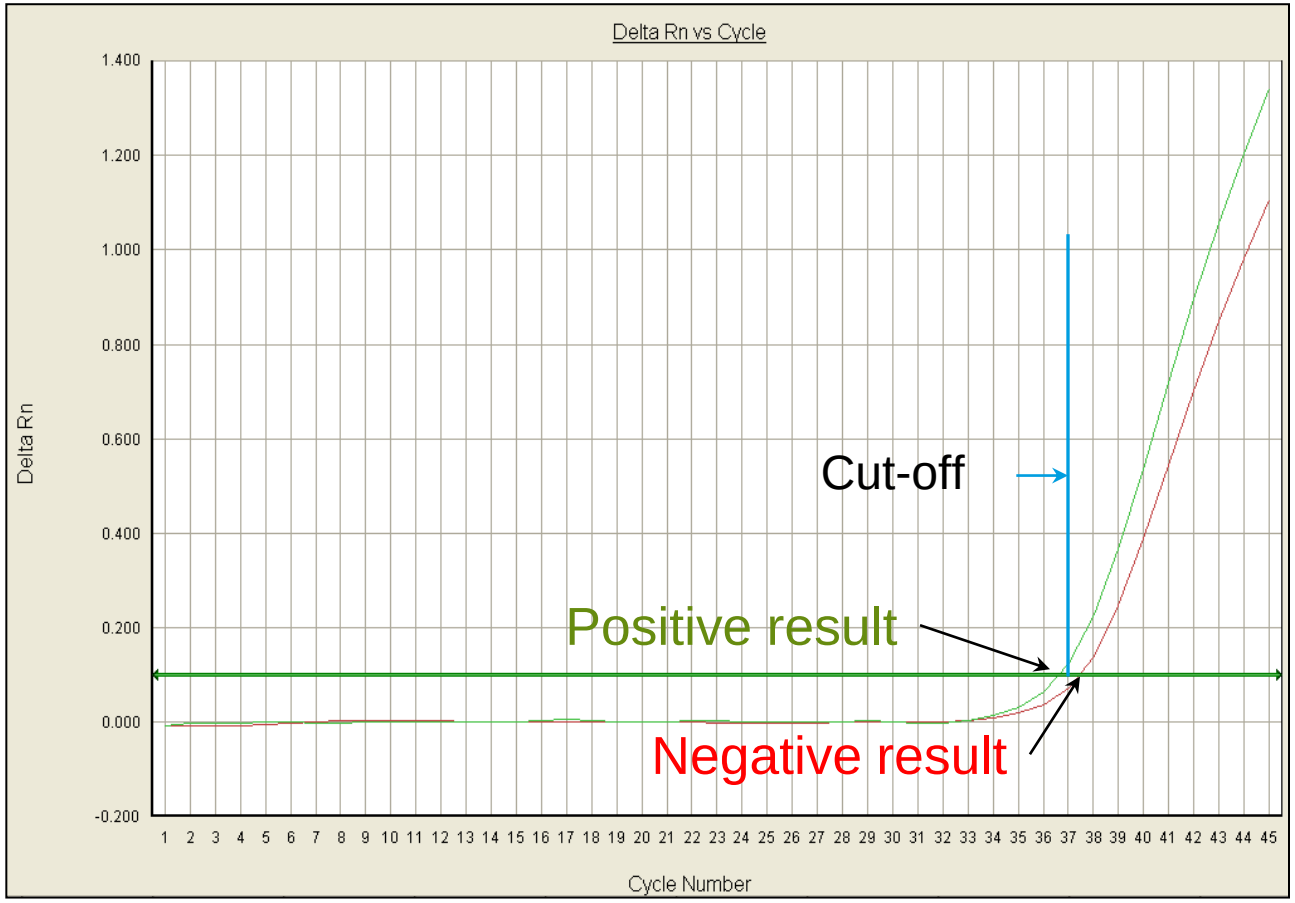
The PCR result interpretation



Copies of the target in the reaction ⇗ - earlier amplification signal

The PCR result interpretation



Problem:
The cut-off is specific of a PCR platform

Protocol to determine the cut –off described in a SOP

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PCR advantages and drawbacks

