



“Validation of objective methods for GM testing – the EU approach”

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Method Validation - Definition

- **ISO/IEC 17025:2005**
 - ◆ **Validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled**
- **ISO 5725-1:1994**
 - ◆ **An inter-laboratory experiment in which the performance of each laboratory is assessed using the same standard measurement method on identical material**



Why Validation Study ?

- We need to get information about a food/feed item by submitting the sample to analysis, applying a specific method
- The analytical problem defines the *purpose* of the method
- Conducting a validation study is a tool to check whether the method is *fit for the purpose*
- The validation study delivers *performance characteristics*
- How to validate the analytical method?
 - ◆ By performing an in-house validation
 - ◆ By conducting a collaborative study



Method Validation

Validation is a confirmation of the EXPECTED performance indexes in a multilaboratory study

a process NOT a result...

Method Validation

Validation is the conclusion of a long process

**Develop.
of a new
method**

**Optimization
of the
method**

**Pre-validation
of the
method**

**Full
validation of
the method**



**Acceptance criteria
(pre-validation requirements)**

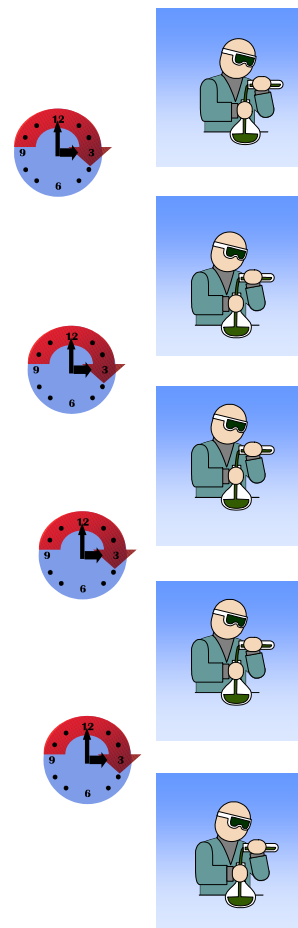


**Performance
requirements**

Repeatability of a method

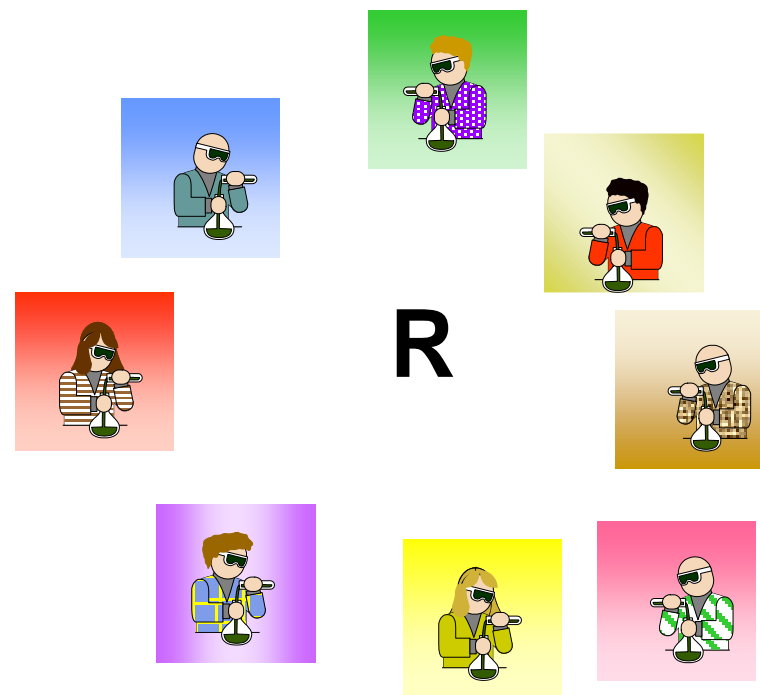
- ↑ Testing the precision under intra-lab conditions
- ↑ same method
- ↑ Testing under repeatability conditions
- ↑ Calculation: ISO 5725 formula 7.4.5.1

