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Requirements and Criteria for Optimization and Validation of Real-time PCR Assays

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- **PCR: Saiki et al., Science 230 (4732): 1350-4 (1985)**
- **Real-time PCR:**
 - **Higuchi et al., Biotechnology 10 (4), 413-7 (1992),
Biotechnology 11 (9): 1026-30 (1993)**
 - **Holland et al., Proc Natl Acad Sci 88 (16): 7276-80 (1991)**
 - **Lee et al., Nucleic Acids Res 21 (16): 3761-6 (1993)**
 - **Livak et al., PCR Methods Appl 4 (6): 357-62 (1995)**

2008:

- **Real-time PCR:**
 - **allows non-laborious, reliable quantification of nucleic acid target sequences**
 - **extensive number of applications in research and in clinical diagnostics**

Real Time PCR 2008

- Majority of applications are non-commercial “in-house” developed assays
- Practical guidelines/recommendations (evidence-based or expert-based) for optimization and validation of commercial and “in-house” molecular diagnostic methods are scarce.
- ISO 15189: demands for verification and validation procedures.

Belgium: ISO 15189 certification is required for actual and future reimbursement

→ we propose a full set of stringent criteria and requirements for optimization and validation of real-time PCR assays.

Checklist for development and validation of Real-time PCR assays

- Based on literature data, existing guidelines and personal experience.
- Different steps for **development** and **validation** of commercial and “in-house” Real-Time PCR assays.
- Validation **process** and **criteria** of validation.
- **Test specific goals** are described in each test specific report of validation.



Document Aanvraag nieuwe versie Druk af Commentaar...

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Checklist voor validatie van real-time PCR testen
Lijst - Moleculaire biologie

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Dit document beschrijft de verschillende stappen bij de keuze en de validatie van real-time PCR testen. De keuze van een nieuwe testmethode voor real-time PCR is gebaseerd op literatuurstudie en andere punten vermeld in punt A. De testmethode wordt overgenomen uit de literatuur of in-house ontwikkeld. Het validatieproces en algemene validatiecriteria worden beschreven onder punt B. De specifieke, test-afhankelijke validatiedoelstellingen worden steeds beschreven in de desbetreffende validatierapporten.

Evidence Based Laboratory Medicine

- Essential part of modern medicine laboratory practice^{1,2}
 - Critically appraised topic (CAT)³
 - Checklist developed for the critical appraisal of collected evidence
 - Evidence can be considered in a hierarchy, containing all elements that are important for making a decision



- Checklist focuses on technical and diagnostic performance

References:

¹ Sackett D.L., Evidence based medicine, 1997

² Sauve et al., Ann R Soc Phys Surg Can, 1995, 28: 396-398

³ Price et al., Clin Chem, 2000, 46: 1041-50

LI.MOL.11

A/ Choice of method

- Evidence based Medicine
- Commercial assay vs in house assay
- Choice of target gene
- Choice of methodology of detection
- Choice of oligonucleotides
 - Length of primer: 18-24 base pairs
 - Length of amplicon: max 400 bp
 - GC content of oligonucleotides: 30-70 %
 - Tm of primers: 58-60 °C
 - Tm of probe: 68-70 °C
 - More C than G in probe
 - Not more than 2 C or G in last 5 positions at 3' end of primer
 - No more than 4 constitutive guanines
 - Avoid primer-dimer
- Choice of sample material and sample processing
- Quantification strategies
 - Standard curve method
 - Comparative method
- Normalisation

B/ Validation

- Verification of design of oligonucleotides (criteria: Expectation value ≤ 0.01)
- Verification of amplification:
 - SYBR GREEN: 1 single peak after melt
 - gel electrophoresis
 - sequencing + blast of amplicon
- Optimization of reaction conditions:
 - Optimization of primers and probe concentration
 - Optimization of annealing temperature
 - Optimization of sample input
- PCR characteristics
 - slope, m: $Ct = \log \text{conc} \times m + y\text{-intercept}$ (criteria: $-3.6 \leq m \leq -3.1$)
 - Efficiency, $E = 10^{-1/\text{slope} - 1}$ (criteria: $0.9 \leq E \leq 1.1$)
 - Coefficient of correlation, r^2 (criteria: $0.99 \leq r^2 \leq 0.999$)
- Analytical verification
 - Precision
 - Linearity, measuring range
 - Trueness
 - Limit of detection ($\geq 95\%$) / limit of quantification
 - Analytical specificity
- Clinical verification
 - Clinical question (CAT)
 - Clinical performance
 - Correlation to disease or disorder
 - negative predictive value
 - positive predictive value
 - Comparison to current methods / standards
- Internal Quality Control:
 - Amplification and inhibition control
 - Negative control
 - Statistical follow-up of a positive control
- Proficiency testing

