



Better Training for Safer Food *Initiative*

Detection of PAPs by light microscopy and PCR

Pascal Veys and Olivier Fumière

Methods of PAPs detection : back in time

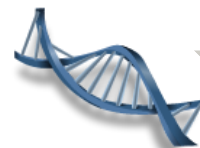


Commission Directive 88/1998 establishing guidelines for the microscopic identification and estimation of constituents of animal origin for the official control of feedingstuffs

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

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





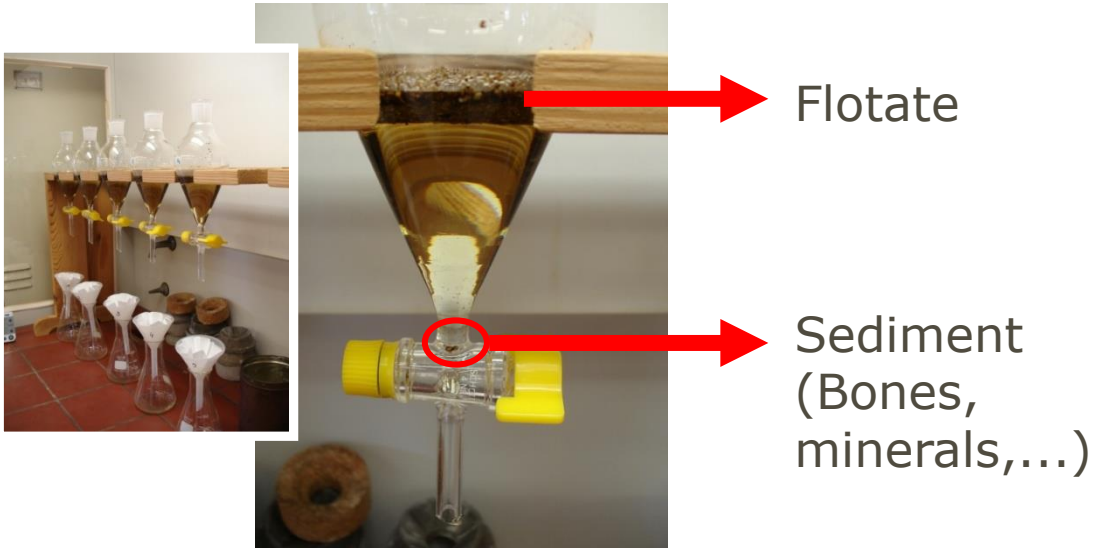
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Analytical methods of PAPs detection

1. Light microscopy

Principle

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



Principle

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



Staining can be used :

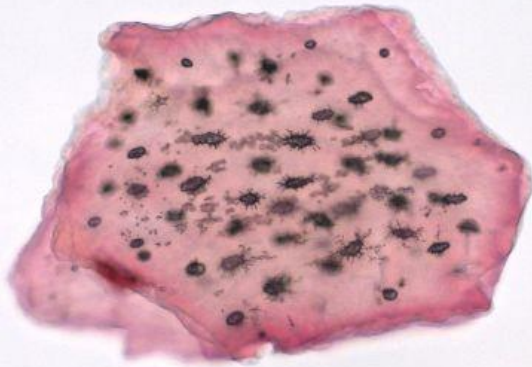
- Alizarin Red → bones, scales
- Cystine reagent → hairs, feathers

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



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Identification : Terrestrial <> Fish



100 μm

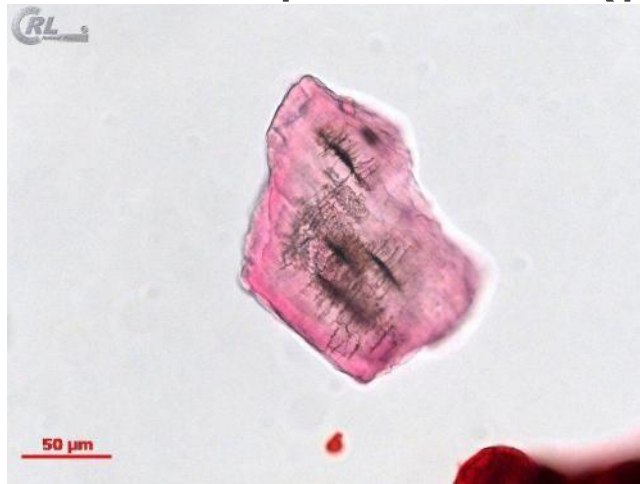


100 μm



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Expertise... (plant or animal ?)



