



Method Verification

Transfer of validated methods into laboratories working routine

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Background

- Danish National Reference Laboratory for GMO in Feed and Seed
- ISO 17025 accredited for GMO analysis since 2006
 - Flexible since 2009
 - 14 methods verified under ISO17025
- 25 event specific methods
- 5 construct or element specific methods



Area: 43.090 km²

Population: 5,4 million people, 12 million pigs





Method verification

- Select method
 - Real time PCR
 - Compendium of validated reference methods
- Make a verification plan

E9	
A	B
1 Gældende fra 25. januar 2011	Dok nr.: I.D.LAB.validering.GMO.26
2 Udarbejdet af: HOU	Side: 1 af 1
3 Godkendt af:	Udg. nr. : 1 Rev. Nr.: 0
4 Valideringsplan	
5 Hvad skal valideres	Soja Mon89788
6 Periode for valideringen og ansvarlig	2010 / 2011
7 Hvor kommer metoden fra (reference)	JRC valideret metode
8 Er metoden valideret i multilaboratorie studie	Ja
9 Reference materiale (udbyder, materialetype)	AOCS
10 Skal der fremstilles andre koncentrationer af referencematerialet end dem der kan købes og evt. hvilke	ja, 10,1 og 0,1%
11 Hvis der skal bruges non GMO materiale hvor kommer det så fra?	AOCS, 0906-A (non GMO soja)
12 Hvilken mastermix er er metoden valideret med og skal den ændres?	Taqman Universal mastermix, ændres til Jumpstart (Sigma)
13 Laves der andre ændringer fra den validerede metode?	Nej
Skabelon A5547 T45 RF3 MON89788 355-tNOS multi CTP2-CP4EP5P5 bar 355-pat	
Klar	



Method selection

- Fit for the purpose
- In line with the capacity for the laboratory
- Validation status
 - Interlaboratory validated methods
 - Single laboratory validated methods
 - Peer review papers
 - Internal developed methods



Make a verification plan

- **Select positive material**
 - Reference material
 - Relevant samples
- **Select changes from the published method**
 - Adjust to laboratory needs and routines
- **Select parameters to verify**
 - QA instruction
- **Select acceptance criteria's**
 - QA instruction



Positive material

- IRMM
 - Certified plant material in different GM levels
- AOCS
 - Certified plant material 0 % or 100 % GM
 - Purified DNA
- FLUCA
 - Purified DNA
- Nippon
 - Multiple target plasmid (quantification)
- EURL-GMFF
 - Single target plasmids (not for dilution curves only identification)



Method changes

- Real Time Instrument
- Mastermix
 - Avoid many different types in a single lab
 - Price
 - Compatible with the real time Instrument
- Reference gene
 - Harmonise with other implemented methods
- Standard curve type
 - Dilutions from a single standard
 - Different GM % in same concentration
 - Δ Ct method



The selected method to verify has to correspond to the method expected to be used in the routine analysis afterwards



Parameters possible to verify for a real time PCR method

- Specificity
- Robustness
- Dynamic range
- Amplification efficiency
- R^2 coefficient
- Trueness
- Precision as relative repeatability standard deviation RSD_r
- Limit of detection (LOD) practical and absolute
- Limit of quantification (LOQ) practical and absolute



