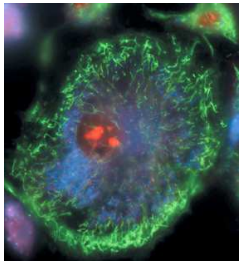

Belgische Vereniging voor Analytische Cytologie vzw.
Association Belge de Cytologie Analytique asbl.
Belgian Society for Analytical Cytology.

SCK•CEN
STUDIECENTRUM VOOR KERNENERGIE
CENTRE D'ETUDE DE L'ENERGIE NUCLEAIRE

Module n°: ABC 503

Title : Validation of PCR based assays

Lecturer : Raymaekers Marijke

Molecular Biology and Cytometry Course, May 7-8 2009, Mol

2008:

- **Guidelines/recommendations Real Time PCR:**
  - NCCLS document MM6-A [ISBN 1-56238-508-9] (2003)
  - Gunson et al. Journal of Clinical Virology 35: 355-367 (2006) (Clinical Virology)
  - Sloan et al. Clinical Microbiology Newsletter 29 (12): 87-95 (2007) (Clinical Microbiology)
- **Guidelines/recommendations (diagnostic) PCR:**
  - Malorny et al. Intern. J. of Food Microbiology 83, 39-48 (2003) (Food Borne Pathogens)
  - Hoorfar et al. APMIS 112, 808-14 (2004) (Food and Veterinary)
  - Noordhoek et al. Werkgroep Moleculaire Diagnostiek, March 2007
  - Rabenau et al. Journal of Clinical Virology 40, 93-97 (2007) (Clinical Virology)
  - Organisation for Economic Co-operation and Development, http://www.oecd.org/dataoecd/43/6/38839788.pdf
  - Amos J, et al. American college of medical genetics: Technical standards and guidelines for CFTR mutation testing. 2006.
  - College of American Pathologists, Laboratory accreditation program, accreditation checklists. 2008.
  - Jennings et al. Arch Pathol Lab Med—Vol 133, May 2009

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Checklist for development and validation of Real-time PCR assays

- Based on literature data, existing guidelines and personal experience.
- Different steps for choice 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...

Klinisch Laboratorium
Virga Jesseziekenhuis
Stadsomvaart 11 3500 Hasselt
TEL 011-30 97 40 - FAX 011-30 97 50

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

DocNr	Versie	Status	Gewijzigd door
LI.MOL.11	2	Definitief	Marijke Raymaekers
Aangemaakt op	**Verstuurd op**	**Geldig van**	**Geldig tot**
27/07/2007	27/07/2007	26/09/2007	26/09/2010

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.

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Evidence Based Laboratory Medicine

- Essential part of modern medicine laboratory practice[1,2]
  - ➢Critically appraised topic (CAT)[3]
    - Checklist developed for the critical appraise 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

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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 ℃
    - Tm of probe: 68-70 ℃
    - 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 conc x m + y-intercept (criteria: - 3.6 ≤ m ≤ - 3.1)
    - Efficiency, E = 10-1/slope – 1 (criteria: 0.9 ≤ E ≤ 1.1)
    - Coefficient of correlation, r^2 (criteria: 0.99 ≤ r2 ≤ 0.999)
- Analytical verification
    - Precision
    - Linearity, measuring range
    - Trueness
    - Limit of detection (≥ 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)

- Choice of assay: CE-IVD/FDA or “in-house”: sometimes no commercial assays available for clinical important parameters
- Choice of nucleic acid target:
  1. General
     - Avoid G+C rich regions
  2. Microbiology
     - Specific and conserved nucleic acid sequence, e.g.: 5’NCR, conserved region of DNA polymerase gene,…
     - Avoid regions of resistance
  3. Haematology
     - region of aberration (literature review)
     - Primers and probes should span exon-exon splice junction for reverse transcriptase (no co-amplification of gDNA)
     - Pseudogenes