Microscopic examination (I)

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

2. Number of slides



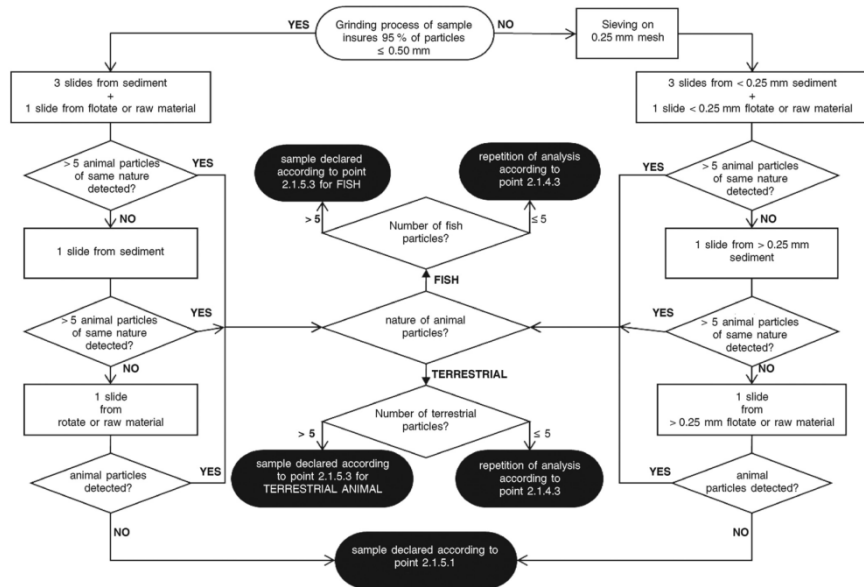
3. Sequence of observations of slides ?
 - cfs. diagrams
 - use of stereomicroscope = optional
 - **strictly** respect diagrams !



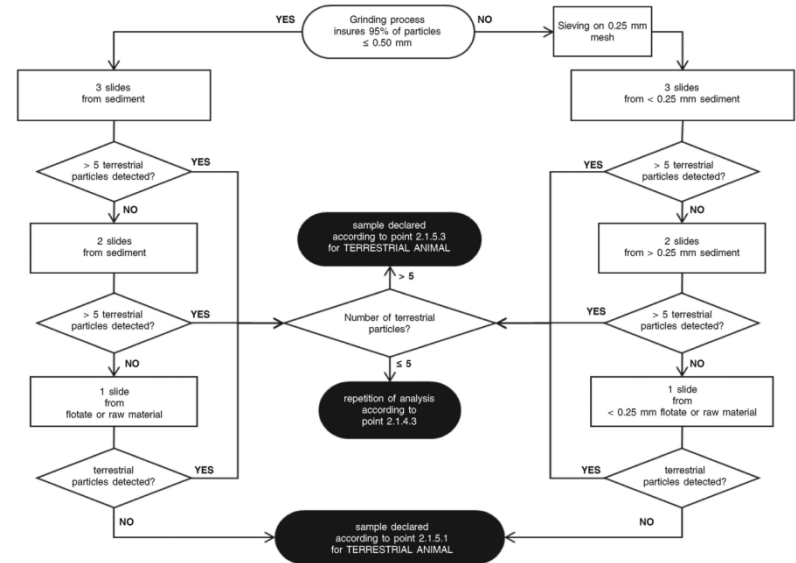
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Microscopic examination (II)

Compound feed & feed materials



Fishmeals



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)