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

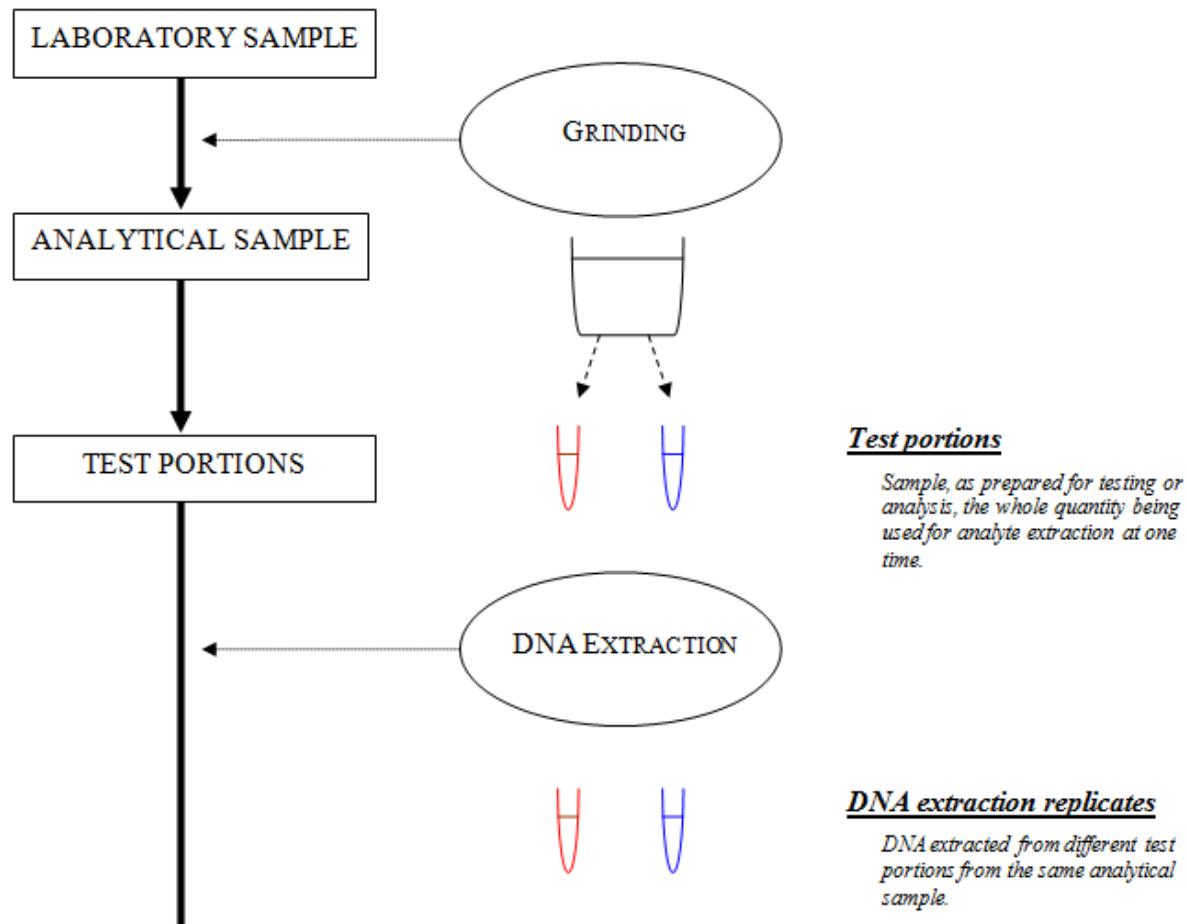
Guidance document from the European Network of GMO
laboratories (ENGL)

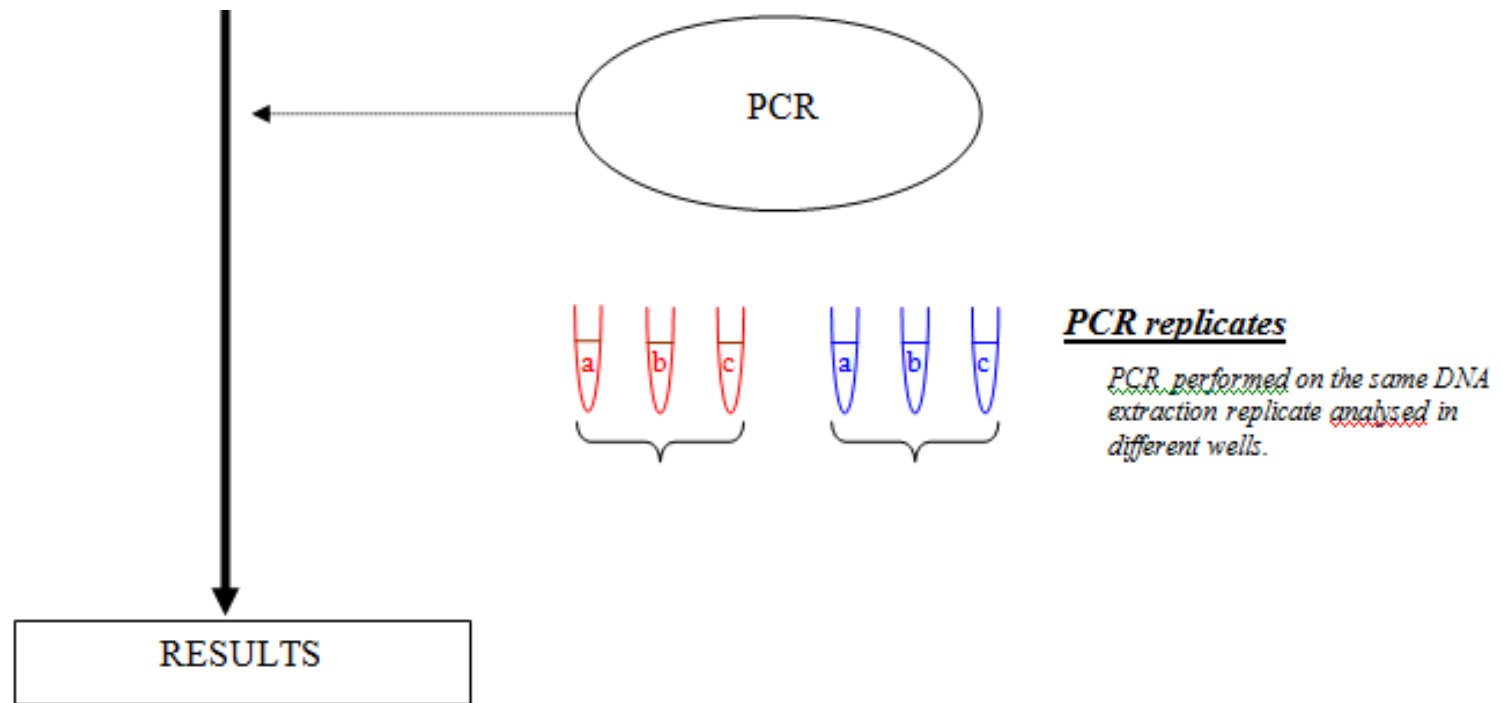
Prepared by the
ENGL working group on “Method Verification”