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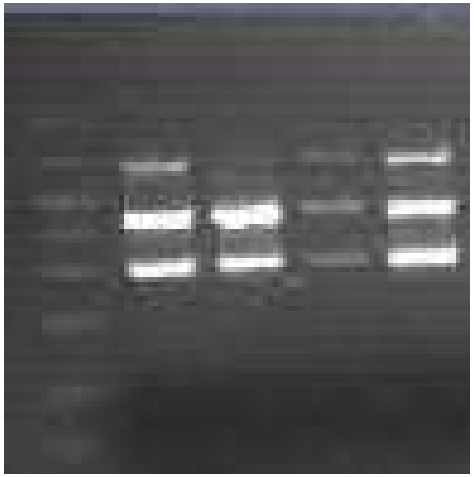
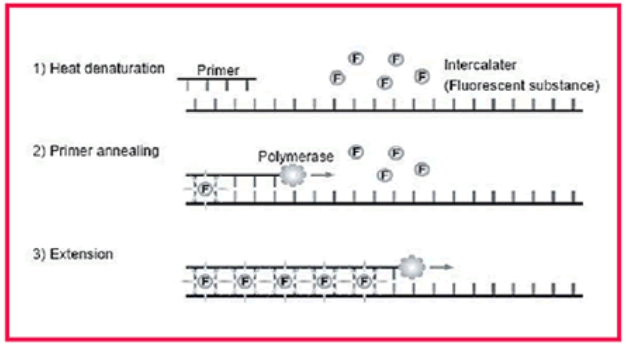
A/ Choice of method (2)



- Choice of methodology of detection

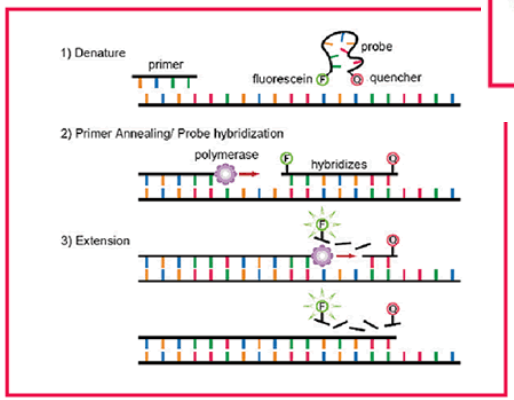
1. Conventional PCR: gelelectrophoresis

2. Real time PCR: monitoring of fluorescence
   - ➢ Non-specific labels



SYBR® Green I Method

   - ➢ Sequence specific probes



TaqMan® Probe Method

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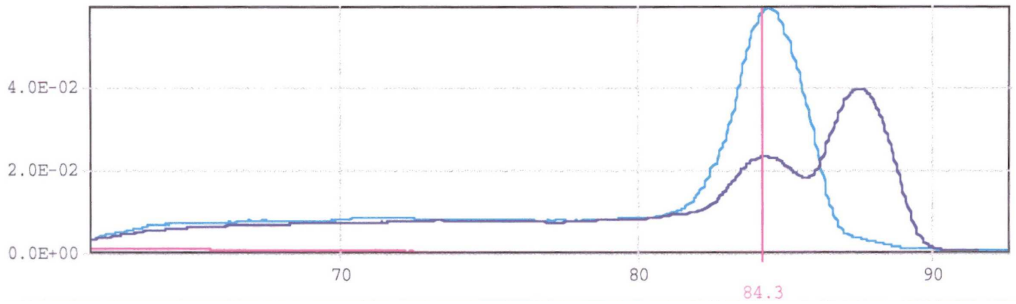
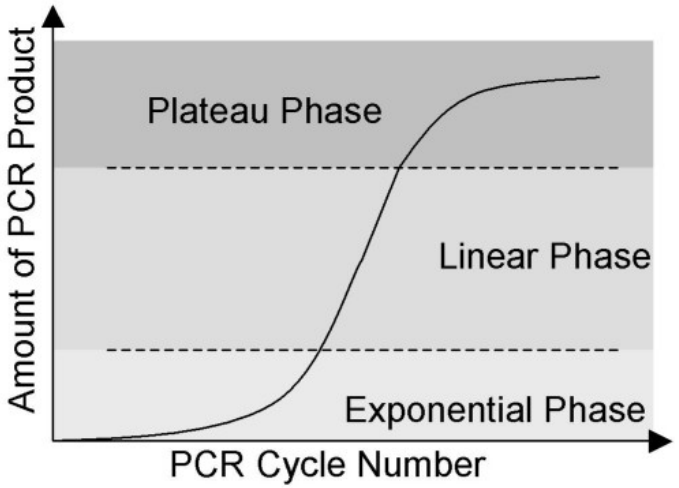
A/ Choice of method (3)

- Melt curve analysis: detection of any ds DNA, mis-priming events!!!