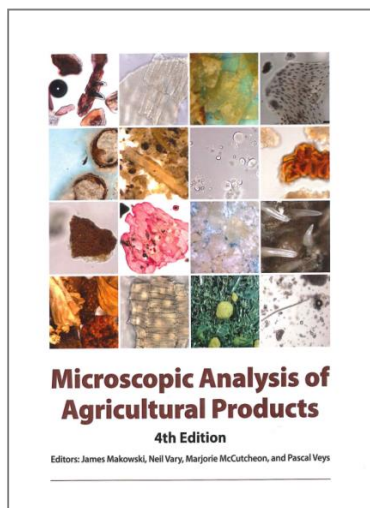
report *shall* mention:

1. Type of material
 - sediment,
 - flotage 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...!

Analytical methods of PAPs detection

2. The PCR (Polymerase Chain Reaction)

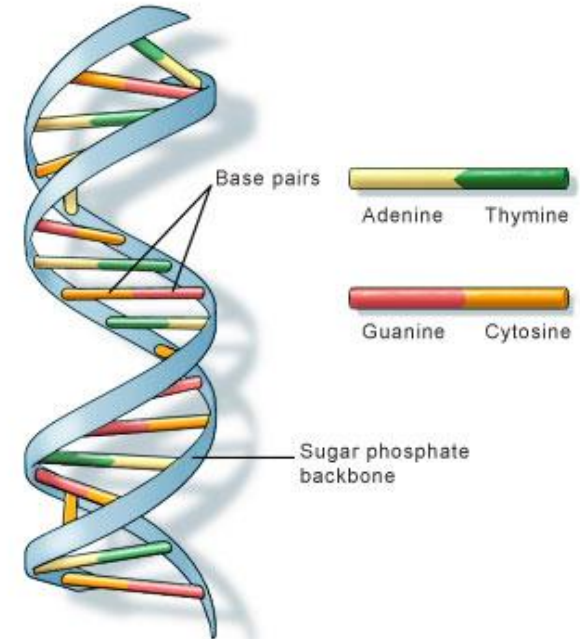
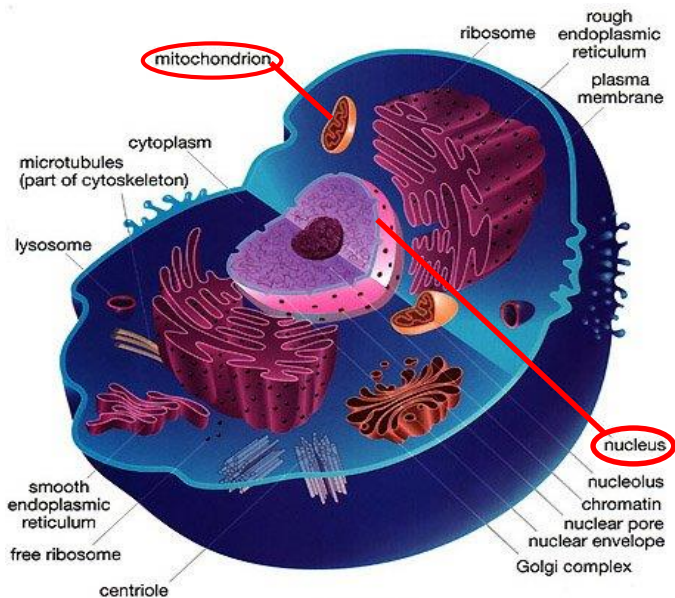


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The target of the PCR : the DNA

