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
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’
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”)
• ISO 24276:2006 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – General requirements and definitions
• ISO 21571:2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic acid extraction
• ISO 21569:2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Qualitative nucleic acid based methods
• ISO 21570:2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Quantitative nucleic acid based methods

ISO EN Standards
• **ISO 21572:2004** Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Protein based methods
- **EN 15568:2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Sampling**
Method validation is a process

Development of a new method → Optimisation of the method → Establishment of the method
Method validation is a process

In-House (single)-validation of the method

Interlaboratory Validation of the method per interlaboratory (collaborative) study

Standardisation Publication

Verification, introduction in different laboratories
Validation is the confirmation by examination and provision of objective evidence that the requirements for a specific intended use or application have been fulfilled (ISO 9000:2005 section 3.8.4). Verification is the confirmation, through the provision of objective evidence that specific requirements have been fulfilled (ISO 9000:2005 section 3.8.4).
“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.
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.
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.
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
© 2002 IUPAC

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

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.

Accuracy

Trueness and precision
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
Available methods

- Booklet: Compendium of reference methods for GMO analysis, developed by EUR-L-GMFF in collaboration with the ENGL
On-line GMO detection method database

http://gmo-crl.jrc.ec.europa.eu/gmomethods/

GMOMETHODS: EU Database of Reference Methods for GMO Analysis

Quantitative GMO detection PCR methods

- GMO specific
  - Event specific
    - Maize
    - Soybean
    - Cotton
    - Oilseed rape
    - Potato
    - Rice
    - Sugar beet
  - Construct specific
  - Element specific
- Taxon specific
  - Validated independently
  - Validated in combination with other method(s)

Qualitative GMO detection PCR methods

- GMO specific
  - Event-specific
  - Construct-specific
  - Element-specific
    - Cauliflower Mosaic Virus 35S promoter (CaMV P-35S)
    - Figwort Mosaic Virus 35S promoter (P-FMV)
    - Neomycin phosphotransferase II gene (nptII)
    - Nopaline synthase terminator (T-nos)
    - Phosphinothricin N-acetyltransferase gene (bar)
- Taxon specific
  - Validated independently
  - Validated in combination with other method(s)
  - Plant-specific

Released the GMOmethods app for iPad on 20-12-2011.
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.


<table>
<thead>
<tr>
<th>Event</th>
<th>Crop</th>
<th>Unique identifier</th>
<th>Applicant</th>
<th>Status/Progress</th>
<th>Reports</th>
<th>Validated Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bt10</td>
<td>maize</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Validation completed</td>
<td><a href="http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm">Validation report</a> Published on: 13/07/2005</td>
<td><a href="http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm">Validated method</a> Published on: 13/07/2005</td>
</tr>
<tr>
<td>NK603</td>
<td>maize</td>
<td>MON-00603-6</td>
<td>Monsanto Company</td>
<td>Validation completed</td>
<td><a href="http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm">Validation report</a> Published on: 10/01/2005</td>
<td><a href="http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm">Validated method</a> Published on: 10/01/2005</td>
</tr>
</tbody>
</table>

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
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
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
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)

<table>
<thead>
<tr>
<th>Control step</th>
<th>Environment control (^b)</th>
<th>Extraction blank control (^c)</th>
<th>Positive extraction control (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenization</td>
<td>Mandatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleic acid extraction</td>
<td>↓(^a)</td>
<td>one per series</td>
<td>mandatory at regular intervals</td>
</tr>
<tr>
<td>Assessment of nucleic acid quality</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Nucleic acid amplification</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Assessment of results of nucleic acid amplification</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Interpretation</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Test report</td>
<td></td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>
## Controls (ISO/EN 24276:2006)

<table>
<thead>
<tr>
<th>Control step</th>
<th>Positive DNA target control</th>
<th>Negative DNA target control</th>
<th>Amplification reagent control</th>
<th>PCR inhibition control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleic acid extraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assessment of nucleic acid quality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleic acid amplification</td>
<td>mandatory</td>
<td>recommended</td>
<td>mandatory</td>
<td>recommended, but mandatory in certain cases</td>
</tr>
<tr>
<td>Assessment of results of nucleic acid amplification</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Interpretation</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Test report</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Material</th>
<th>Certified value Roundup Ready mass fraction (g/kg)</th>
<th>Uncertainty (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERM-BF410a</td>
<td>&lt; 0.3</td>
<td>-</td>
</tr>
<tr>
<td>ERM-BF410ak</td>
<td>&lt; 0.7</td>
<td>-</td>
</tr>
<tr>
<td>ERM-BF410b</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>ERM-BF410bk</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>ERM-BF410c</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>ERM-BF410dk</td>
<td>10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>ERM-BF410e</td>
<td>20.0</td>
<td>2.6</td>
</tr>
<tr>
<td>ERM-BF410gk</td>
<td>100</td>
<td>7</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf Tissue DNA 10 µg</td>
<td>0711A Certificate</td>
<td>Report</td>
<td>Bayer CropScience</td>
<td>Ms1</td>
<td>0711-A</td>
</tr>
<tr>
<td>Leaf Tissue DNA 10 µg</td>
<td>0711B Certificate</td>
<td>Report</td>
<td>Bayer CropScience</td>
<td>Rf1</td>
<td>0711-B</td>
</tr>
</tbody>
</table>
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/default.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

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

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
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.

<table>
<thead>
<tr>
<th>Performance Characteristic</th>
<th>Identification 1</th>
<th>Analyte at Low Concentration Quantitative 2</th>
<th>Analyte at Low Concentration Limit Test 3</th>
<th>Analyte at High Concentration Quantitative 4</th>
<th>Analyte at High Concentration Limit Test 5</th>
<th>Qualitative 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Precision</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Specificity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LOD</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes/No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>LOQ</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes/No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Ruggedness</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Linearity/Range</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Performance Characteristic</td>
<td>Verification</td>
<td>Verification Activities</td>
<td>Reason for Verification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>No— if the lab’s samples are identical to those in the standard method and if any differences in instrumentation do not impact specificity.</td>
<td>NA</td>
<td>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.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes— if the lab’s samples differ from those in the standard method.</td>
<td>Same as those required for validation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes— if differences between instruments could affect specificity.</td>
<td>The activity need only deal with the unique aspect’s of the lab’s samples or instrumentation.</td>
<td>Specificity can be impacted by differences in instrumentation.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Example: **Verification of validated real-time PCR methods**

Guidance document:

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

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”
Example: Verification of validated real time PCR methods

Possible performance characteristics to be considered in the verification:
- Specificity
- Robustness
- Dynamic range
- Amplification efficiency
- $R^2$ coefficient
- Trueness
- Precision
- LOD
- LOQ

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
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”
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

Picture by Dr. Manuela Schulze