- *Species* or *taxa* identification (e.g. ruminant, pig,…)
- Very *sensitive* (~ 0.1%)
- *Common* technique
- Able to detect *DNA degraded* by heating processes

- *Indirect* detection
- Not able to determine the *source* of the DNA (e.g. milk vs bovine PAP)
- Only *qualitative*…!
- *Trained people*
- *Specific and costly equipment*

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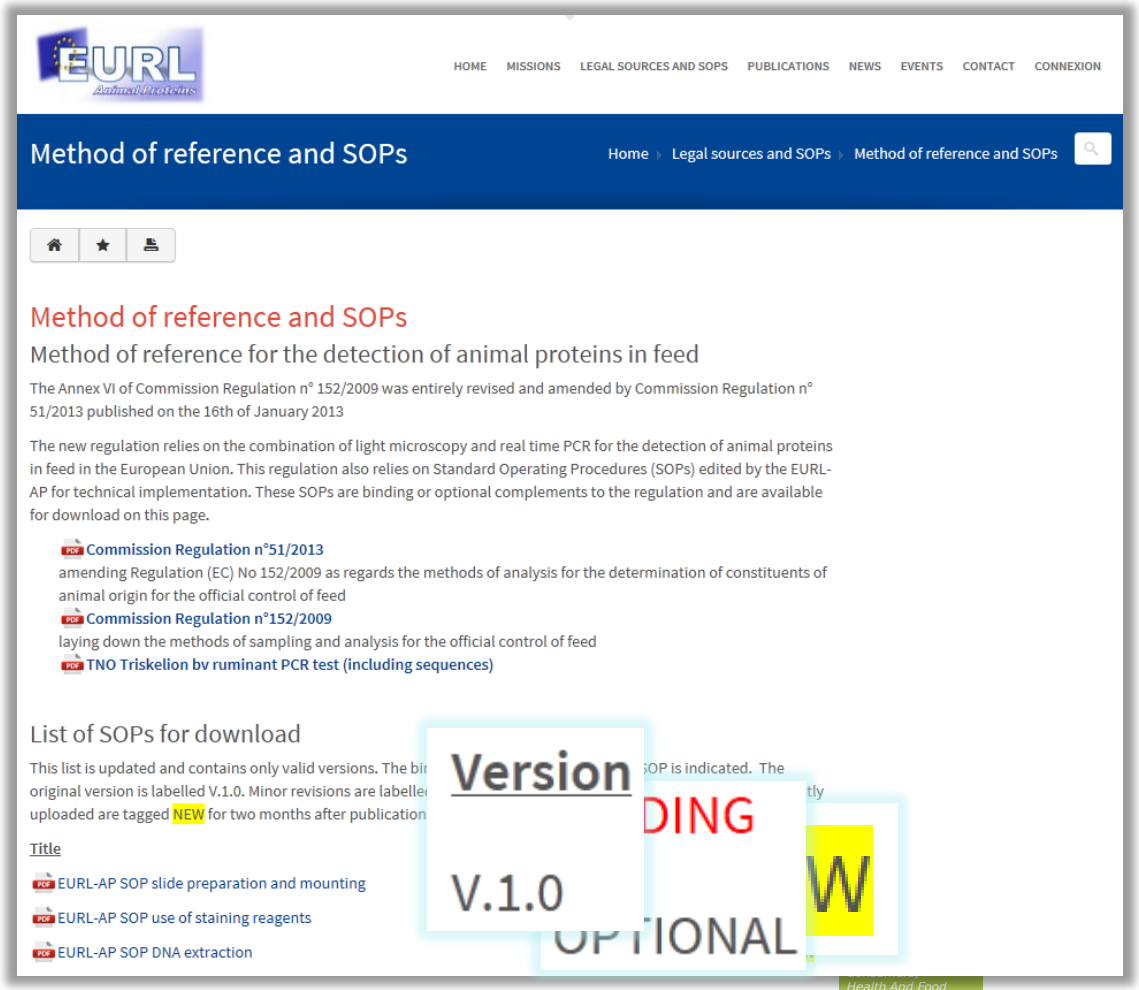
Legal framework