The DNA = desoxyribonucleic acid

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

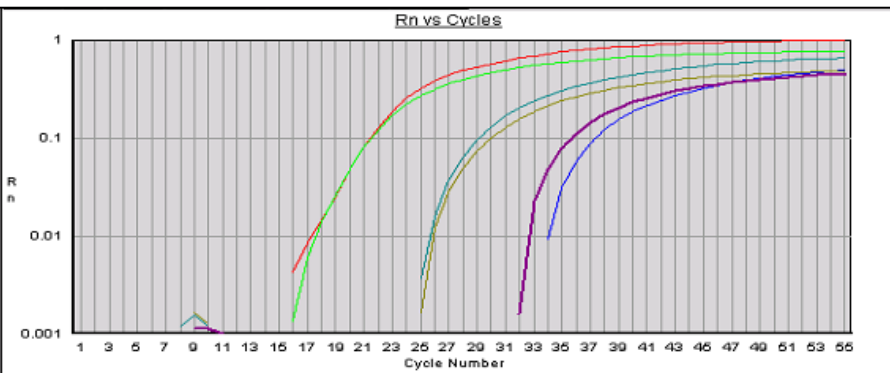
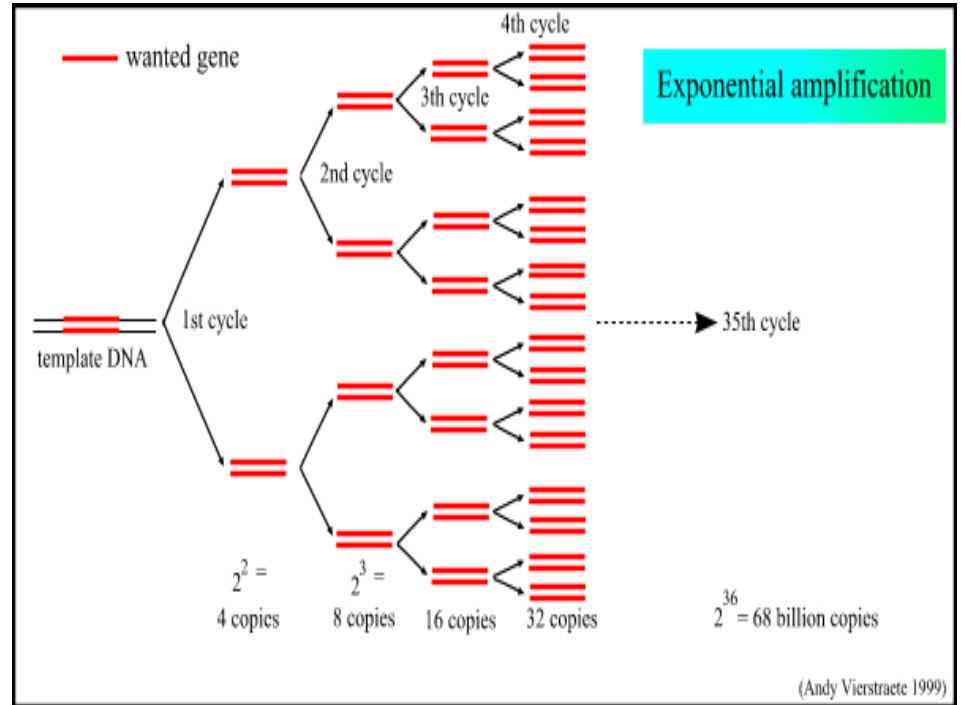
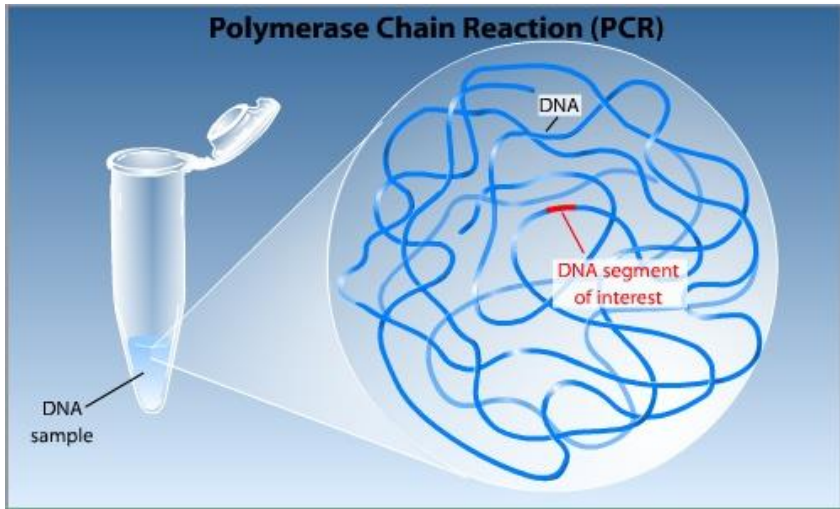


