# Methods verification

Transfer of validated methods into laboratories working routine

1. Introduction

2. Definitions and differences
validation – verification

3. How to perform verification in
GMO detection laboratories

# Regulation (EC) No 882/2004 Recital 17

## General obligations with regard to the analysis of official samples

Laboratories involved in the analysis of official samples should work in accordance with internationally approved procedures or criteria-based performance standards and use methods of analysis that have, as far as possible, been validated.

VALIDATED METHODS are needed !!!

# Regulation (EC) No 882/2004
# Article 12
## General obligations with regard to the analysis of Official samples

Laboratories involved in the analysis of official samples should operate and should be accredited in accordance with the

EN ISO/IEC 17025 on ‘General requirements for the competence of testing and calibration laboratories’

Accreditation of laboratories is mandatory !!!

# ISO/IEC 17025:2005
# General requirements for the competence of testing and calibration laboratories

5.4.2 Selection of methods

5.4.5 Validation of methods

# Advantages of validated methods

Providing

- reliable and accurate test results

- comparable results between different laboratories

requirement for the accreditation according to ISO/IEC 17025 Chapter 5.4.5 ‘validation of methods’

# ISO/IEC 17025: 2005

## Section 5.4.2 Selection of methods

1. Methods published in international, regional or national standards shall (i.e. must) preferably be used.
Latest valid edition

2. Published by
   reputable technical organisations,
   in relevant scientific texts or journals or
   as specified by the manufacturer of the equipment

3. Laboratory-developed methods or methods adopted by the laboratory

# Regulation (EC) No 882/2004 Article 11

## Methods of sampling and analysis

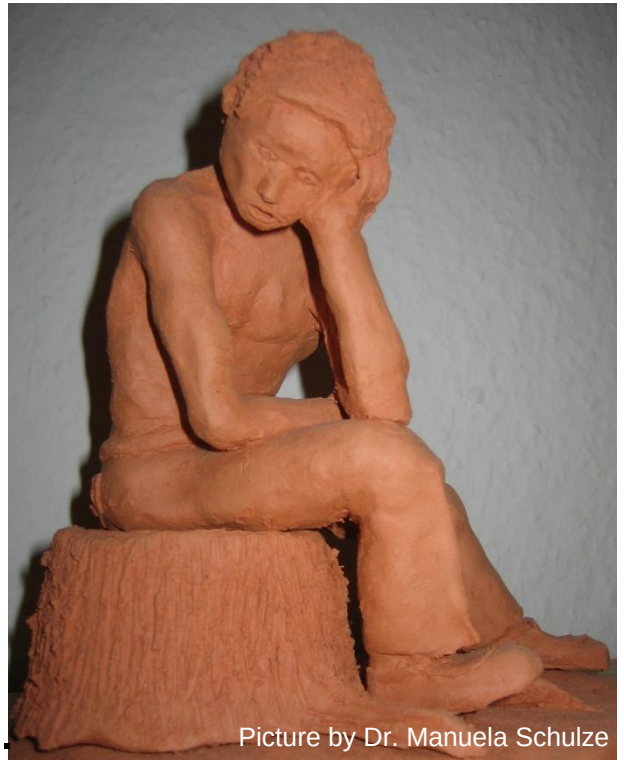
1. Sampling and analysis methods used in the context of official controls shall comply with relevant Community rules or,
(a) if no such rules exist, with internationally recognised rules or protocols,
for example those that the European Committee for Standardisation (CEN) has accepted or those agreed in national legislation;
or,
(b) in the absence of the above, with other methods fit for the intended purpose or developed in accordance with scientific protocols.

1. Methods fixed in legislation
2. ISO methods / CEN methods
3. Other appropriate methods (“fit for purpose”)

# Regulation (EC) No 882/2004
# Annex III

## CHARACTERISATION OF METHODS OF ANALYSIS

1. Methods of analysis should be characterised by the following criteria:
(a) accuracy;
(b) applicability (matrix and concentration range);
(c) limit of detection;
(d) limit of determination;
(e) precision;
(f) repeatability;
(g) reproducibility;
(h) recovery;
(i) selectivity;
(j) sensitivity;
(k) linearity;
(l) measurement uncertainty;
(m) other criteria that may be selected as required.


Picture by Dr. Manuela Schulze

# Validation
# (ISO 17025, 5.4.5.3 note 3)

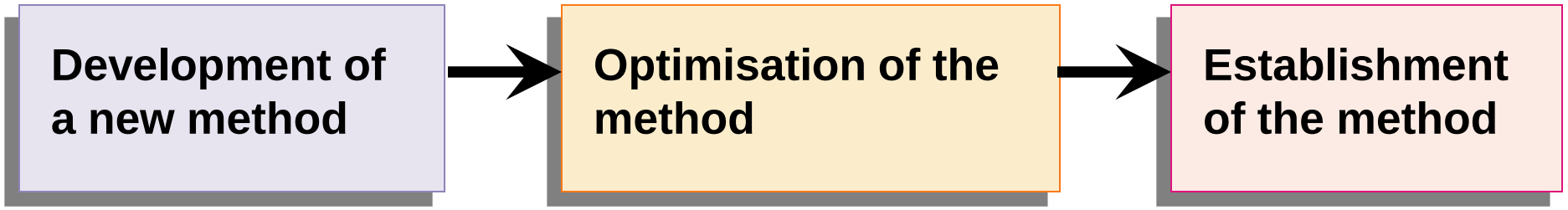
Validation is always a balance between

costs,
risks and
technical possibilities.

There are many cases in which the range and uncertainty of the values can only be given in a simplified way due to lack of information.