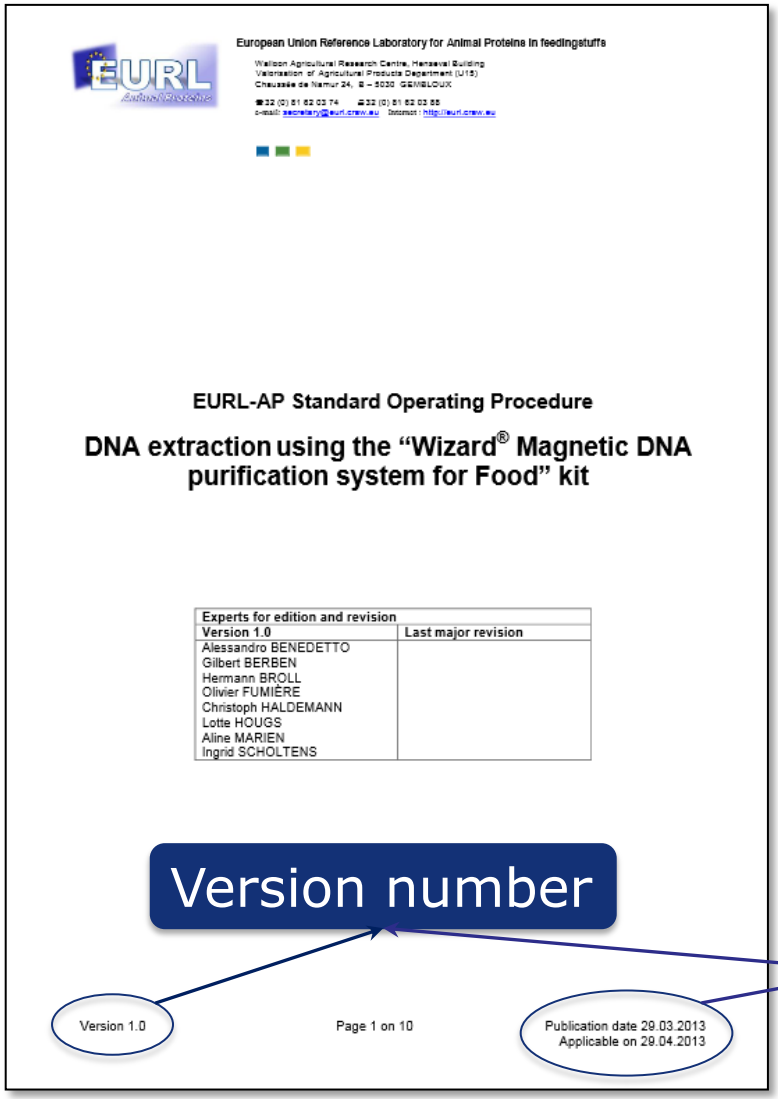
- Legislation
  - *Commission Regulation 51/2013*
  - *Annex VI to Commission Regulation (EC) No 152/2009 as lastly amended by Commission Regulation (EU) No 51/2013*
- Standard Operating Procedures
  - *Complements to the regulations*
  - *Better flexibility in case of changes in the protocols*

SOPs : where ?