Version 1

- Real time PCR based methods
- Implementation of EURL validated methods without changes
- Modular approach
- Adapted by the ENGL steering committee 23. February 2011
- Based on “Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing”
European Network of GMO Laboratories (ENGL)







Experiments

1. Optional: Preliminary test to define appropriate DNA concentrations

Test at least 3 target concentrations in the range of 300 ng – 0.1 ng (dependent on plant species).

300 ng Maize DNA corresponds to approx. 110 000 endogenous gene copies

0.1 ng corresponds to approximately 37 copies.

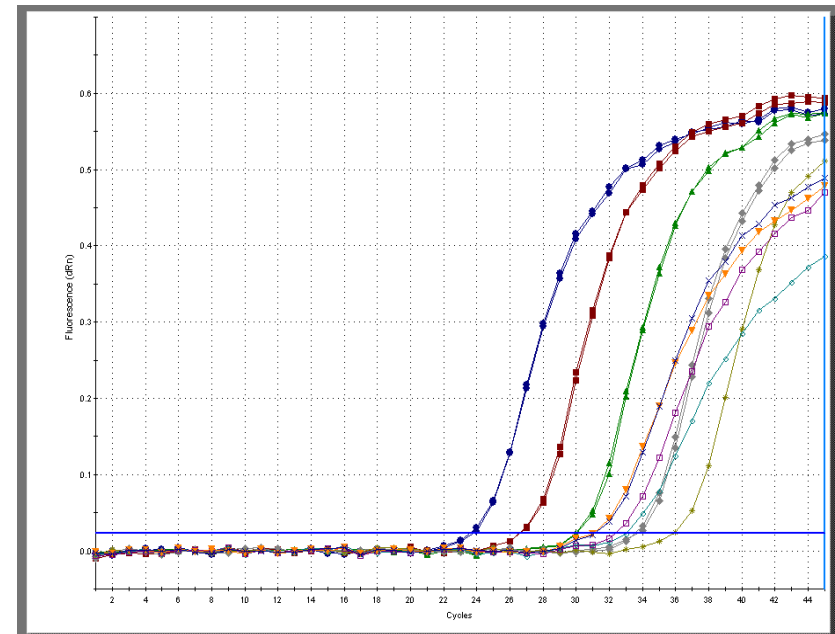
P. Hübner et al. Validation of PCR methods for quantification of genetically modified plants in food. Journal of AOAC International, 84 (6): 1855-1864, 2001.



Acceptance

1. Optional: Preliminary test to define appropriate DNA concentrations

- Parallel amplification curves
- Satisfactory shape
- Slope/efficiency
- R^2





Definitions

- Dynamic range
 - The range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision.
- Amplification efficiency,
 - The rate of PCR amplification
 - A theoretical slope of -3.32 corresponds to an efficiency of 100 % in each cycle.
 - The efficiency (in %) can be calculated by the following equation:

$$\text{Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$$



Definitions

- R^2 coefficient
 - R^2 is the coefficient of determination, which is calculated as the square of the correlation coefficient between the measured Ct-value and the logarithm of the concentration of a standard curve obtained by linear regression analysis.



Experiments

2. **Dynamic range, R^2 coefficient, and amplification efficiency**

- Example 1: 2 calibration curves minimum requirements
- 5 calibration points with 3 PCR replicates each (triplicates)



Experiments

2. **Dynamic range, R^2 coefficient, and amplification efficiency**

- Example 2: 4 calibration curves;
- 5 calibration points with 2 PCR replicates each (duplicates)
average of 4 slopes and R^2 are used to verify the acceptance.



Experiments

2. **Dynamic range, R^2 coefficient, and amplification efficiency**

- Example 3: 2 calibration curves;
- 8 calibration points in 5 PCR replicates (pentaplicates) also covering the low concentrations for LOD and LOQ. Average of the part above LOQ for slope and R^2 are used to verify the acceptance.