U.S. National Library of Medicine



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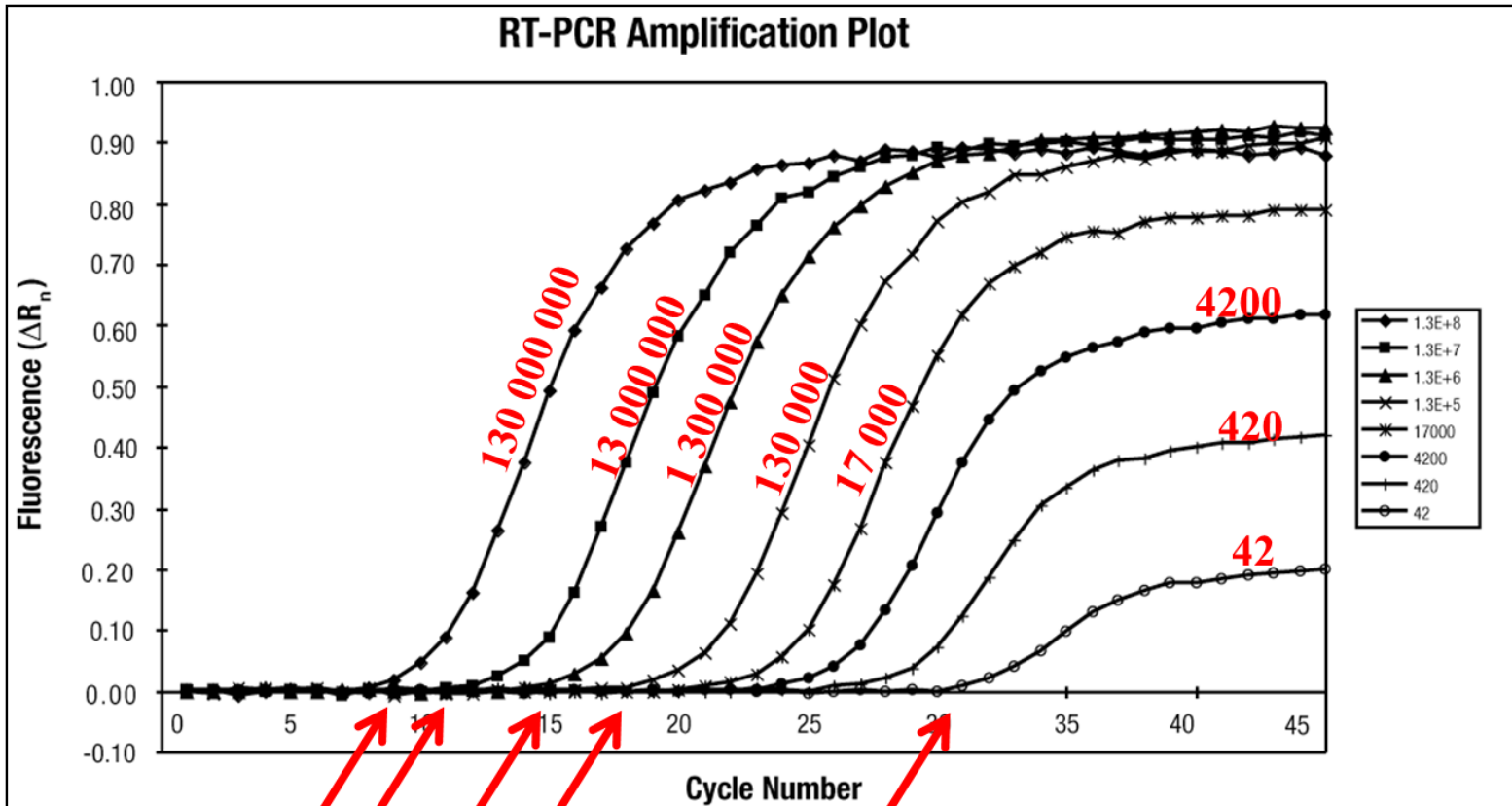
The target of the PCR : the DNA





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The PCR result interpretation