To download from EURL-AP Website

SOPs



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EURL-AP Standard Operating Procedure

DNA extraction using the "Wizard® Magnetic DNA purification system for Food" kit

Experts for edition and revision	
Version 1.0	**Last major revision**
Alessandro BENEDETTO Gilbert BERBEN Hermann BROLL Olivier FUMIÈRE Christoph HALDEMANN Lotte HOUGS Aline MARIEN Ingrid SCHOLTENS	

Version number

Version 1.0

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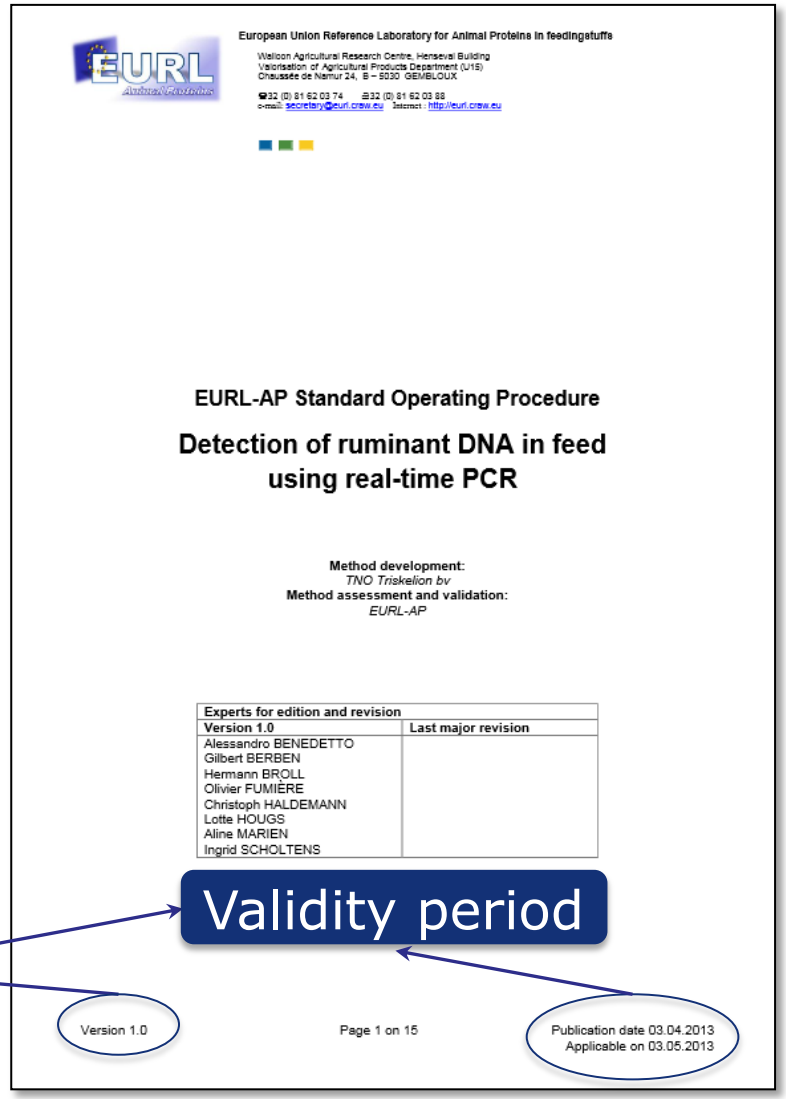
Applicable on 29.04.2013



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EURL-AP Standard Operating Procedure

Detection of ruminant DNA in feed using real-time PCR

Method development:
TNO Triskelion bv
Method assessment and validation:
EURL-AP

Experts for edition and revision	
Version 1.0	**Last major revision**
Alessandro BENEDETTO Gilbert BERBEN Hermann BROLL Olivier FUMIÈRE Christoph HALDEMANN Lotte HOUGS Aline MARIEN Ingrid SCHOLTENS	

Validity period

Version 1.0

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Publication date 03.04.2013
Applicable on 03.05.2013

DNA extraction

- *Binding complement of the legislation*
- *kit "Wizard ® Magnetic DNA purification system for Food" (Promega, Madison, WI, USA - www.promega.com)*
- *Validated method*