Acceptance

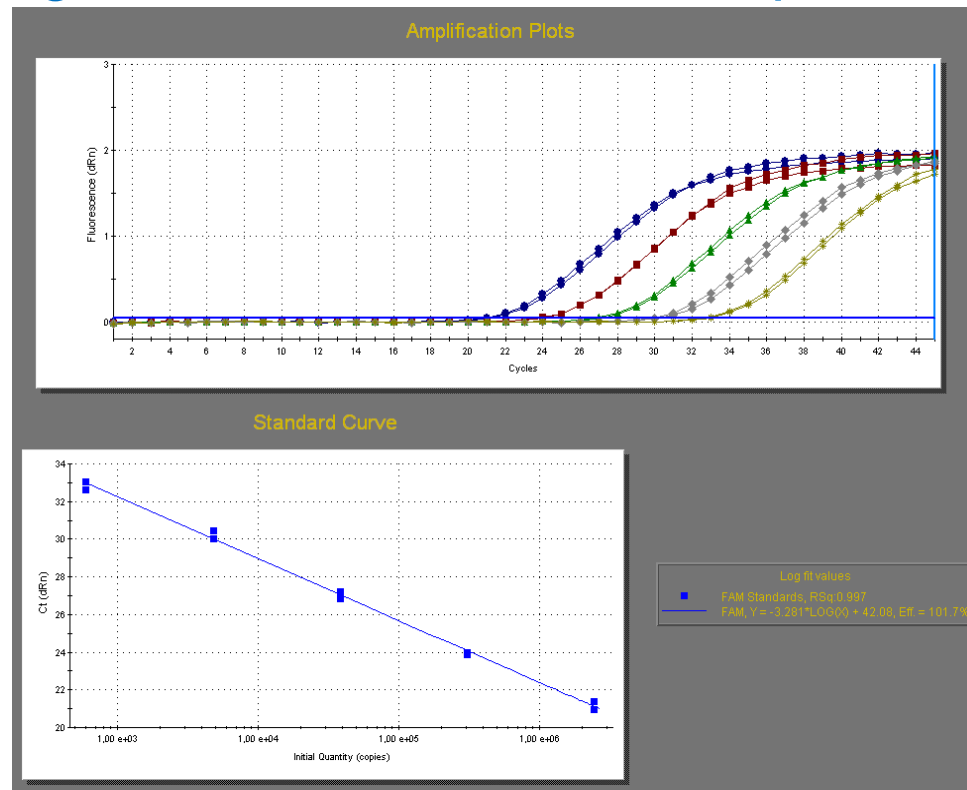
2. **Dynamic range, R^2 coefficient, and amplification efficiency**

- Cover the range of interest e.g. 10x and 1/10x of threshold (Threshold 0.9 %, range 4.5 % - 0.09 %)
- R^2 values should be ≥ 0.98
- Slope in the range of $-3.6 \leq \text{slope} \leq -3.1$
- Efficiency 90-110%