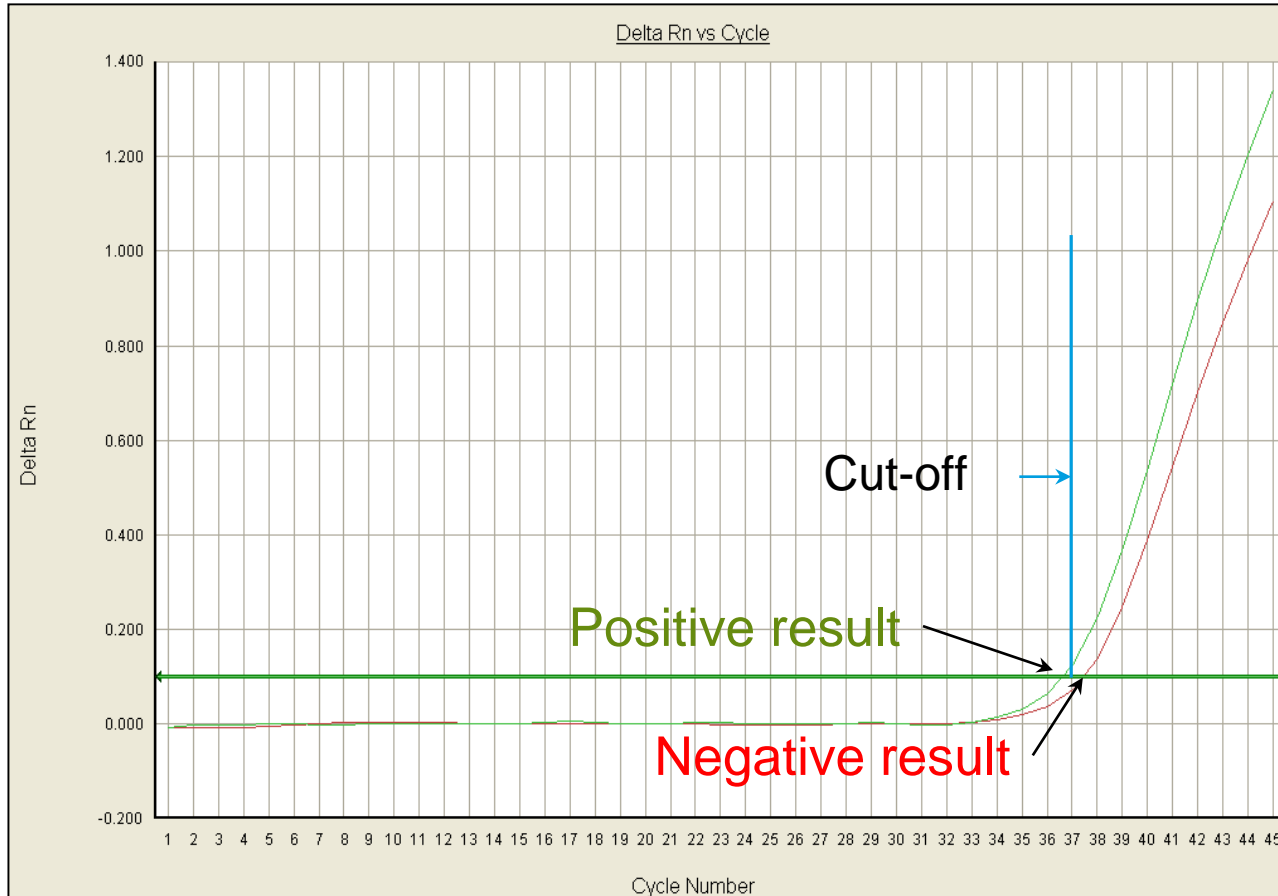


Copies of the target in the reaction ↗ - earlier amplification signal



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The PCR result interpretation



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

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*



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*



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SOPs : where ?