  ⇨ *No other DNA extraction method is allowed*
- *Two test portions per sample ⇨ 2 independent DNA extracts*
- *Controls to validate the extraction step*
  - *Positive DNA extraction control*
  - *Extraction blank control*
- *Two protocols*
  - *Manual*
  - *Semi-automated*

Ruminant PCR

- *Binding complement of the legislation*
- *Real-time PCR procedure*
- *Nuclear multicopy target developed by TNO Triskelion bv*
- *Validated method*

  ⇨ *No other PCR method is allowed*
- *Master mix ⇨ list of approved master mixes*
- *Controls to validate the PCR step*

  Positive PCR control

  PCR blank control
- *Rules of interpretation of the results*

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Analytical methods of PAPs detection

3. Operational schemes for the combination of light microscopy and PCR

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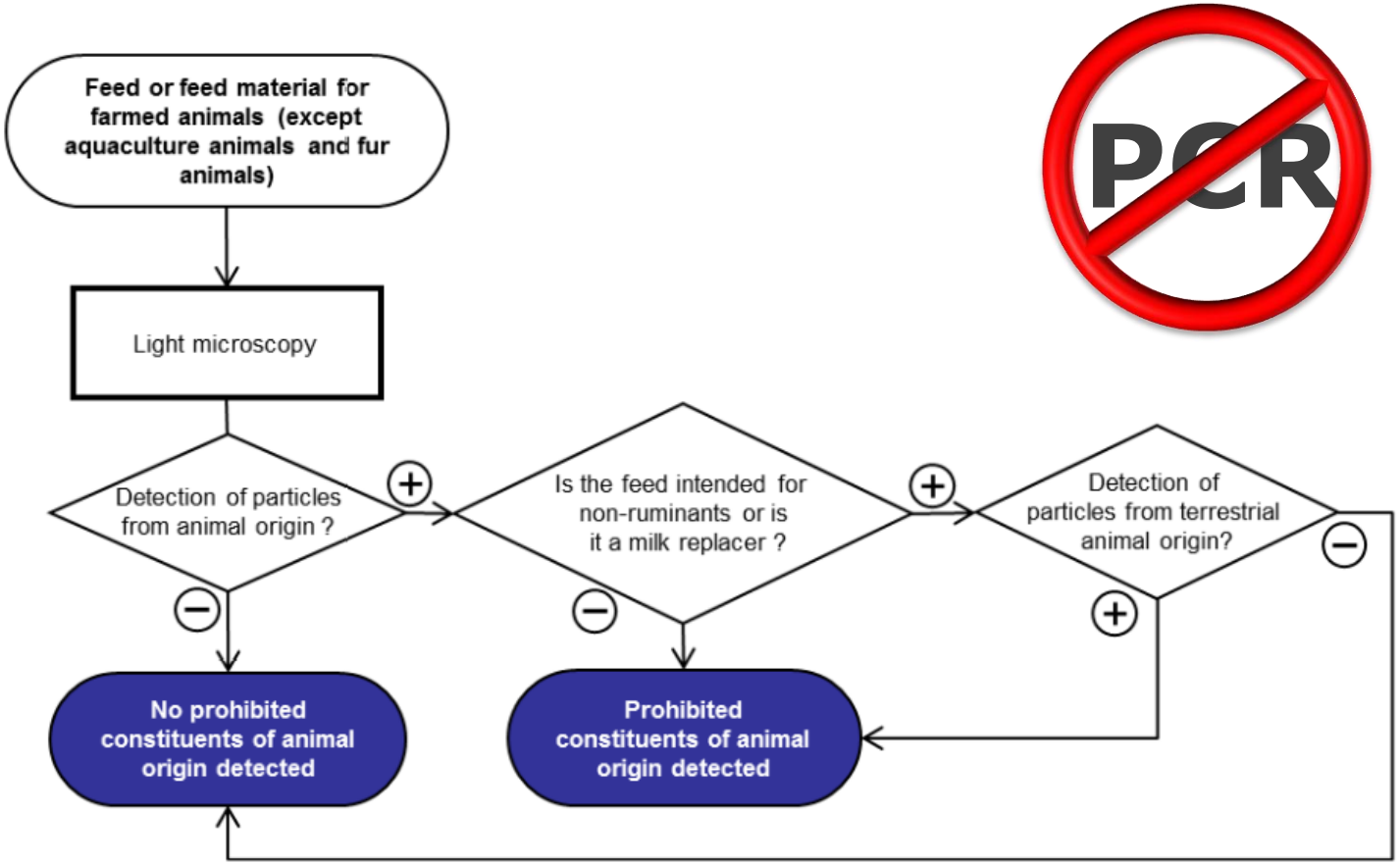
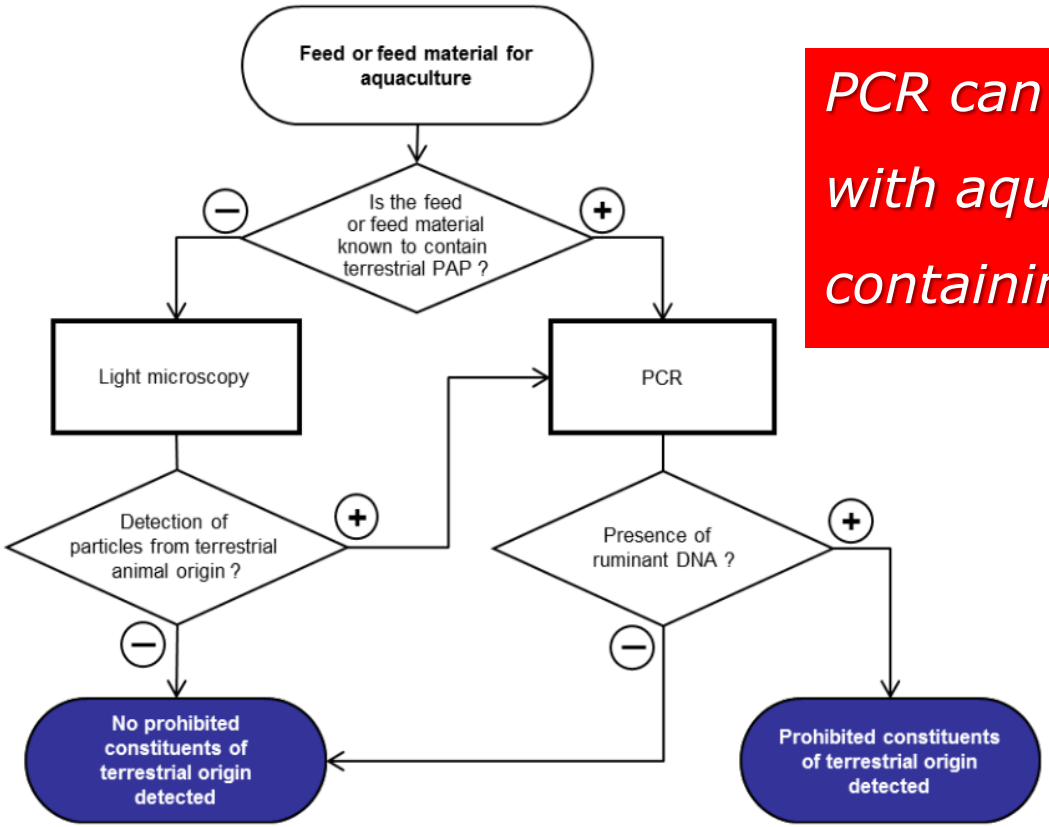
1. Feed or feed material intended for farmed animals other than aquaculture animals and fur animals



2. Feed or feed material intended for aquaculture animals



PCR can be used only with aquafeed and aquafeed material containing terrestrial PAP

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