r



Reproducibility of a method

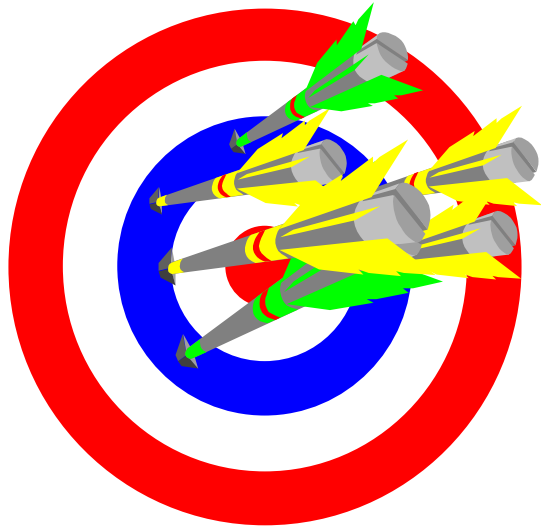
- ↑ **Testing the precision under reproducible conditions**
- ↑ **same method**
- ↑ **different laboratories**
- ↑ **preferably on international level rather than on national level**
- ↑ **calculation: ISO 5725 formula 7.4.5.2 and 7.4.5.5**



Accuracy (trueness and precision)

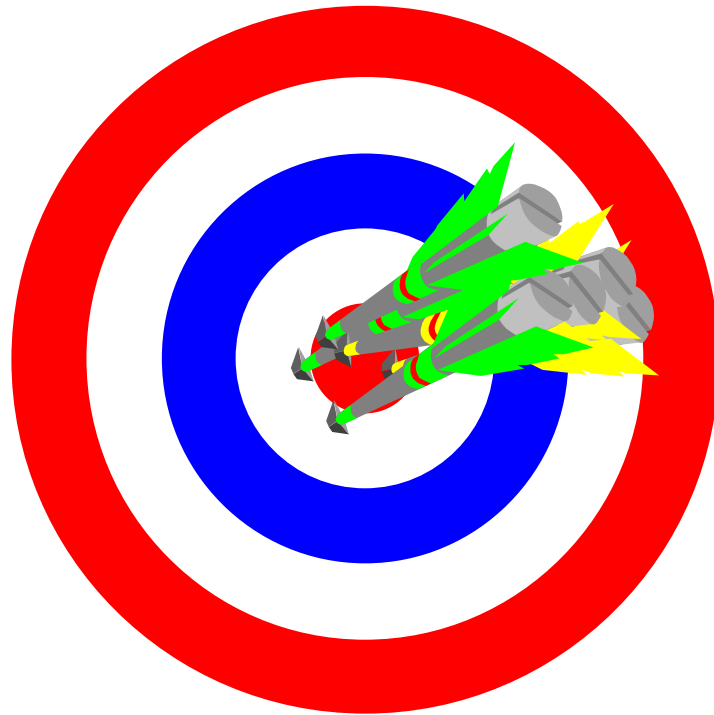


**Precise
But low trueness (far
from the true value)**



**High trueness, low
precision**

Accuracy (trueness and precision)



**Close to the true value
and high precision**

TESTING METHOD INTER-LABORATORY PERFORMANCE: Example of the CRL validation of a method for regulatory compliance: TC1507 *Herculex* maize “fit for the purpose”

Validation 1507 (*Herculex*TM I – Pioneer) maize

Sample	0,00	0,1	0,5	0,9	2	5
Number of laboratories	14	14	14	14	14	14
Number of outliers	0	0	1	2	1	0
Number of laboratories retained after eliminating outliers	14	14	13	12	13	14
Mean value	0,000	0,106	0,480	0,933	1,966	5,420
Bias (%)	0	6	-4	4	-2	8
Repeatability standard deviation s_r	0,00	0,02	0,06	0,07	0,17	0,78
Repeatability relative standard deviation RSD_r (%)	0,00	18,11	11,70	7,68	8,48	14,41
Repeatability limit r ($r = 2.8 \times s_r$)	0,00	0,05	0,16	0,20	0,47	2,19
Reproducibility standard deviation s_R	0,00	0,02	0,07	0,10	0,42	1,17
Reproducibility relative standard deviation RSD_R (%)	0,00	19,91	14,78	10,24	21,19	21,65
Reproducibility limit R ($R=2,8 \times s_R$)	0,00	0,06	0,20	0,27	1,17	3,29