Acceptance

2. Dynamic range, R^2 coefficient, and amplification efficiency



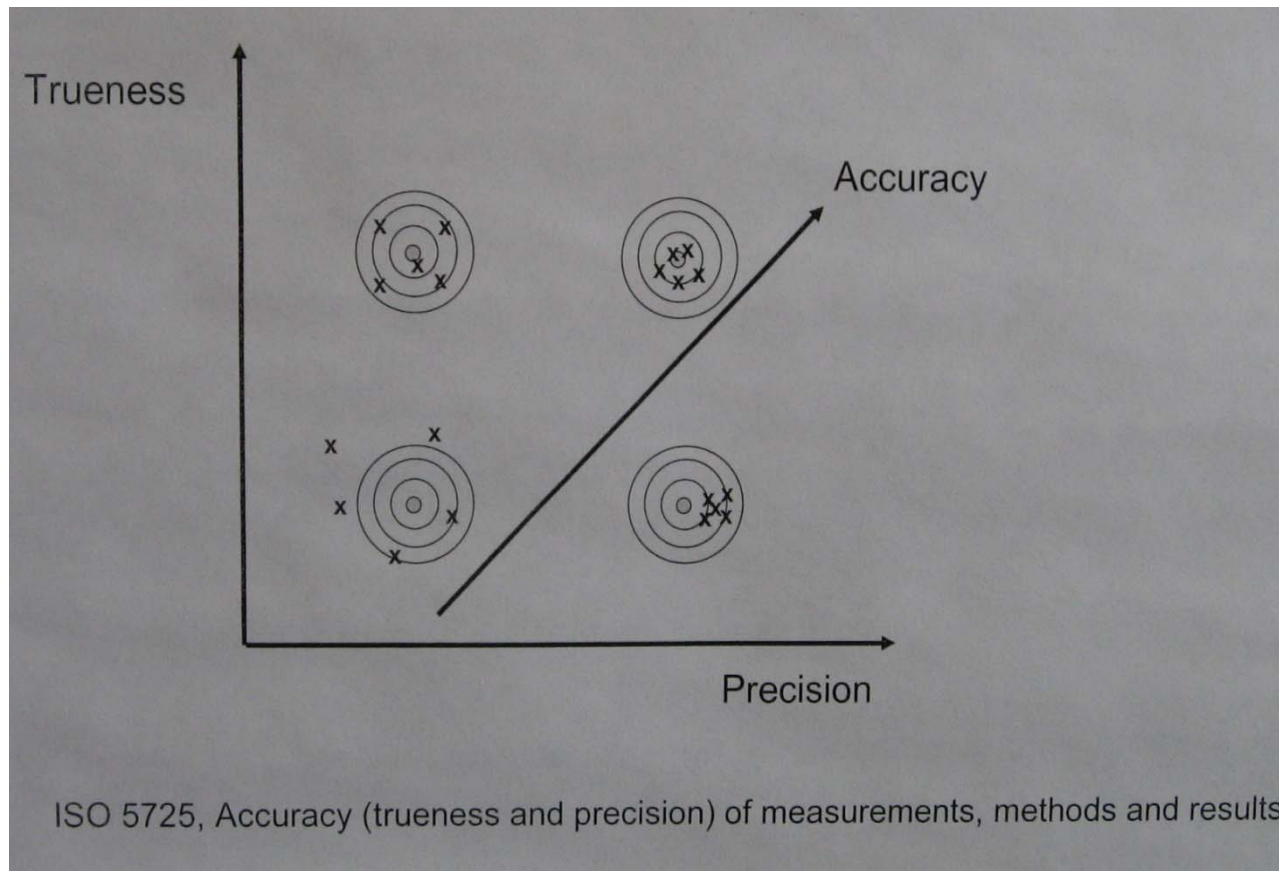


Definitions

- **Trueness**
 - The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.
- **Precision -relative repeatability standard deviation RSD_r**
 - The relative standard deviation of test results obtained under repeatability conditions.
- **Repeatability**
 - The conditions where test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.



Definitions



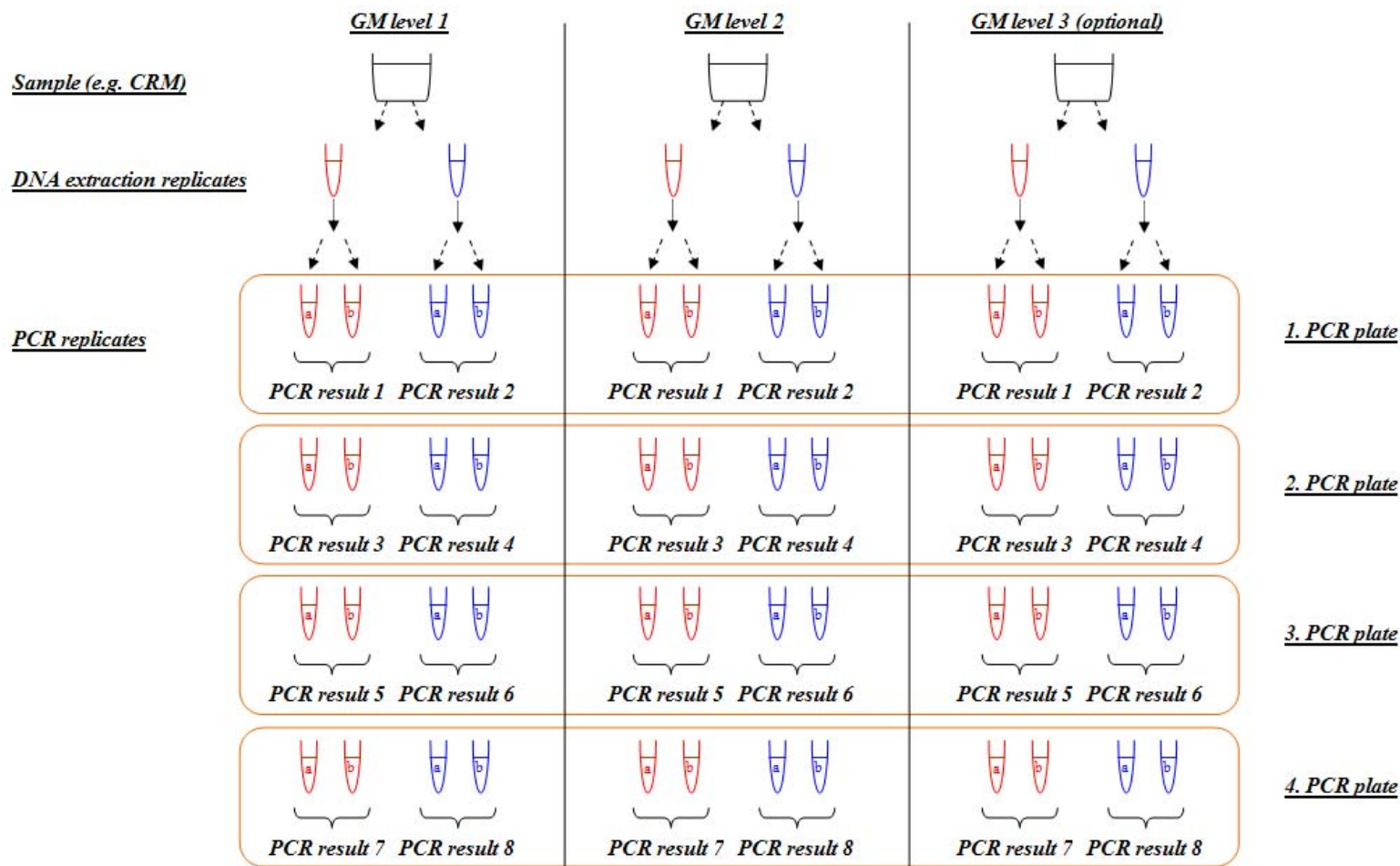


Experiments

3. **Trueness, Precision, RSD_r**

- At least 2 GM levels
 1. labeling threshold
 2. LOQ
- Example 1: 2 DNA extraction replicates per GM level, 2 PCR replicates per extraction/plate, 4 plates
- Intermediate precision for PCR runs require:
 - PCR on out at least on two days
 - Same operator or by an additional operator.