- Continuous detection: exponential phase: increase of fluorescence is directly proportional to amount of starting material



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A/ Choice of method (4)

- Choice of oligonucleotides[1,2,3,4,5,6,7]: a critical step for a successful PCR
  1. Tm is indicator for hybridisation strength of oligonucleotides (nearest-neighbour model)

     For annealing and extension at 60℃:
     - Tm of primers: 58-60 ℃
     - Tm of probe: 68-70 ℃ (5-10℃ higher than for prime rs)
  2. GC content of oligonucleotides: 30-70 %
  3. Length of amplicon
  4. (Length of primer: 18-24 base pairs)
  5. Not more than 2 C or G in last 5 positions at 3' end of primer
  6. No more than 4 constitutive guanines
  7. Avoid primer-dimer
  8. 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

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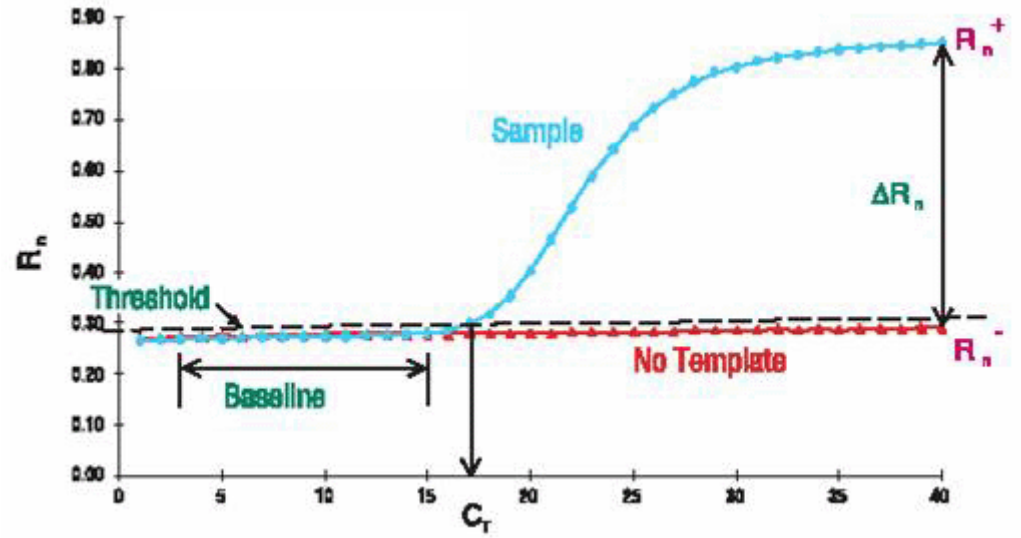
A/ Choice of method (5)



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A/ Choice of method (6)

1. Choice of sample matrix and sample processing[1,2,3]
   - ➢ specimen must be representative of disease (literature):
     - describe population
     - describe sample type: sample specific validation (matrix induced effects)
   - ➢ Efficient sample processing:
     - Remove PCR inhibitors
     - Concentrate NA
2. Quantification strategies[4]
   - ➢ Standard curve method (exponential phase)
     - Absolute
     - Relative
   - ➢ Comparative method[5,6]

     ΔΔCt or method of Pfafll
3. Normalisation[7] (compensate for the differences in amount of biological material)

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

A/ Choice of method (7)

Relative changes in expression of the target gene:

- When PCR efficiencies are the same:

  ΔCt= Ct (target sequence) - Ct ("housekeeping" gene)

  $\Delta\Delta Ct = \Delta Ct$ (calibrator) - ΔCt (staal)

  $R = 2^{-\Delta\Delta Ct}$ R= relatieve expressie

- When PCR efficiencies are different:

$$R = \frac{(1 + E_{\text{target sequence}})^{\Delta Ct\ \text{target sequence (control - sample)}}}{(1 + E_{\text{reference gene}})^{\Delta Ct\ \text{reference (control - sample)}}}$$

E= efficiency

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 + blast[1] of amplicon sequence
- Optimization of reaction conditions: efficient an specific PCR
  - Optimization of primers and probe concentration: reduce primer-dimer: sensitive and efficient PCR
  - (Optimization of dNTP, Taq polymerase and $MgCl_{2)}$
  - Optimization of annealing temperature
  - Optimization of sample input: maximal sensitivity, minimal inhibition

References

[1] http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

B/ Validation (2)