# Method validation

**Method validation is a process**



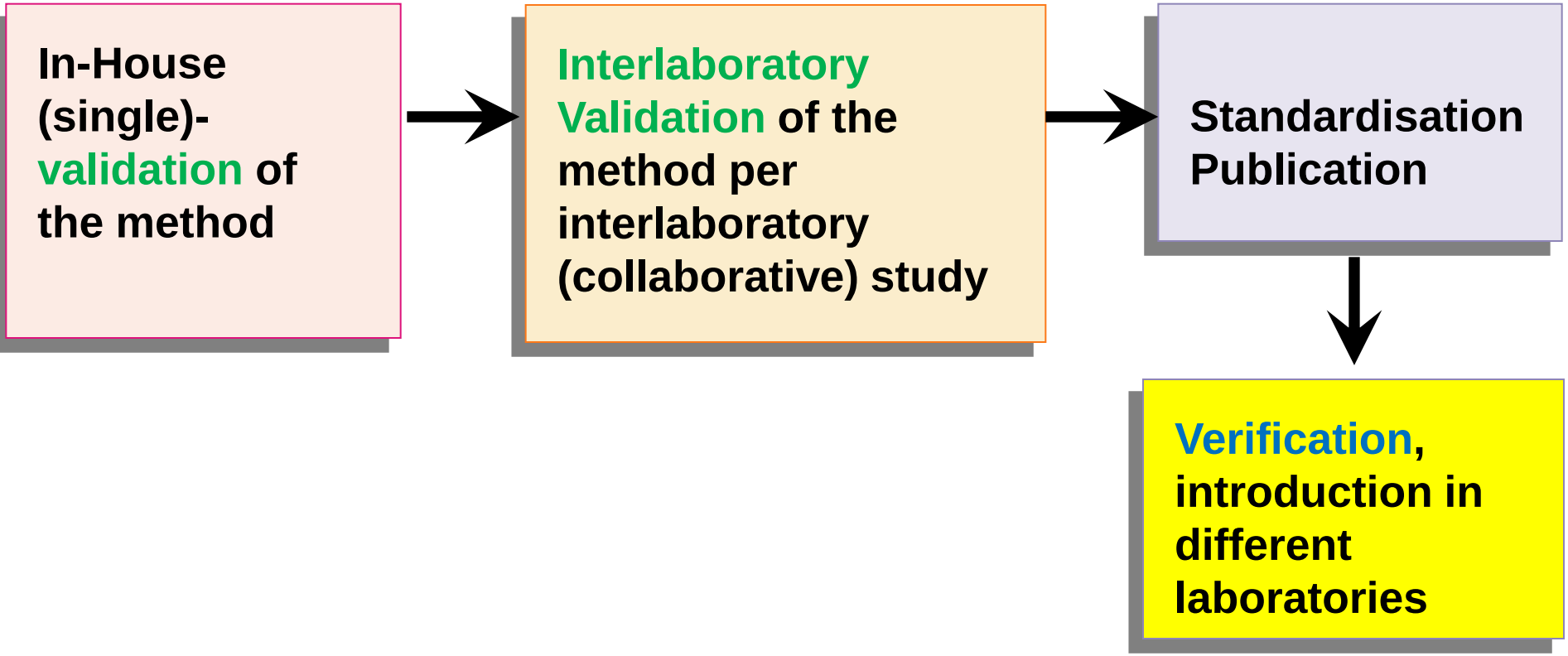
# HARMONIZED GUIDELINES FOR SINGLE LABORATORY VALIDATION OF METHODS OF ANALYSIS

**(IUPAC Technical Report)**

*Prepared for publication by*
MICHAEL THOMPSON, STEPHEN L. R. ELLISON AND ROGER WOOD
*In: Pure Appl. Chem., Vol. 74, No. 5, pp. 835–855, 2002.*

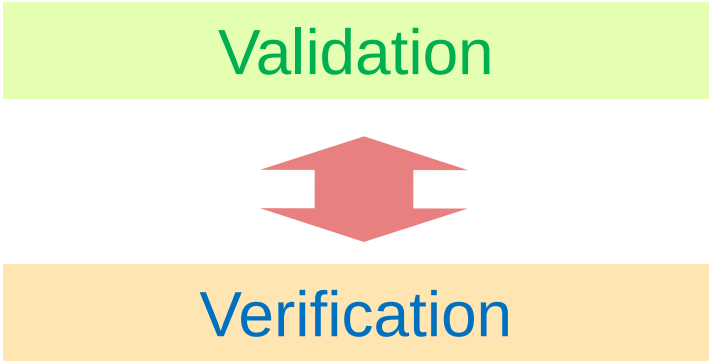

Guideline which provide minimum recommendations on procedures that should be employed to ensure adequate validation of analytical methods.

**Method validation is a process**



# Definitions (1)

Validation is the confirmation by examination and provision of objective evidence that the
(ISO 9000:2005 section 3.8.4) ...requirements for a specific intended use or application have been fulfilled
(ISO 17025:2005 section 5.4.5.1)... particular requirements for a specific intended use are fulfilled




Verification is the confirmation, through the provision of objective evidence that specific requirements have been fulfilled (ISO 9000:2005 section 3.8.4)

# Definitions (2)

Validation

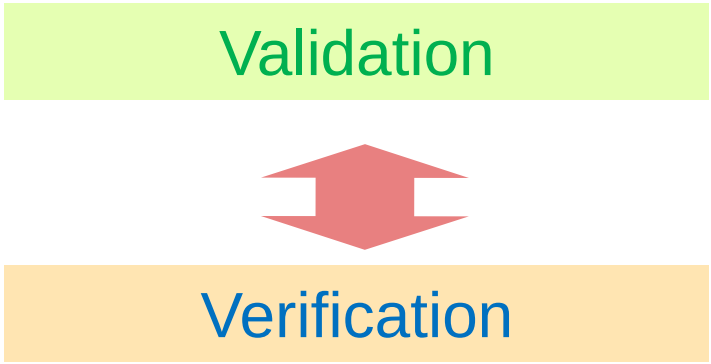
Verification

“Full” validation “

usually by interlaboratory method performance study (collaborative study)