Experimental design for Trueness/Precision (example 1)



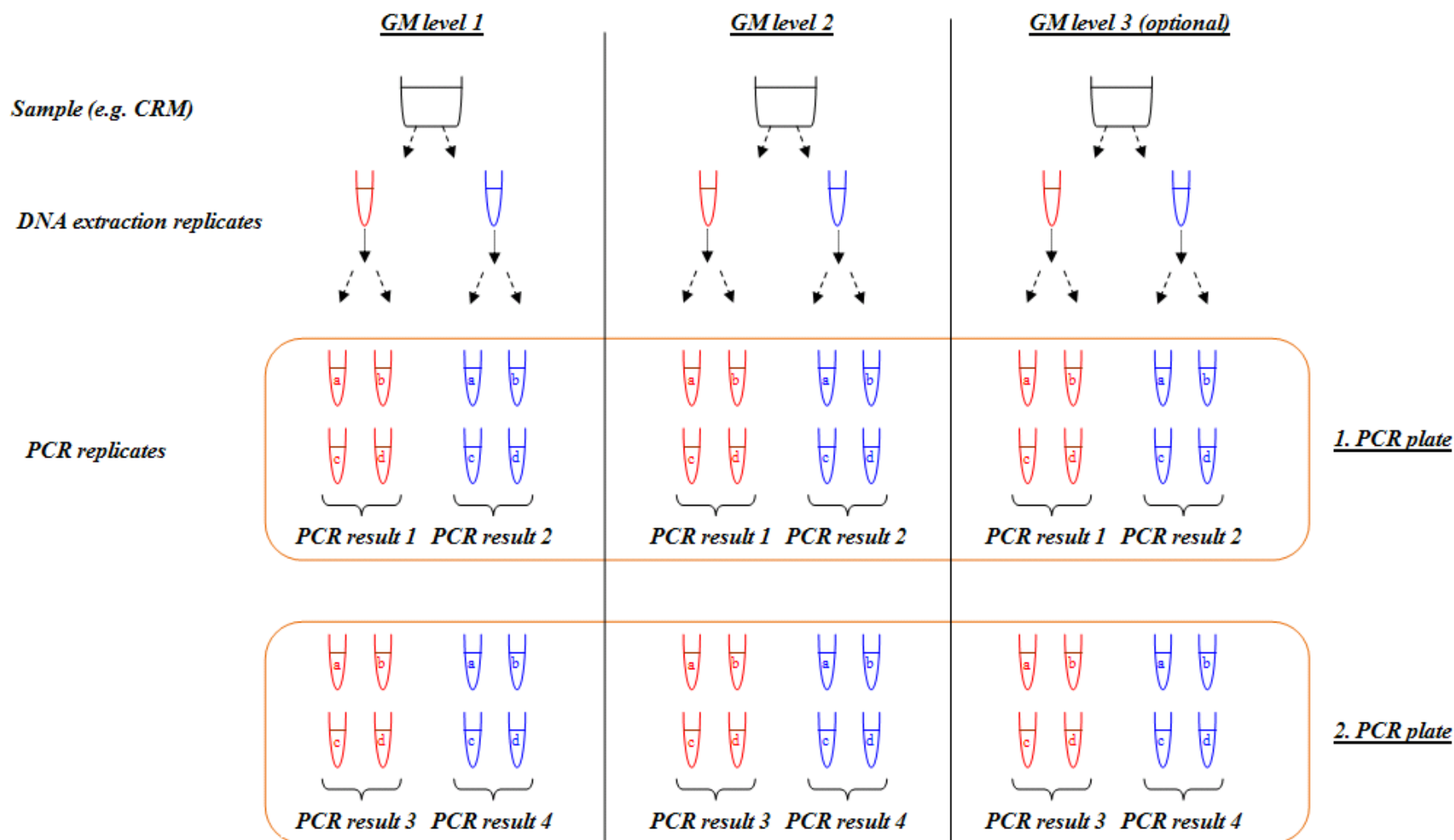


Experiments

3. **Trueness, Precision, RSD_r**

- At least 2 GM levels
 1. labeling threshold
 2. LOQ
- Example 1: 2 DNA extraction replicates per GM level, 4 PCR replicates per extraction/plate, 2 plates
- Intermediate precision for PCR runs require:
 - PCR on out at least on two days
 - Same or different operator.