1. Optimization of primer and probe concencentrations:

- optimization matrix, using 2 ten-fold dilutions of a pos control, near the LOD
  voor dual labeled probes:
  Primers: 50, 300 en 900 nM in mix
  Probe: 50, 100, 150, 200 nM in mix
  voor SYBR green: Primers: 50, 100, 200 en 300 nM in mix
  - optimal combination: lowest treshold cycle, highest Rn, difference in Ct value~ 3

2. Materials :

- Reference material (clear statement)
  - proficiency panels
  - panels from commercial companies
  - Cell lines
  - Standards from NIBSC
- Samples analysed with a second method (third method):
  - patient samples
  - (spiked samples)

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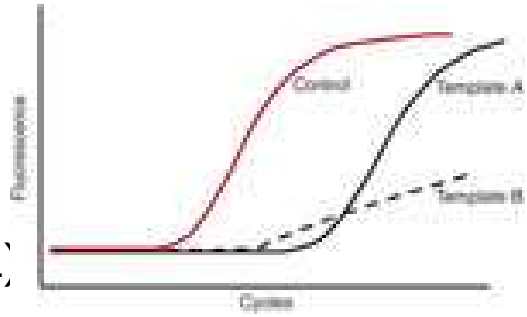
B/ Validation (3)

- PCR characteristics[1](standard curves based on serial dilutions, each in triplicate)
  - slope, m: Ct = log conc x m + y-intercept (criteria: - 3.6 ≤ m ≤ - 3.1)
    - calculated by linear regression
    - inflection point present
    - m= - 3.3219 give efficiency of 1 (ideal)
  - Efficiency, E = $10^{-1/slope}$ - 1 (criteria: 0.9 ≤ E ≤ 1.1)
    - equal amplification efficiencies between quantification standards and unknown test samples
    - sample-specific amplification efficiencies: sigmoidal[2] or logistic[3] curve fitting (theoretically 2)
  - Coefficient of correlation, r^2 (criteria: 0.99 ≤ r^2 ≤ 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

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B/ Validation (4)

- Analytical verification
  - Precision
  - Linearity, measuring range
  - Trueness
  - Limit of detection (CI ≥ 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 (5)

- Analytical verification
  - ➢ Precision
    - CLSI MM6-A: at least 2 concentrations, between run, between day
    - CLSI EP15: one run per day with three replicate samples at each of two concentrations daily for five days (commercial assays).
    - Rabenau:
      - ✓ 2 – 6 (intra-assay, commercial); 2 – 9 (intra-assay, in-house)
      - ✓ 2 – 2 (inter-assay, commercial); 2 – 3 (inter-assay)

B/ Validation (6)

- Analytical verification
  - ➢Linearity, measuring range
    - CLSI MM6-A: performed for the overall system, from nucleic acid extraction to detection and quantitation
    - CLSI EP6:
      - ✓Commercial assays 5 to 7 concentrations chosen throughout the stated linear range, 2 replicates at each level
      - ✓ Developers of new methods should use 7 to 11 concentrations, 2 to 4 replicates
    - Rabenau:
      - ✓ 0 – 1 (commercial assays)
      - ✓ 0 – 2 (in-house assays)

B/ Validation (7)

- Analytical verification
  - Trueness
    - CLSI EP15: 20 patient samples in duplicate distributed evenly over the entire measuring interval (commercial assays).
    - Rabenau:
      - ✓ 9 samples (commercial assays)
      - ✓ 9 samples (in-house assays)
  - Limit of detection / quantification
    - CLSI EP17: a minimum of 20 results at the claimed level
    - Rabenau: 20 – 20 (in-house assays)
  - Analytical specificity
    - Rabenau: 20 – 20 (in-house assays)

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B/ Validation (8)

- 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 concentrations[1]
    - target value: 20 measurements on different days[2]
- 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

- Requirements and criteria for optimization and validation of real-time PCR assays, Raymaekers M, Smets R, Maes B, Cartuyvels R.
  Published in J. Clin. Lab. Anal. 23:145-151, 2009
- Reflections and proposals on quality assurance of molecular diagnostic tests in Belgium, Raymaekers M, Bakkus M, Boones E, Derijke B, Descheemaeker P, Deschouwer P, El Housni H, Franke S, Hillen F, Nollet F, Soetens O, Vankeerberghen A, on behalf of the moleculardiagnostics.be working group.