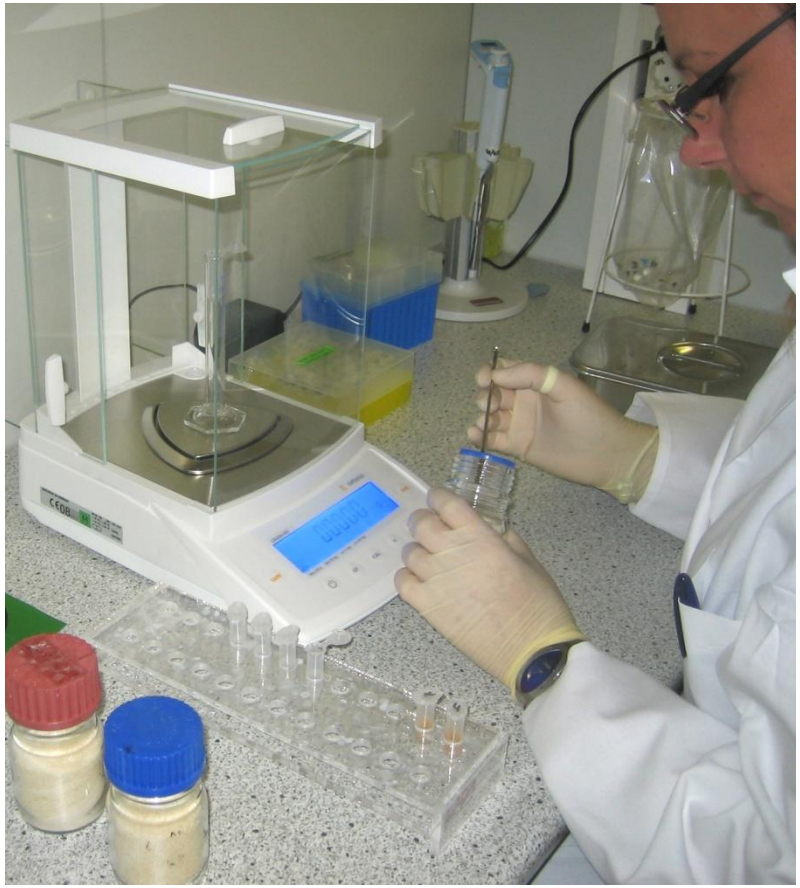
A laboratory using a collaboratively studied method, which has been found to be fit for the intended purpose, needs only to demonstrate that it can achieve the performance characteristics stated in the method by verification.

# Definitions (3)




Validation and verification apply
to a defined protocol
for the determination of a specified analyte
for a specific range of concentrations
in a particular type of test material
for a specific purpose

# Validation / Verification



Realistic operation situations

- matrix
- analyte concentration
- staff
- equipment
- reagents
- environment

# Verification

Verification is applicable only for standard/accepted methods which have been validated before.

The laboratory has to

- demonstrate that the performance parameters specified in the method have been met with the matrices to which the method is being applied.

Most often the critical parameter are the trueness and the precision (ALACC Guide 2007).

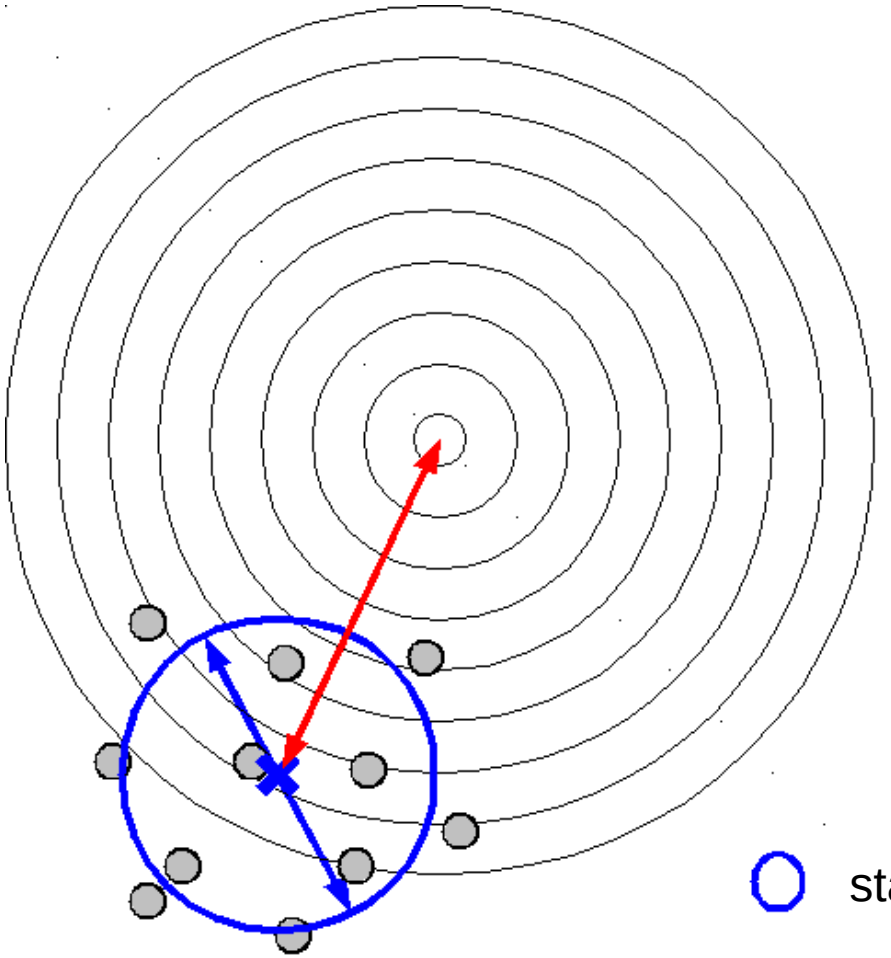
# Definitions

accuracy

trueness and precision

Trueness: Closeness of agreement between the average value obtained from a series from test results and an accepted value