Experimental design for Trueness/Precision (example 2)





Acceptance

3. **Trueness, Precision, RSD_r**

- Trueness:
 - within 25 % of the accepted reference value
 - Z-score in the range 2 and -2
- Precision determined as RSD_r
 - Relative repeatability standard deviation $RSD_r \leq 25\%$, over the dynamic range of the method.



Definitions

- Limit of detection (LOD) practical and absolute
 - The practical LOD is the lowest relative quantity of the target DNA that can be reliably (e.g. with $\geq 95\%$ probability) detected, given a known (determined/estimated) number of target taxon genome copies
 - The absolute LOD is the lowest amount or concentration of analyte in a sample, which can be reliably detected but not necessarily quantified. Experimentally, methods should detect the presence of the analyte at least 95% of the times at the LOD, ensuring $\leq 5\%$ false negative results.



Definitions

- Limit of quantification (LOQ) practical and absolute
 - The practical LOQ is the lowest **relative quantity** of the target DNA that can be reliably quantified, given a known (determined/estimated) number of target taxon genome copies
 - The absolute LOQ is the lowest **absolute quantity** of analyte in a sample, which can be reliably quantified with an acceptable level of precision and trueness.



Experiments

4. LOQ, LOD

- LOQ: 10 PCR replicates at a low concentration (e.g. 80, 60, 40, 20, 10, 5, and 1 copies). LOQ is the lowest concentration of a series where RSD of the copy number measurements are below 25% and the point is covered by the standard curve.
- LOD: 10 PCR replicates at a low concentration (e.g. 20, 10, 5, and 1 copies). LOD is then the lowest concentration in a series where all replicates are positive.



Acceptance

4. LOQ, LOD

- LOQ
 - In line with the validation data
 - 1/10 of threshold
- LOD
 - In line with the validation data
 - 1/20 of threshold



Definitions

- **Specificity**
 - The property of the method to respond exclusively to the characteristic or the analyte of interest
- **Robustness**
 - The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.



Specificity

- When to test?
 - When e.g. the master mix is changed
- What to test
 - Test available GM reference materials in high concentration
 - Relevant non GM species
- Acceptance
 - Results in line with validation data
 - No unexpected cross reactivity



Robustness

- When to test
 - When changes are expected to influence on the robustness
 - When there is no robustness data available
- What to test
 - Compare standard curves made with and without method changes
 - Vary primer concentration
 - Vary master mix concentration (e.g. +/- 20 %)
- Acceptance
 - Small variations should not influence on the assay



Annexes

- **Annex 1**
 - Effect of DNA content on the practical LOD
- **Annex 2**
 - Inhibition test for DNA preparations
- **Annex 3**
 - Preparation of new concentrations of positive material
- **Annex 4**
 - Estimation of mean, SD and RSD for randomly related replicates to calculate the GM content



Annex 1

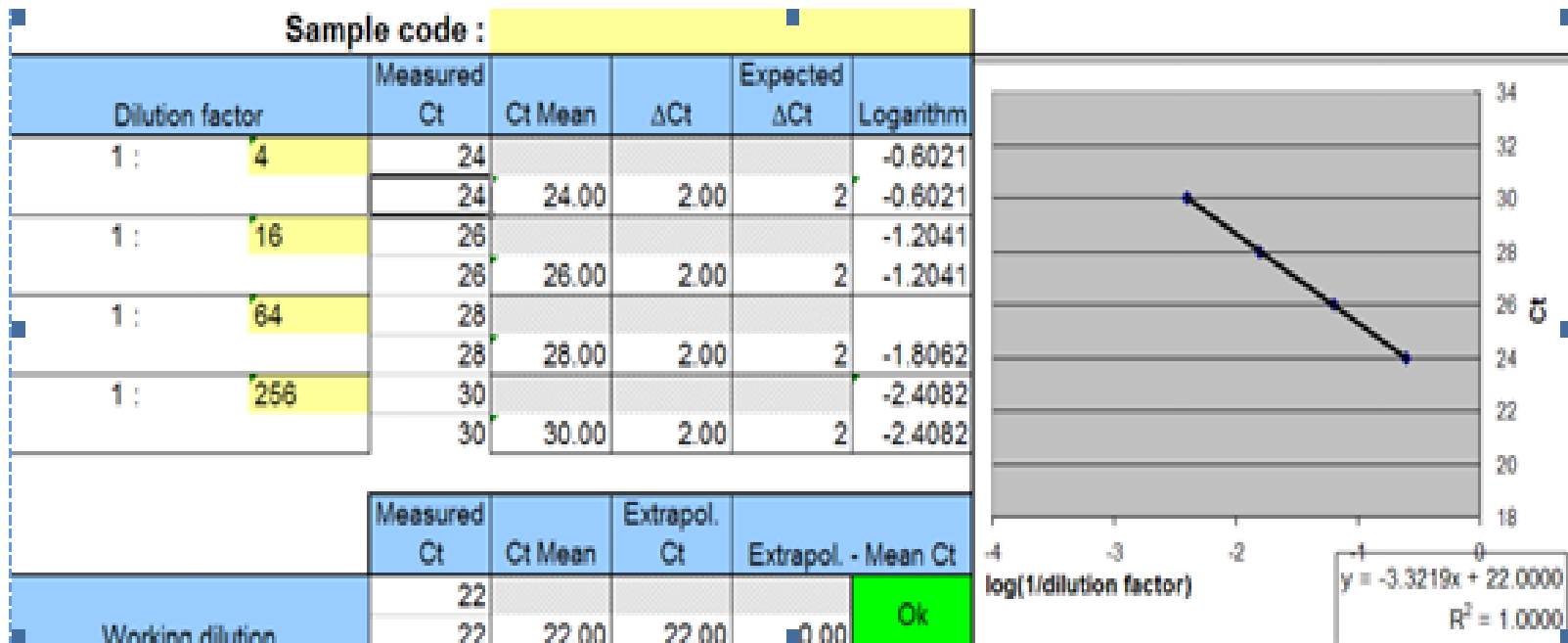
- Effect of DNA content on the practical LOD