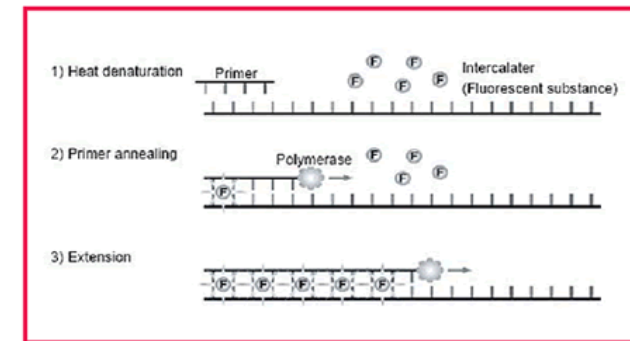
A/ Choice of method (1)

- Commercial assay or “in-house” assay
- Choice of target gene
 - specific and conserved nucleic acid sequence
 - region of aberration
 - exon-exon splice junction
 - pseudogenes

A/ Choice of method (2)

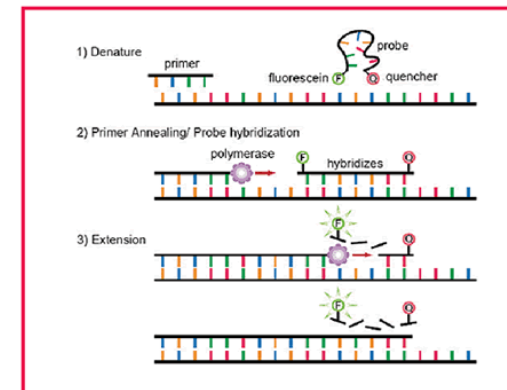
- Choice of methodology of detection¹

- Non-specific labels



SYBR® Green I Method

- Sequence specific probes



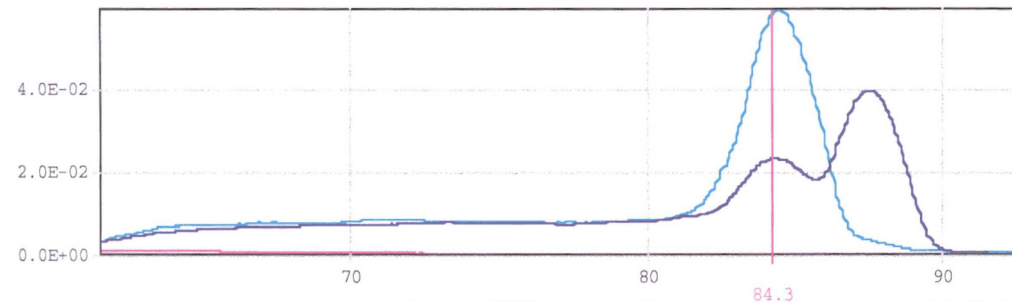
TaqMan® Probe Method

References:

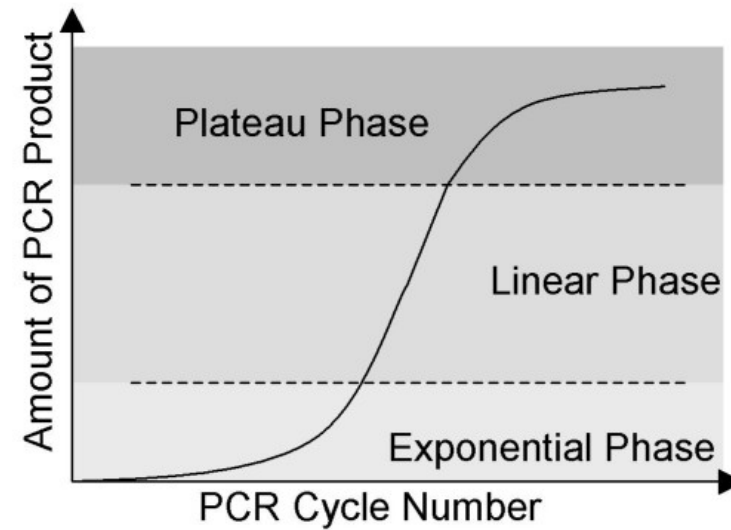
¹ Gunson et al. J Clin Virol, 2006, 35 (4): 355-67

A/ Choice of method (3)

- Melt curve analysis



- Continuous detection



A/ Choice of method (4)

- Choice of oligonucleotides^{1,2,3,4,5,6,7}
 - T_m of primers: 58-60 °C
 - T_m of probe: 68-70 °C
 - GC content of oligonucleotides: 30-70 %
 - Length of amplicon: max 400 bp
 - (Length of primer: 18-24 base pairs)
 - Not more than 2 C or G in last 5 positions at 3' end of primer
 - No more than 4 constitutive guanines
 - Avoid primer-dimer
 - More C than G in probe