Accuracy: Closeness of agreement between a test result and the accepted reference value



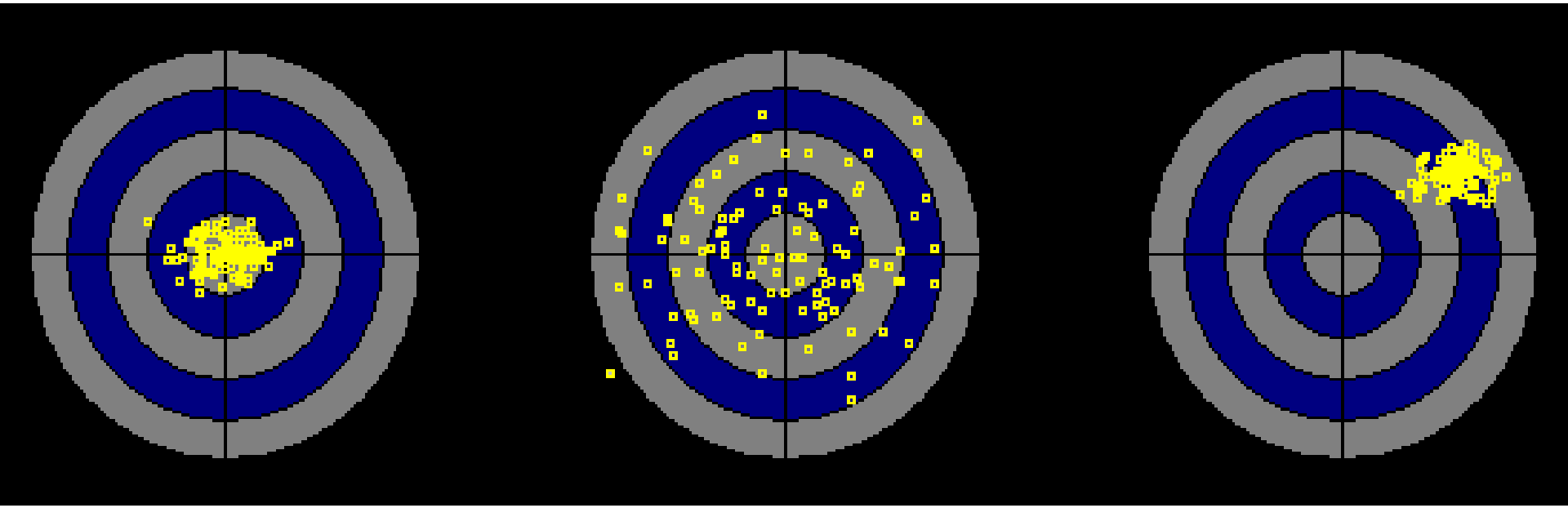
trueness (bias)

precision

average value

standard deviation

[Source: wikipedia.com]



high trueness
high precision

high trueness
low precision

low trueness
high precision

(note: no picture for low trueness and low precision)

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

Accuracy involves a combination of
random components and
a common systematic error or bias component.

The validation/verification parameter which have to be considered are dependent on

- the data already available.

The extent of validation/verification is dependent on

- the parameters which have already been tested.

Laboratory own methods need the “whole validation”.

Procedures and responsibilities for validation/verification have to be described in the quality management system of the laboratory and records about the process of validation/verification have to be kept.

# Strategy

Selection of method(s) needed

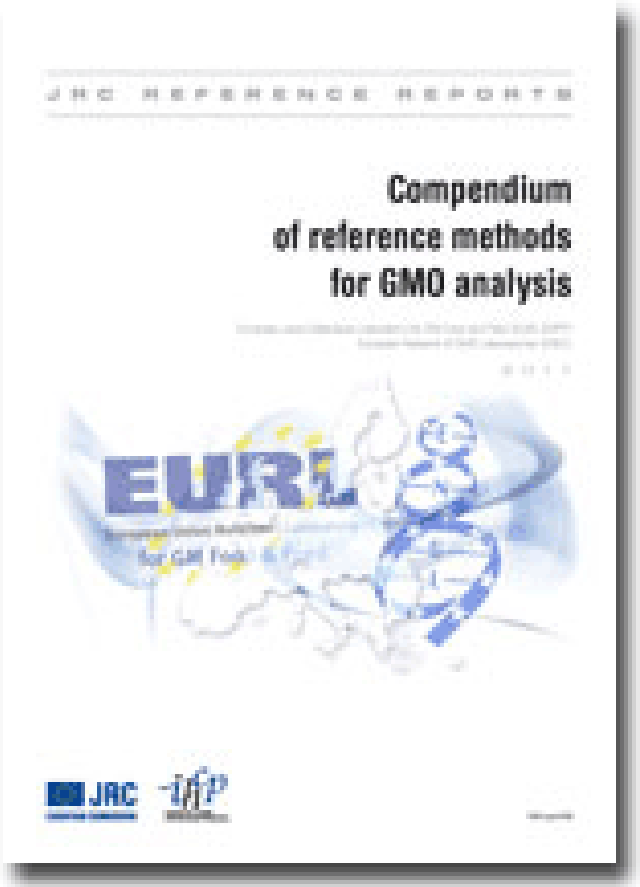
Check of available methods
- fit for purpose for the laboratory
- validation status of the methods

Consider the modular approach
- step by step
- extraction method
- PCR method
- real time PCR


Picture by Dr. Manuela Schulze

# Available methods

- Booklet: Compendium of reference methods for GMO analysis, developed by EURL-GMFF in collaboration with the ENGL



# On-line GMO detection method database

# http://gmo-jrl.jrc.ec.europa.eu/gmomethods/

The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) is responsible for GMO detection methods for food, animal feed and seeds in the EU´s authorisation process.