To download from
EURL-AP Website

The screenshot shows the EURL-AP website interface. At the top left is the EURL logo with the text 'Animal Proteins'. A navigation menu includes 'HOME', 'MISSIONS', 'LEGAL SOURCES AND SOPS', 'PUBLICATIONS', 'NEWS', 'EVENTS', 'CONTACT', and 'CONNEXION'. The main header area displays 'Method of reference and SOPs' with a search icon. Below this is a sub-header 'Method of reference and SOPs' and a breadcrumb trail: 'Home > Legal sources and SOPs > Method of reference and SOPs'. The main content area features a title 'Method of reference and SOPs' and a subtitle 'Method of reference for the detection of animal proteins in feed'. A paragraph explains that the Annex VI of Commission Regulation n° 152/2009 was revised by Commission Regulation n° 51/2013. Another paragraph states that the new regulation relies on light microscopy and real time PCR. A list of links for download is provided, including 'Commission Regulation n°51/2013', 'Commission Regulation n°152/2009', and 'TNO Triskelion by ruminant PCR test (including sequences)'. A section titled 'List of SOPs for download' includes a table with columns for 'Version' and 'Title'. The table lists three SOPs: 'EURL-AP SOP slide preparation and mounting', 'EURL-AP SOP use of staining reagents', and 'EURL-AP SOP DNA extraction'. A large, semi-transparent graphic with the text 'Version', 'OPTIONAL', and 'W' is overlaid on the bottom right of the screenshot.



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SOPs

EURL
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☎ 32 (0) 81 62 03 74 ☎ 32 (0) 81 62 03 88
e-mail: secretary@eurl.craw.eu Internet: <http://eurl.craw.eu>

■ ■ ■ ■

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 FUMIERE Christoph HALDEMANN Lotte HOUQS Aline MARIEN Ingrid SCHOLTENS	

Version number

Version 1.0 Page 1 on 10 Publication date 29.03.2013
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 Triskellon 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 FUMIERE Christoph HALDEMANN Lotte HOUQS Aline MARIEN Ingrid SCHOLTENS	