References:

- ¹ Primer Express software (AppliedBiosystems)
- ² Hyndman et al., Methods Mol Biol, 2003, 226: 81-8
- ³ Breslauer et al., Proc Natl Acad Sci, 1986, 83 (11): 3746-50
- ⁴ Holland et al., Proc Natl Acad Sci, 1991, 88 (16): 7276-80
- ⁵ Mitsuhashi et al., J Clin Lab Anal, 1996, 10 (5): 285-93
- ⁶ Kubista et al., Mol Aspects Med, 2006, 27 (2-3): 95-125
- ⁷ Gunson et al. J Clin Virol, 2006, 35 (4): 355-67

A/ Choice of method (5)

- Choice of sample material and sample processing^{1,2,3}
- Quantification strategies⁴
 - Standard curve method
 - Comparative method^{5,6}
- Normalisation⁷

References:

¹ Espy et al., Clin Microbiol Rev, 2006, 19 (1): 165-256

² Hoorfar et al., APMIS, 2004, 112 (11-12): 808-14

³ Radstrom et al., Mol Biotechnol, 2004, 26 (2): 133-46

⁴ Bustin et al., J Mol Endocrinol, 2000, 25 (2): 169-93

⁵ Livak et al., Methods, 2001, 25 (4): 402-8

⁶ Pfaffl et al., Nucleic Acids Res, 2001, 29 (9): e45

⁷ Kubista et al., Mol Aspects Med, 2006, 27 (2-3): 85-125

B/ Validation (1)

- Verification of design of oligonucleotides (criteria: Expectation value ≤ 0.01)¹
- Verification of amplification:
 - SYBR GREEN: 1 single peak after melt
 - gel electrophoresis
 - sequencing + blast¹ of amplicon sequence
- Optimization of reaction conditions:
 - Optimization of primers and probe concentration
 - Optimization of annealing temperature
 - Optimization of sample input

References

¹ <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>

B/ Validation (2)

- PCR characteristics¹
 - slope, m: $Ct = \log \text{conc} \times m + y\text{-intercept}$ (criteria: $-3.6 \leq m \leq -3.1$)
 - calculated by linear regression
 - inflection point present
 - $m = -3.3219$ give efficiency of 1 (ideal)
 - Efficiency, $E = 10^{-1/\text{slope}} - 1$ (criteria: $0.9 \leq E \leq 1.1$)
 - equal amplification efficiencies between quantification standards and unknown test samples
 - sample-specific amplification efficiencies: sigmoidal² or logistic³ curve fitting (theoretically 2)
 - Coefficient of correlation, r^2 (criteria: $0.99 \leq r^2 \leq 0.999$)

References

¹ Bustin et al., J Biomol Tech, 2004, 15 (3): 155-66

² Rutledge et al., Nucleic Acid Res, 2004, 32 (22): e178

³ Tichopad et al., Nucleic Acid Res, 2003, 31 (20): e122

B/ Validation (3)

- Analytical verification
 - Precision
 - Linearity, measuring range
 - Trueness
 - Limit of detection (CI \geq 95 %) / limit of quantification
 - Analytical specificity
- Clinical verification
 - Clinical question (CAT)
 - Clinical performance
 - Correlation to disease or disorder
 - negative predictive value
 - positive predictive value
 - Comparison to current methods / standards

B/ Validation (4)

- Internal quality control
 - Amplification and inhibition control
 - cell rich specimens: human gene
 - cell free specimens: synthetic internal control
 - negative control
 - extraction and amplification
 - statistical follow-up of a positive control (reference material)
 - near the limit of detection
 - reliable results
 - Quantitative assays: at least 2 concentrations¹
 - target value: 20 measurements on different days²
- Proficiency testing
 - QCMD, INSTAND, UKNEQAS, College of American Pathologists,...
 - Ring test (twice a year)³

References:

¹ CLSI MM6-A

² Westgard Tietz textbook of clinical chemistry, 1999

³ CLSI GP29A

Conclusion (1)

1. We hereby propose **practical** guidelines for the optimization and validation of commercial and “in-house” developed Real-Time PCR Assays. The proposed checklist describes **requirements and stringent criteria** that allow **standardisation** of the complete process.
2. In our experience, the use of the proposed guidelines leads to a more **efficient optimization and validation** of commercial and “in-house” diagnostic assays.
3. Since it is independent of **environment, equipment and specific applications**, it is **exchangeable** between laboratories worldwide.
4. Ultimately, a **worldwide consensus** on this kind of checklist should be aimed at.

Conclusion (2)

The proposed checklist is a **crucial** step in **harmonization of differing methodologies**. These recommendations include also use of matrix for analysis, appropriate standards, reference material, calibrators and international scale of measurement.