Regulation (EC) 1829/2003 on Genetically Modified Food and Feed

- Risk assessment under responsibility of EFSA
- Covers food and feed produced from a GMO
- Includes GM additives, flavourings, enzymes
- Not products from animals fed GM feed
- Provisions for **labelling, threshold** for adventitious presence
 - ◆ **0.9 % threshold for adventitious presence of approved GMOs**
 - ◆ **0.5% threshold for adventitious presence of non-approved GMOs but positively risk assessed (transitional rule applicable for 3 years)**
- Methods for sampling, identification and detection of GM food and feed should be provided by the applicant
- Methods should be validated by the **Community Reference Laboratory for GM Food and Feed**





The Community Reference Laboratory for GM Food and Feed

Regulation (EC) No1829/2003 on GM Food and Feed

Regulation 1829/03 on GM Food and Feed (Annex):

“ The **Community Reference Laboratory** referred to in Article 32 is the Commission's Joint Research Centre.”

And:

“ For the tasks outlined in this Annex, the Commission's Joint Research Centre shall be assisted by a consortium of National Reference Laboratories, which will be referred to as the ‘**European Network of GMO laboratories.**’



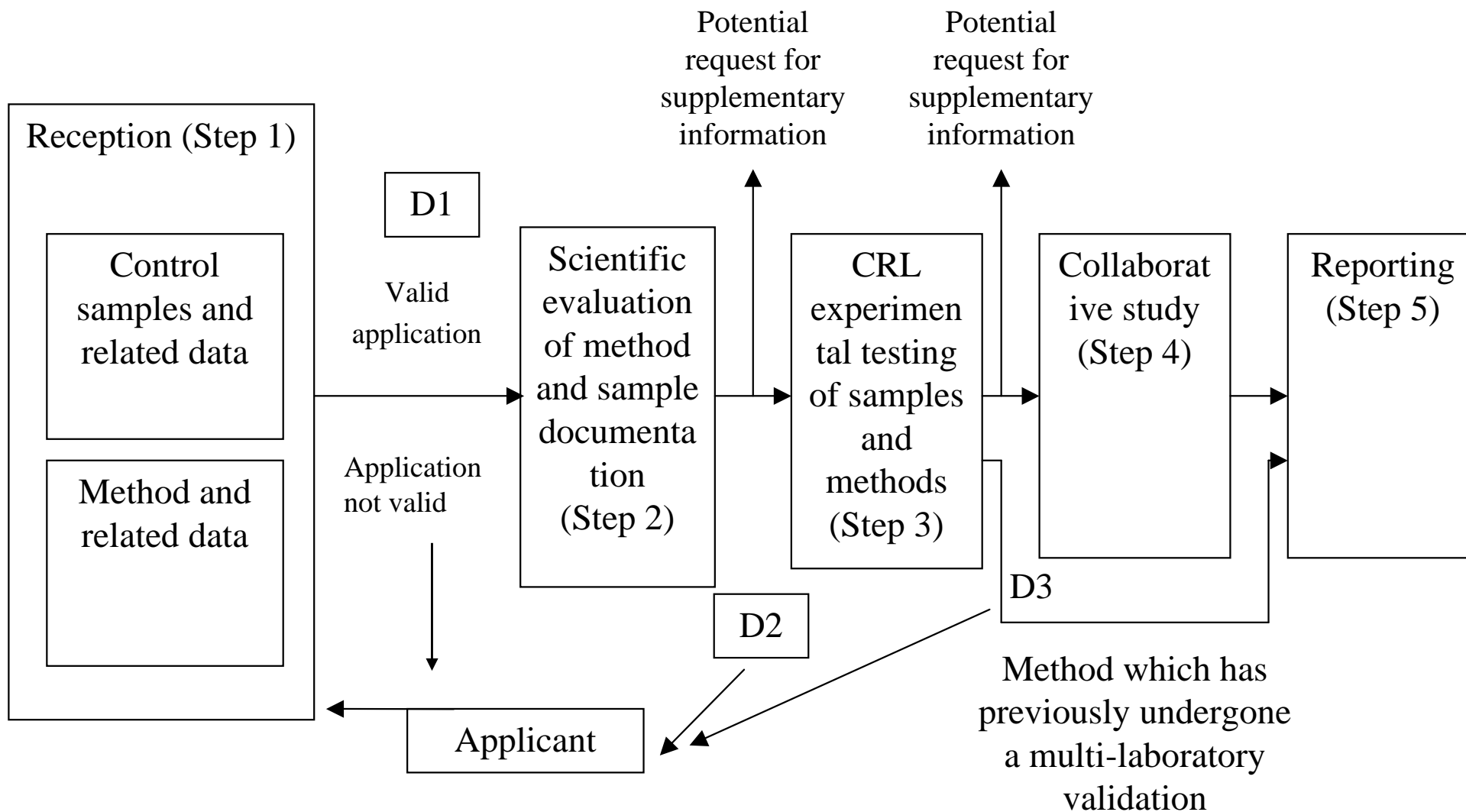
Duties and tasks of the CRL as defined by Reg. (EC)1829/2003