The EU-RL GMFF regularly publishes information on the status of validation dossiers on the internet. gmo-crl.jrc.ec.europa.eu/statusofdoss.htm

Last updated 21/09/2011 Watch this page for changes

| Event | Unique identifier | Applicant | Status/Progress | Reports | Validated Method |
|---|---|---|---|---|---|
| Bt10 maize | - | - | Validation completed | Validation report Published on: 13/07/2005 | Validated method Published on: 13/07/2005 |
| Bt11 sweet maize | SYN-BT011-1 | Syngenta Seeds | Validation completed | Validation report Published on: 05/08/2004 | Validated method Published on: 05/08/2004 |
| NK603 maize | MON-00603-6 | Monsanto | Validation | Validation | Validated |

Once the validation process is complete, the full validation report and the protocol of detection and quantification are published on the EU-RL GMFF web site.

# Preparation of the verification (1)

Prerequisite: An appropriate validated method is available!

1. Do you have the same matrix to analyse?

Extraction: Sometimes different materials from the same plant need additional steps


e.g. potato leaf: CTAB extraction method without CTAB precipitation step works

potato tuber: a CTAB precipitation step should be included

# Preparation of the verification (2)

Prerequisite: An appropriate validated method is available!

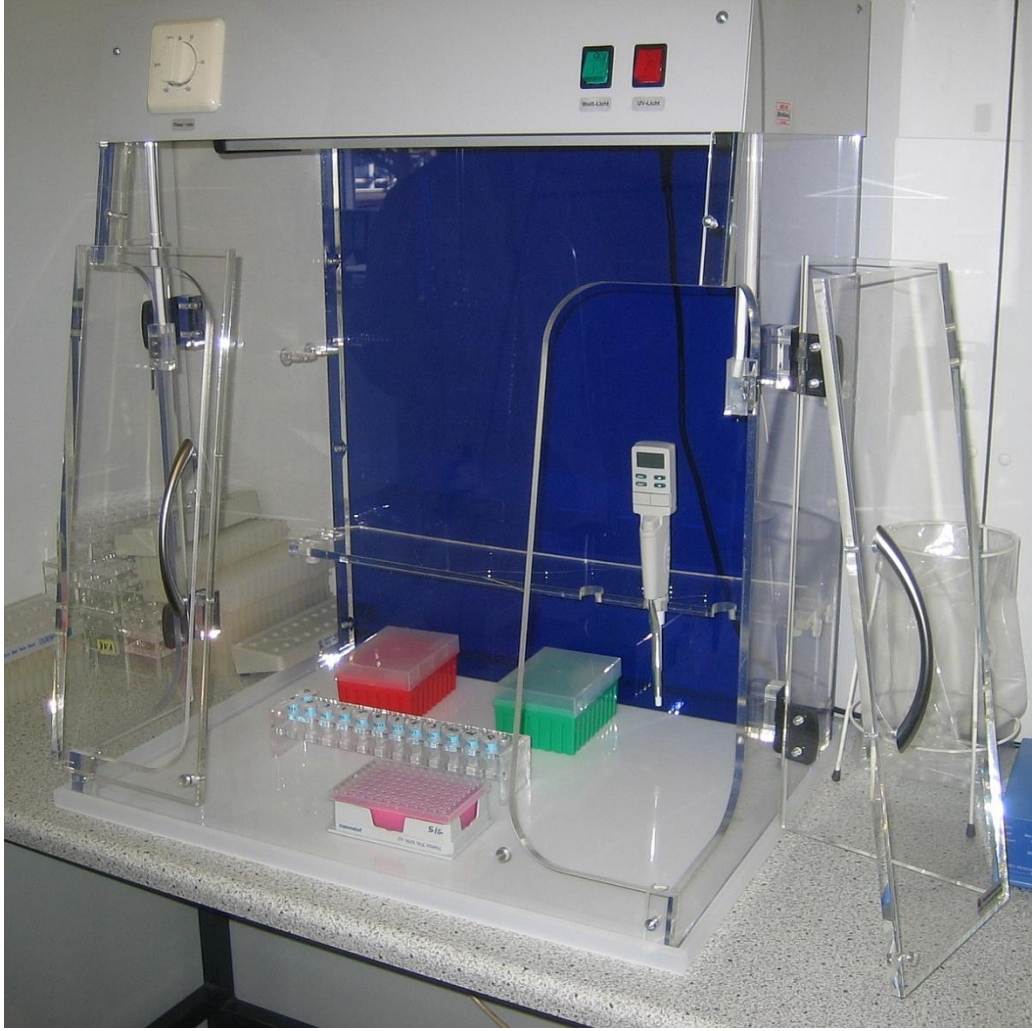
2. Check the possibility to introduce the validated method without changes in your laboratory
   - do you have all the equipment?
     - If not, do you intend to introduce the equipment?
     - If not, how can you achieve the result by a modification or adaptation?
   - Do you have all chemicals needed?
     - If not, which chemicals are mandatory for a first test?
     - How can you achieve the result by a modification or adaptation?

     Also the price can be a reason for a modification.

# Chemicals

PCR-Mastermix: Compare especially the $MgCl_2$ - concentration of the validated method and your own mastermix.
The mastermix intended to be used must be compatible with your thermal cycler.