Validity period

Version 1.0 Page 1 on 15 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*

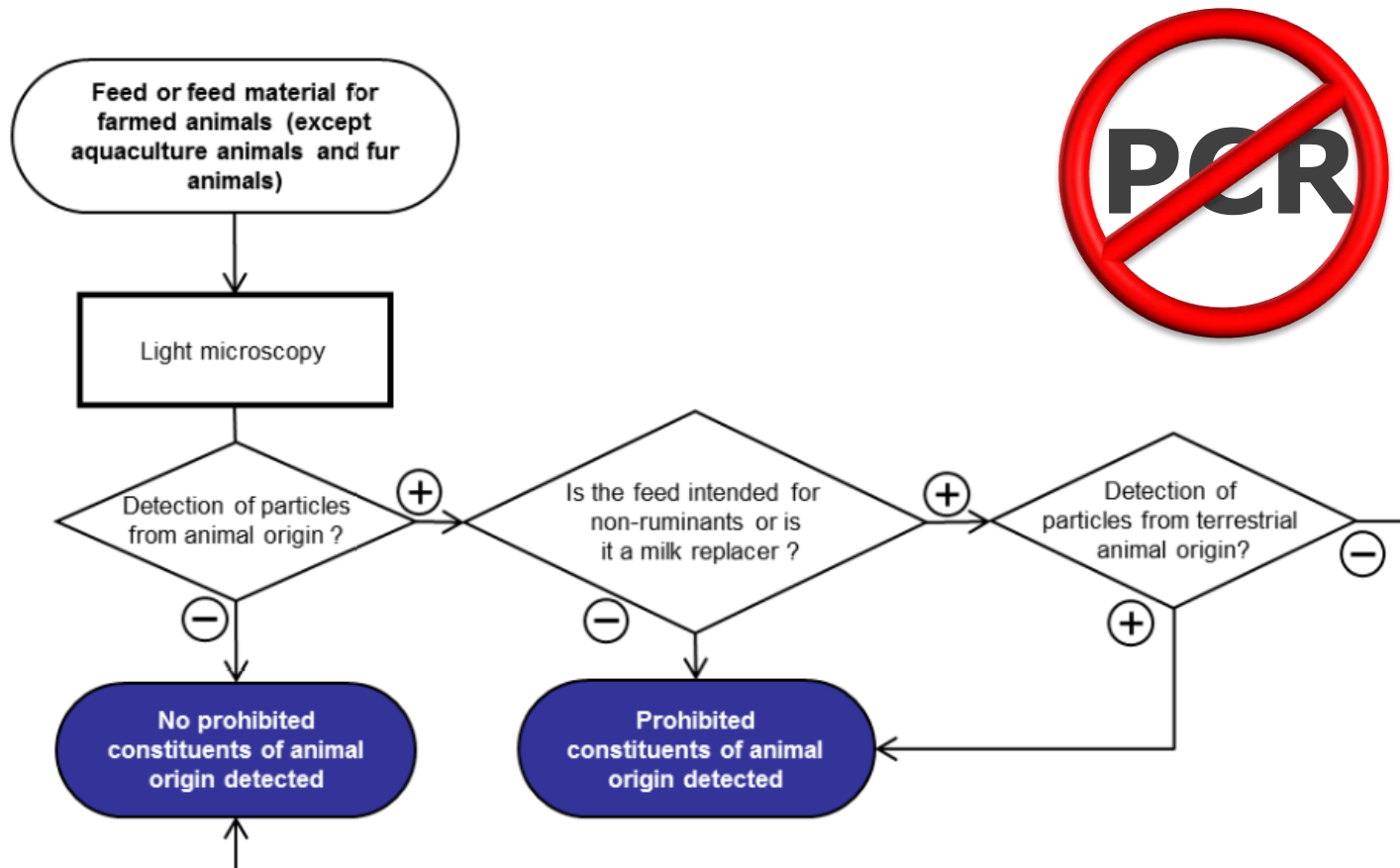
Ruminant PCR

- *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*

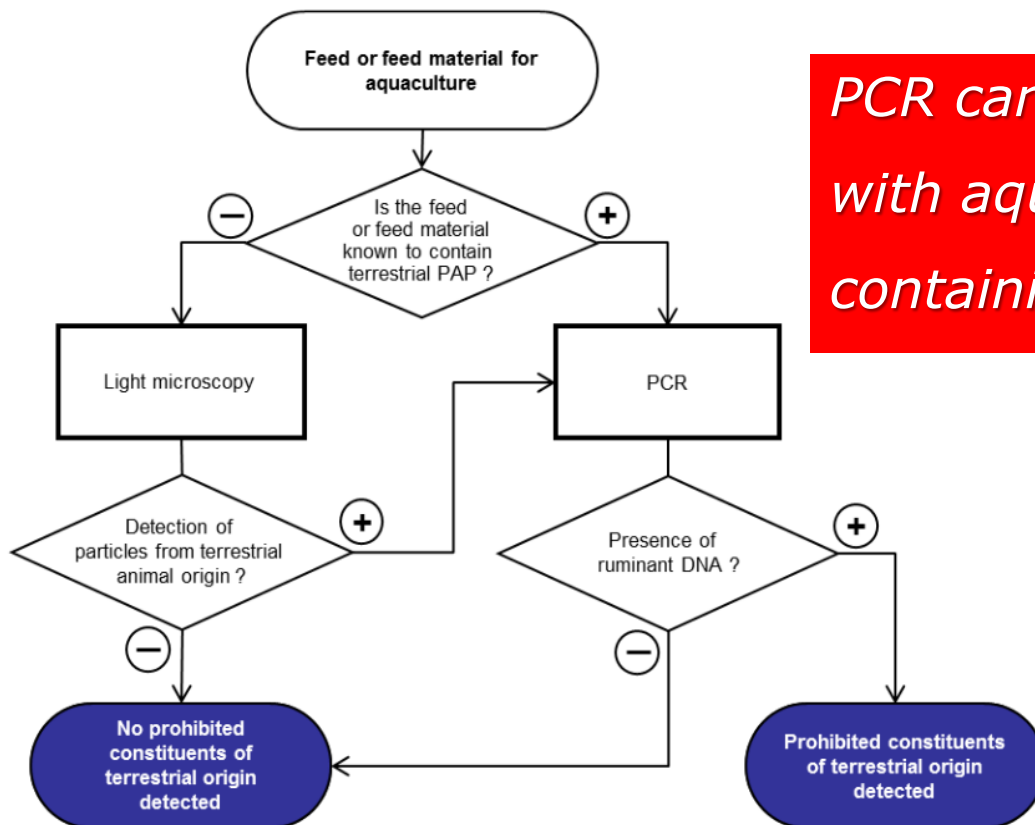
Analytical methods of PAPs detection

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

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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Better Training for Safer Food BTSF

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