- Reception, preparation, storage, maintenance and distribution to national reference laboratories of the appropriate positive and negative control samples
- Evaluating the data provided by the applicant for authorisation for placing the food or feed on the market
- Testing and validation of the method for detection, including sampling and identification of the transformation event.....in the food or feed
- Submitting full evaluation reports to the Authority
- The Community reference laboratory shall play a role in dispute settlements between MS



CRL operational procedures



The process is a step-by-step procedure and can be stopped or re-initiated as required

Standards for Method Validation

- ENGL: Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing – Version 25/01/2005
- ISO 5725 – Accuracy (trueness and precision) of measurements methods and results
- IUPAC, 1995 – Protocol for the Design, Conduct and Interpretation of Method-Performance Studies
- Codex Alimentarius Commission - Consideration of the methods for the detection and identification of foods derived from biotechnology general approach and criteria for the methods. Discussion paper.
- Codex Alimentarius Commission – Single Laboratory Validation – Consideration of Harmonized IUPAC guidelines for Single-Laboratory Validation of Methods of Analysis



Methods minimum performance requirements: CRL acceptance criteria and performance requirements

Applicability	Scope of the method, interferences with analytes etc.
Practicability	Equipment, timing, practical difficulties
Specificity	Event-specificity
Dynamic Range	Include the 1/10 and at least 5 times the target concentration
Accuracy	Within $\pm 25\%$ of the reference value
R² Coefficient	≥ 0.98
PCR efficiency	$-3.1 \geq \text{slope} \geq 3.6$
RSDr	Below 25% over the whole dynamic range
LOQ	Less than 1/10th of the value of the target concentration with an RSDr $\leq 25\%$
LOD	Less than 1/20th of the target concentration
Robustness	Deviate not more than $\pm 30\%$
RSDR	Below 35% at the target concentration; < 50% below 0.2%
Trueness	Within ± 25 of the accepted reference value over the whole range



The importance of a reference gene

Real-time relative quantitation of GM-event is based upon the notion of species-specific reference gene

An optimal reference gene should be:

1. single (or low and known) copy number
2. stable across varieties of the same species
3. specific to the species of interest