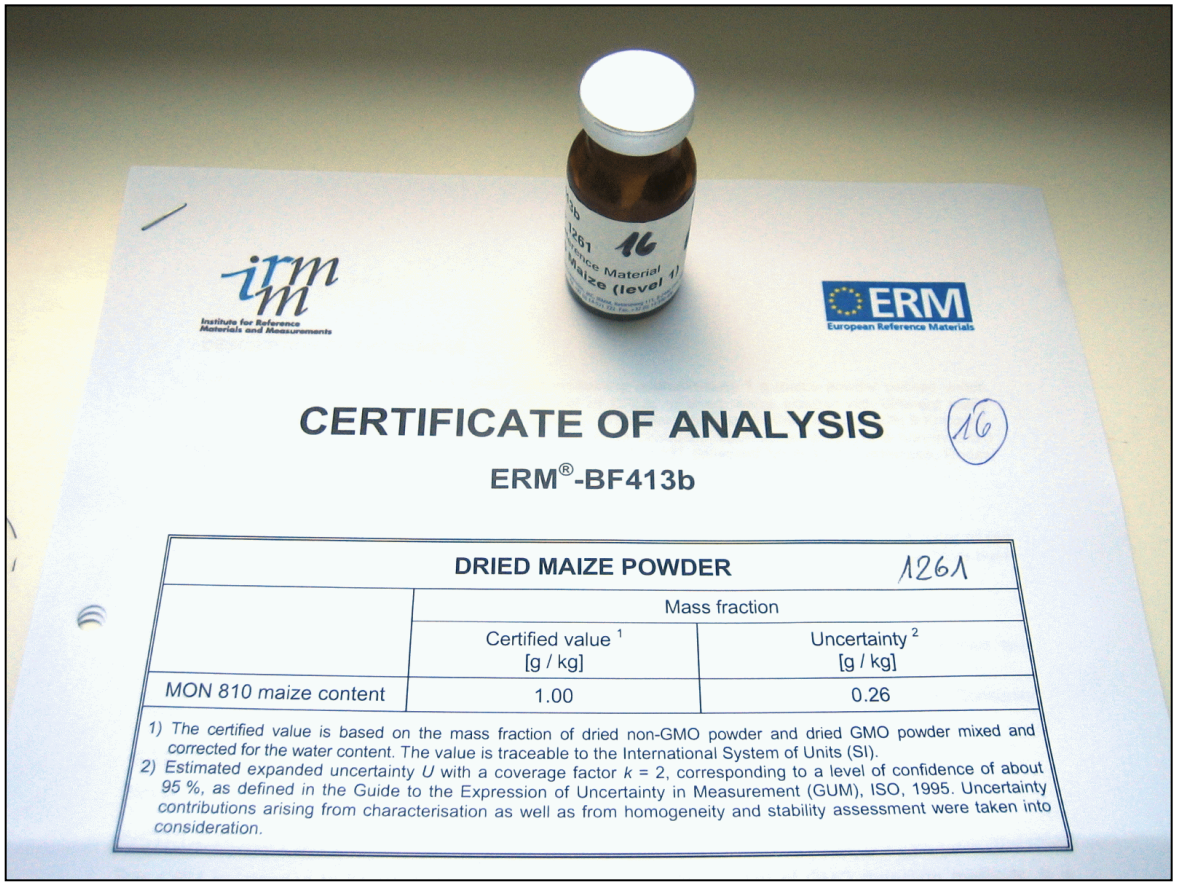
Note: Try to minimize the different numbers of mastermixes in your laboratory.



Probe: If necessary, adapt the detection wavelength if you have to modify the label of the probe

# Preparation of the verification (3)

3. Check the availability of positive and negative material



Reference material

Certified Reference material

RM and CRM provide essential traceability in measurements and are used for, e.g.

- demonstrate the accuracy of results
- calibration of equipment
- monitoring of laboratory performance (quality control)
- Validation of methods
- Comparison of methods

Reference material

Certified Reference material

ISO Guide 30:1992/Amd.1:2008

Certified reference material

CRM

Reference material

- characterized by a metrological valid procedure for one or more specified properties,
- accompanied by a certificate that provides
  - the value of the specified property,
  - its associated uncertainty, and
  - a statement of metrological traceability

Reference material

Certified Reference material

ISO Guide 30:1992/Amd.1:2008

Reference material

RM

Material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process

Reference material

Certified Reference material

ISO Guide 31:2000

Reference materials – Contents of certificates and labels

ISO Guide 34:2009

General requirements for the competence of reference material producers

# Positive Materials

Check available certified reference materials

Check available reference materials

Check available positive material

For the verification of an extraction method a matrix matched material is useful, for the verification of a PCR method a DNA-Standard can be chosen.

For the verification of an extraction method there is no need for genetically modified material.

Sometimes you have to consider to use spiked material.

# Controls (ISO/EN 24276:2006, drafted update/actual revision)

| Control step | Environment control [b] | Extraction blank control [c] | Positive extraction control [d] |
|---|---|---|---|
| Homogenization | Mandatory | | |
| Nucleic acid extraction | ↓[a] | one per series | mandatory at regular intervals |
| Assessment of nucleic acid quality | ↓ | ↓ | ↓ |
| Nucleic acid amplification | ↓ | ↓ | ↓ |
| Assessment of results of nucleic acid amplification | ↓ | ↓ | ↓ |
| Interpretation | | ↓ | ↓ |
| Test report | | ↓ | ↓ |

# Controls (ISO/EN 24276:2006)

| Control step | Positive DNA target control [e] | Negative DNA target control [f] | Amplification reagent control [g] | PCR inhibition control [h] |
|---|---|---|---|---|
| Homogenization | | | | |
| Nucleic acid extraction | | | | |
| Assessment of nucleic acid quality | | | | |
| Nucleic acid amplification | mandatory | recommended | mandatory | recommended, but mandatory in certain cases[i] |
| Assessment of results of nucleic acid amplification | ↓ | ↓ | ↓ | ↓ |
| Interpretation | ↓ | ↓ | ↓ | ↓ |
| Test report | ↓ | ↓ | ↓ | ↓ |


Picture by Dr. Manuela Schulze

Certified Reference material producers e.g. European Commission Institute for Reference Materials and Measurements (IRMM)
irmm.jrc.europa.eu/reference_materials_catalogue/catalogue/Pages/index.aspx

## 2.2 MATRIX MATERIALS

### *2.2.1 CERTIFIED FOR GMO CONTENT*

The materials were prepared by quantitative mixing of non genetically modified powder and genetically modified powder, produced from ground seed with the help of a dry-mixing technique, and are intended for the calibration of methods for the detection of genetically modified food.