Copies of taxon specific gene	Absolute LOD (copies of GMO target)	Practical LOD (%)
100,000	10	0.01
10,000	10	0.1
1000	10	1



Annex 2

- Inhibition test for DNA preparations



Difference between expected and extrapolated Ct should be less than 0.5



Annex 3

- **Preparation of new concentrations of positive material**

$$X = (A/B) * (Y-1) + 1$$

X = the practical dilution factor (how much the GM material has to be diluted compensated for difference in concentration)

A = copy number of reference gene for the GM positive DNA preparation

B = copy number of the reference gene for the GM negative DNA preparation

Y = the theoretical dilution factor e.g. from 100 % GM to 10 % GM = 10x



Preparation of new concentrations of positive material

DNA A = 100% GMO, DNA B = 0%

5 µL DNA is added per PCR well for A and B

Quantification as unknown sample on reference gene calibration curve:

A (from DNA A): 10 copies/5 µL

B (from DNA B): 8 copies/5 µL

To make 10 % GMO from 100 % GM corresponds to 10 times dilution (theoretically $Y = 10$).

practical dilution factor X:

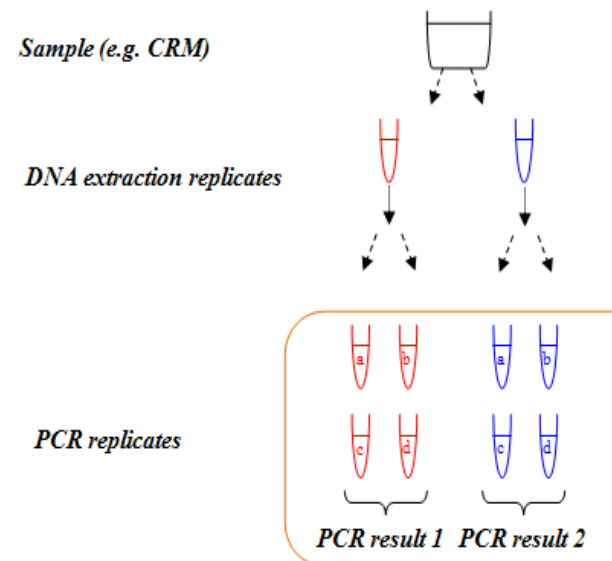
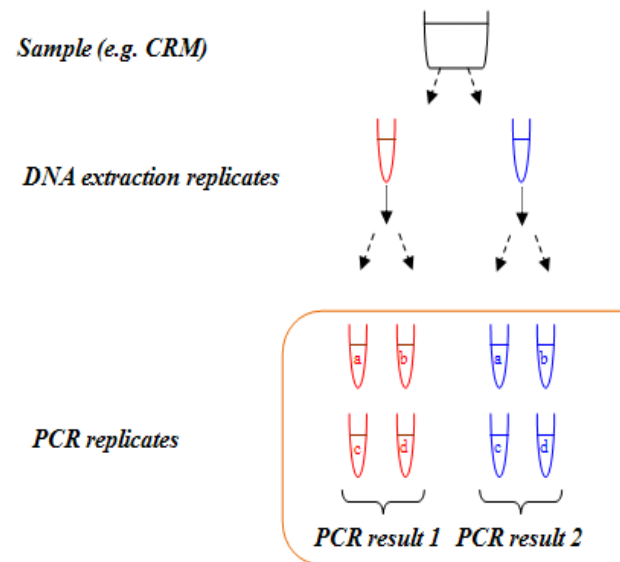
$$(10/8) \cdot (10-1) + 1 = ((10/8) \cdot 9) + 1 = 90/8 + 1 = 11.25 + 1 = 12.25$$

so 1 µl A has to be mixed with 11.25 µl B.



Annexes

- **Annex 4**
 - **Estimation of mean, SD and RSD for randomly related replicates to calculate the GM contend**





Thank you for your attention