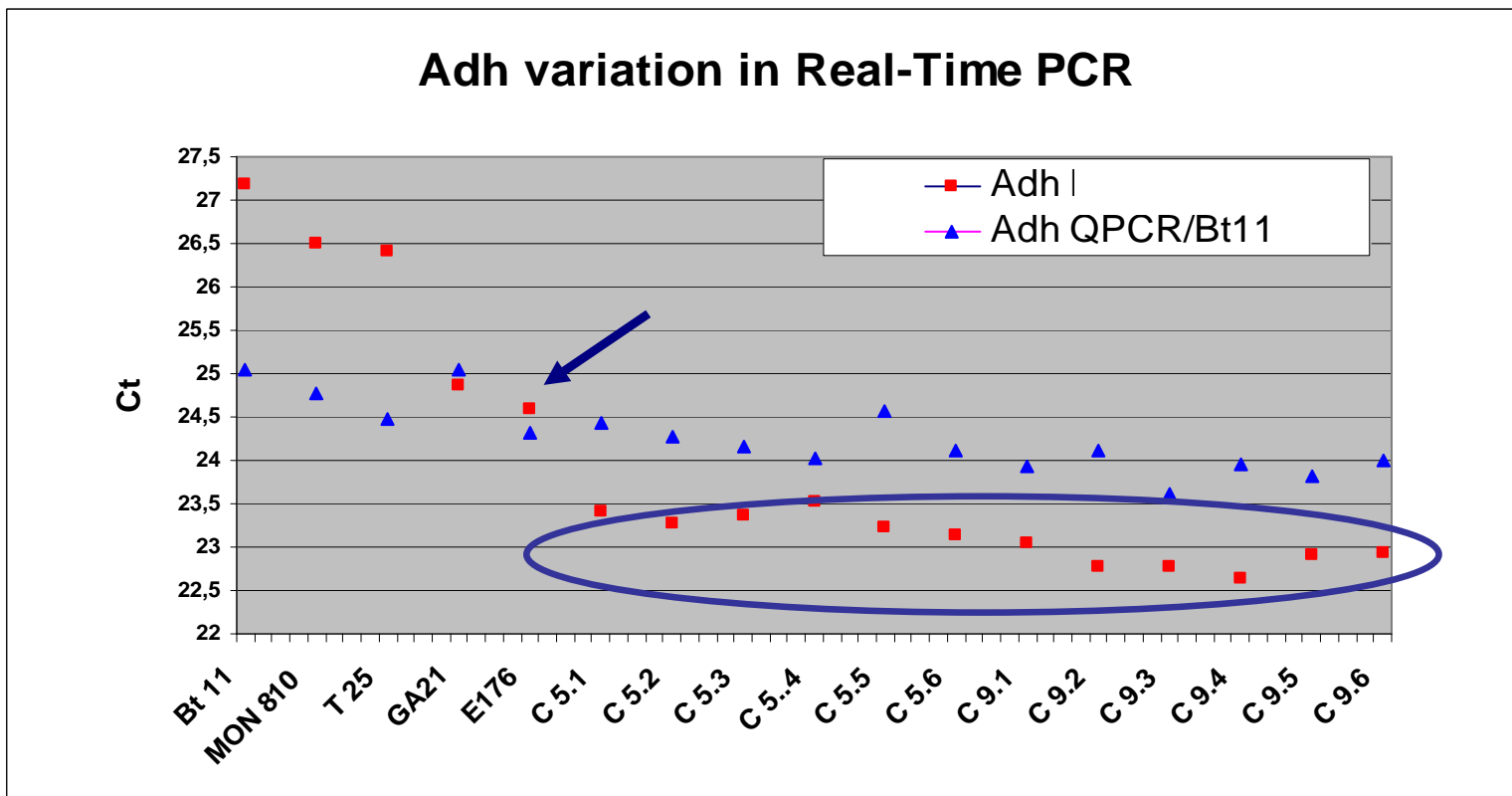
should not be:

4. target of genetic transformation

Ideally, reference gene should be proposed by the scientific community as golden standard for a given taxon



Reference gene *Adh1*



Sample	Measured % GA21	Expected % GA21
5.1	0.33 +/- 0.055	0.5
5.2	0.2 +/- 0.2	0.5
5.3	2.48 +/- 0.187	5
5.4	0.05 +/- 0.036	0.1
5.5	2.25 +/- 0.435	5
5.6	0.05 +/- 0.017	0.1

Sample	Measured % GA21	Expected % GA21
9.1	0	0
9.2	0	0
9.3	0.26 +/- 0.055	0.5
9.4	0.62 +/- 0.072	1.5
9.5	0.74 +/- 0.131	1.5
9.6	0.23 +/- 0.012	0.5



How many reference genes ?