**CRMs for genetically modified Roundup Ready™ soya beans (ERM-BF410)**

Six CRMs of dried soya bean powder with different mass fractions of genetically modified (Roundup Ready™) soya beans were produced by IRMM.

| | Certified value Roundup Ready mass fraction (g/kg) | Uncertainty (g/kg) |
|---|---|---|
| ERM-BF410a | < 0.3 | - |
| ERM-BF410ak | < 0.7 | - |
| ERM-BF410b | 1.0 | 0.5 |
| ERM-BF410bk | 1.0 | 0.5 |
| ERM-BF410c | 5.0 | 1.0 |
| ERM-BF410dk | 10.0 | 1.0 |
| ERM-BF410e | 20.0 | 2.6 |
| ERM-BF410gk | 100 | 7 |

Availability: Vials containing about 1 g of soya bean powder.

# Positive Materials

American Oil Chemists´ Society (AOCS)
https:secure.aocs.org/crm/index.cfm

AOCS
Your Global Fats and Oils Connection

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GO
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## Certified Reference Materials (CRM)

**Prepared according to ISO Guidelines 30–35, are intended to serve as control material for third party qualitative testing of transformation events.**

**The tariff code for Certified Reference Materials is 3822.00.6000.**

**CRM's are available for:**

- Canola
- Cotton
- Maize
- Potato
- Rice
- Soybean
- Sugarbeet

Purchase now online | Request *Proforma* invoice | Create printable order form for faxing or mailing

| Canola | Certificate (.pdf) | Report (.pdf) | Company | Event | Product Code |
|---|---|---|---|---|---|
| **Leaf Tissue DNA 10 µg** | 0711A Certificate | Report | Bayer CropScience | Ms1 | 0711-A |
| **Leaf Tissue DNA 10 µg** | 0711B Certificate | Report | Bayer CropScience | Rf1 | 0711-B |
| **Leaf Tissue DNA 10 µg** | 0711C Certificate | Report | Bayer CropScience | Rf2 | 0711-C |

# Positive Materials

Possible sources:

- botanical garden
Millennium  Seed Bank Kew Royal botanic garden www.kew.org
or DNA-bank e.g. botanical garden/German Science Foundation Berlin-Dahlem ww2.bgbm.org/herbarium/dna/defaut.cfm

- Commercial companies: e.g. divider of IRMM material

- Commercial companies producing genetically modified organisms

- Official authorities responsible for the control of deliberate release studies

- Research institutes

- EU-RL GMFF

# Positive Materials?

Picture by Dr. Manuela Schulze


Picture by Dr. Manuela Schulze

Dr. Manuela Schulze

# Positive Materials?

Picture by Dr. Manuela Schulze

Picture by Dr. Manuela Schulze

Picture by Dr. Manuela Schulze

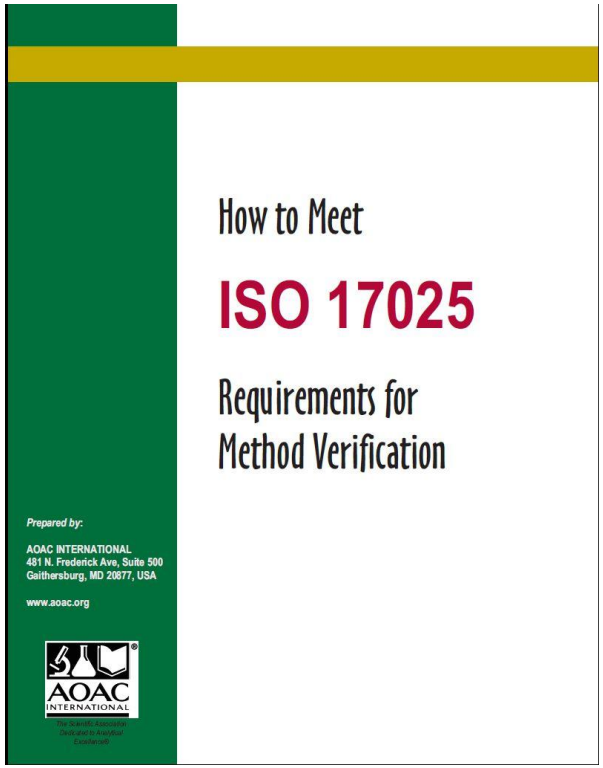
Picture by Dr. Manuela Schulze

# Preparation of the verification (4)

4. Document all the changes you have to consider in your verification e.g. because you have a different thermal cycler

5. Select the parameters which you have to verify

# How to meet ISO 17025 requirements for method verification




*prepared by*

*Analytical Laboratory Accreditation Criteria Committee*
*on request of AOAC*

Guideline available as

http://www.aoac.org/alacc_guide_2008.pdf

**Table 1. Categories of Chemical Test Methods: Since the activities needed for method verification are a subset of those needed for validation, the required performance characteristics for validation are presented in this table**

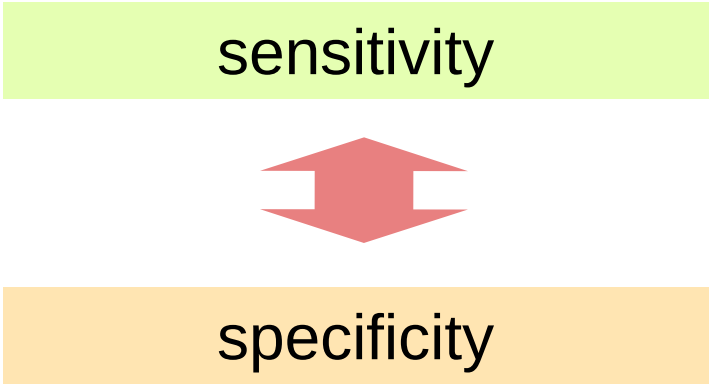
| | Performance Characteristics Included in a Validation | | | | | |
|---|---|---|---|---|---|---|
| Performance Characteristic | Identification 1 | Analyte at Low Concentration Quantitative 2 | Analyte at Low Concentration Limit Test 3 | Analyte at High Concentration Quantitative 4 | Analyte at High Concentration Limit Test 5 | Qualitative 6 |
| Accuracy | No | Yes | No | Yes | Yes | No |
| Precision | No | Yes | No | Yes | Yes | No |
| Specificity | Yes | Yes | Yes | Yes | Yes | Yes |
| LOD | No | Yes | Yes | Yes/No | No | No |
| LOQ | No | Yes | No | Yes/No | No | No |
| Ruggedness | No | Yes | No | Yes | No | No |
| Linearity/Range | No | Yes | No | Yes | No | No |