Maize Hmg, adh-70bp, adh-136bp, Adh-136”

Cotton SAH7, Adh, Acp1, Sad1 (L. Yang et al)

Rice Phospholipase D

Oilseed rape Cruciferin, Fat A

Soybean Lectin 100 bp, lectin 72bp

Sugar beet Glutamine synthetase

Potato UGP-ase

Reference systems: need to rationalise

Most taxon-specific reference systems are far from being ideal



1. Acceptance criteria should be implemented taking into account the need a) to determine the reference system copy number; b) to produce Ct figures from testing of varieties and related species

3. ideally, one reference system per species



2. Best candidates reference system should be proposed for all taxon-related GM-quantitative methods



Performance Requirements for DNA Extraction

Validation process

- Food and feed samples
- 18 DNA extractions / sample over 3 days (i.e. 6 per day)
- Fluorometric measurement, 2 replicates per sample
- Gel electrophoresis
- Test for PCR inhibitors



Extraction yield: acceptance criteria



- Repeatability
 - ◆ RSDr of all measurements: < 25%
- Quantity
 - ◆ Concentration mean:
 - In agreement with the one declared by the applicant
 - > working concentration according to the PCR method to be used
 - ◆ Total quantity: > minimum quantity needed for tests in control laboratories (Min. quantity?)



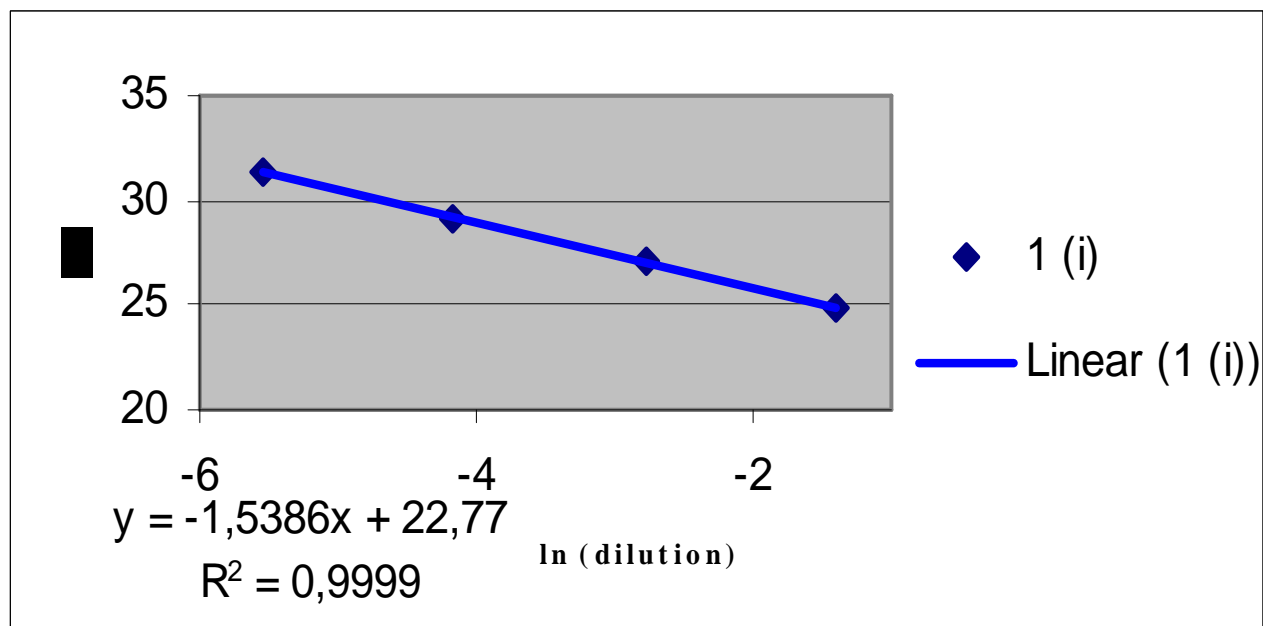
DNA integrity: Proposal for acceptance criteria

- High molecular weight DNA
- Low fragmentation state
- Visual inspection in agarose gel electrophoresis
- Side comparison with marker of molecular weight