**Table 2. Category 1: Confirmation of Identity—A method that ensures a material is what it purports to be or confirms the detection of the target analyte**

| Performance Characteristic | Verification | Verification Activities | Reason for Verification |
|---|---|---|---|
| Specificity | No—if the lab's samples are identical to those in the standard method and if any differences in instrumentation do not impact specificity. | NA | If the samples have the same matrix, the specificity which is based on basic principles, will not be impacted. Basic principles are chemical reactions, e.g. reaction of Ag with Cl to create a precipitate. |
| | Yes—if the lab's samples differ from those in the standard method. | Same as those required for validation. | |
| | Yes–if differences between instruments could affect specificity. | The activity need only deal with the unique aspect's of the lab's samples or instrumentation. | Specificity can be impacted by differences in instrumentation. |

# Definitions

Sensitivity relates to the ability of the test to identify positive results.




Specificity relates to the ability of the test to identify negative results.

**Table 3. Category 2: Analyte at Low Concentration, Quantitative**

| Performance Characteristic | Verification | Verification Activity | Reason for Verification |
|---|---|---|---|
| Accuracy | Yes | If the concentration range for which the method is validated is narrow (<1 order of magnitude), analyze one reference material/standard/spike at one concentration. Otherwise, demonstrate accuracy at each concentration level (low, middle and high) by analyzing one reference material/standard/spike at each level. | Over a narrow concentration range, the accuracy and precision should not vary, therefore, the demonstration at one concentration is sufficient. Over a wide concentration range, the accuracy and precision can vary, thus they need to be verified at the different concentration levels. |
| Precision | Yes | Perform the repeatability test once. If the method covers a concentration range >1 order of magnitude, then the repeatability test must include low, middle and high concentrations. | Over a narrow concentration range, the accuracy and precision should not vary, therefore, the demonstration at one concentration is sufficient. Over a wide concentration range, the accuracy and precision can vary, thus they need to be verified at the different concentration levels. Intermediate precision, between analysts, is handled by making sure the analysts are trained and can adequately perform the method. |
| Specificity | No/Yes | *See* Specificity in General Requirements | *See* Specificity in General Requirements |
| LOD | Yes | Run a sample close to LOD | LOD is very likely to be matrix and instrument specific |
| LOQ | Yes | Run a sample close to LOQ | LOQ is very likely to be matrix and instrument specific |

**Table 4. Category 3: Analyte is present above or below a specified, low concentration (Limit Test)**

| Performance Characteristic | Verification | Verification Activity | Reason for Verification |
|---|---|---|---|
| LOD | Yes | Run a sample close to LOD | LOD is very likely to be matrix and instrument specific |
| LOQ | Yes | Run a sample close to LOQ | LOQ is very likely to be matrix and instrument specific |
| Specificity | No/Yes | *See* Specificity in General Requirements | *See* Specificity in General Requirements |

# Example: Verification of validated real time PCR methods

Guidance document:

Verification of analytical methods for GMO testing when implementing interlaboratory validated methods

http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm

JRC Scientific and Technical Reports

# Verification of analytical methods for GMO testing when implementing interlaboratory validated methods

Guidance document from the European Network of GMO laboratories (ENGL)

Prepared by the ENGL working group on "Method Verification"

EUR 24790 EN - 2011

JRC EUROPEAN COMMISSION

ihcp Institute for Health and Consumer Protection

# Example: Verification of validated real time PCR methods

Possible performance characteristics to be considered in the verification:

- Specifity
- Robustness
- Dynamic range
- Amplification efficiency
- $R^2$ coefficient
- Trueness
- Precision
- LOD
- LOQ

http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm

# Preparation of the verification (5)

6. Set up a verification plan (documentation of the result of the considerations)
   - draft a version of the method as it will be performed in the laboratory
   - write down the persons foreseen
   - fix the number of experiments, replicates,
   - make sure that the word “replicates” or repetitions is unambiguous

   (analytical sample, test sample, DNA-extractions replicates, PCR replicates, …)

# Determination of 1000 corn weight, documentation of subsamples

# Documentation

Base for transparency, tracing back

- *When* did *?*
- *Who ?*
- *What ?*
- By using
  - *Which* material, reagents *?*
  - *Which* equipment *?*
  - *Which* methods and *why ?*

**Not documented** means **not done!**

Collaborative study

Test of the same method, Ring trial

Proficiency test

Test of the laboratory proficiency, test of the method used as in the routine by the laboratory

- inter-laboratory studies
- analysis of unknown samples provided by an external source

Collaborative study

Proficiency test

Interpretation of PT outcome

Key evaluator: z-score

Acceptable: +-2

Indicates that results are equal to those of the other laboratories participated in the PT

What if falling out of the score?

- Compare with other failing laboratories (similar methods, equipments...)
- Handle as “non-conformity”

Collaborative study

Proficiency test

**Benefits for laboratories**

Participation in proficiency testing programs allows laboratories to:

- identify areas where improvement in their testing and measurement methods is needed
- identify further training of their staff
- foster confidence in the performance of their testing and measurements
- assure laboratory performance in their accredited test and calibration results
- is mandatory for accredited laboratories
- is a confirmation of the correct verification of methods

# After verification