DNA purity: Proposal for acceptance criteria

- Absence of PCR inhibitors
- $260/280 > 1.8$ (protein) and $260/230 > 2.0$ (polysaccharides)
- Four 4-fold serial dilutions
- Real-time PCR
- $Ct_{dilutions} = f [\ln(\text{concentration})]$
- For the undiluted sample:
 - $(\text{Measured Ct}) - (\text{Extrapolated Ct}) < 0.5$

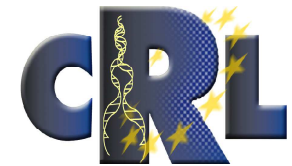


Comparison of extrapolated Ct values versus measured Ct values:

delta Ct = abs(Ct extrapolated - Ct measured)

DNA extract	R ²	Ct extrapolated	C _T measured	delta Ct
1 (i)	0,9999	22,77	22,73	0,04
1 (ii)	0,9976	23,42	23,19	0,22

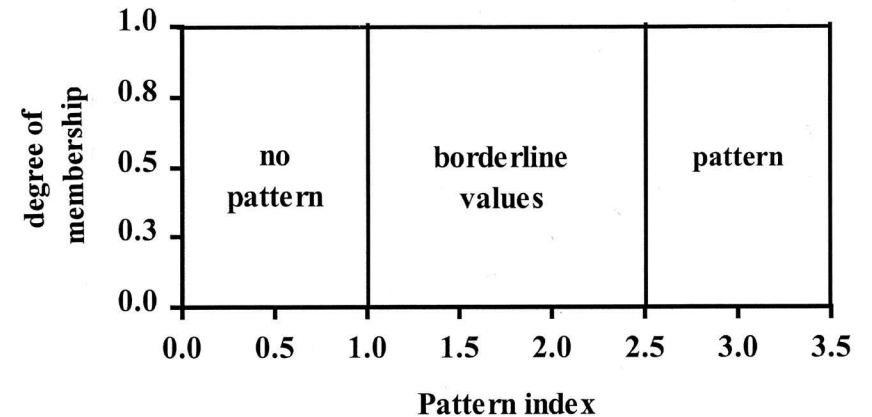
Joint evaluation of performance indices



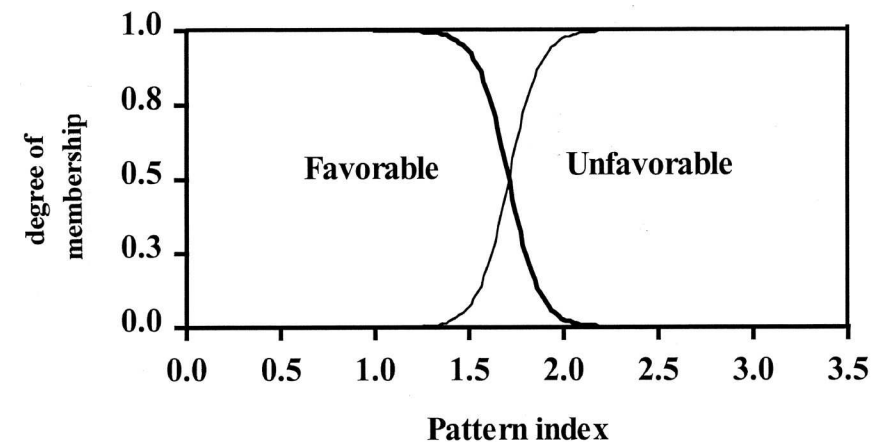
- Approaches based on crispy thresholds are difficult to handle:
 - ◆ thresholds suitable for all methods do not exist
 - ◆ if the thresholds are tight no method is acceptable, if thresholds are not stringent all methods are acceptable

- Fuzzy-based aggregation of indices is proposed to:
 - ◆ include experts knowledge (judgement) in classification
 - ◆ classify global “quality” of different methods for the same analysis
 - ◆ test the stability of the solution under different judgment of importance of the various indices

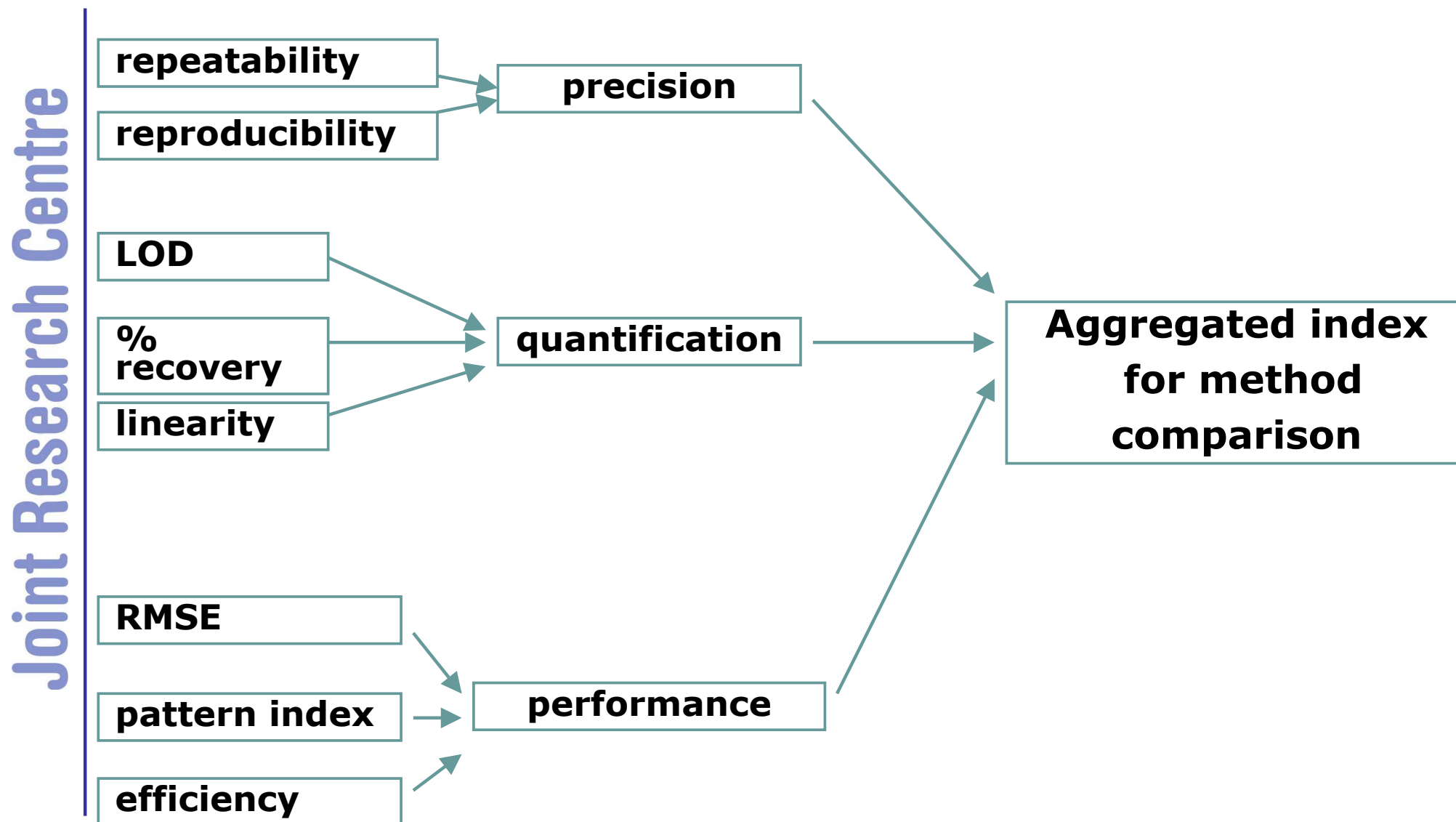
A - CRISP SETS



B - FUZZY SETS



Fuzzy-based aggregation



On Web Further Information

- <http://gmo-crl.jrc.it> (the CRL web-site)
- <http://biotech.jrc.it> (overview of the BGMOS Unit activities)

