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PCR validation

August 2014

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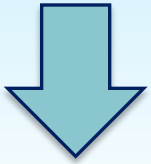
TopLab

Validation of PCR test

Primers, probes has been designed. PCR protocol developed.

- Does the new developed assay meet all performance criteria?
- Is the outcome clinically relevant?



VALIDATION OF THE PCR TEST

Validation of PCR test

1. First line: infra structure lab, performance and management lab
2. Second line: design PCR for specific target(s)
3. Second line: technical validation (IQA)
4. Second line: clinical validation, concordance test (gold standard method)
5. Third line: EQA

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The PCR reference curve

technical validation part 1

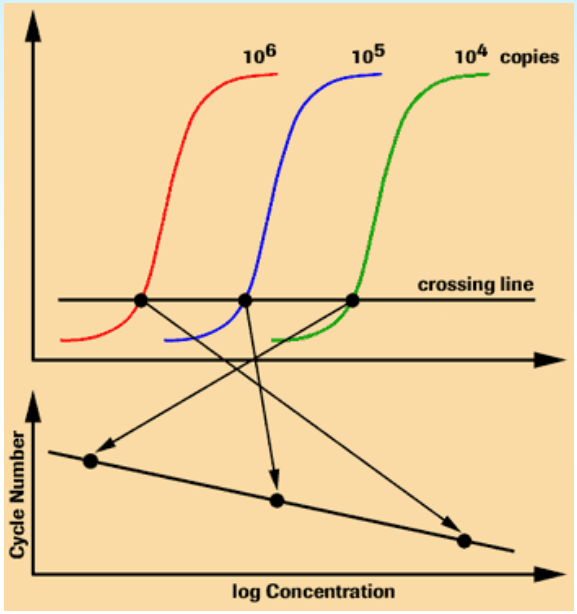
TopLab

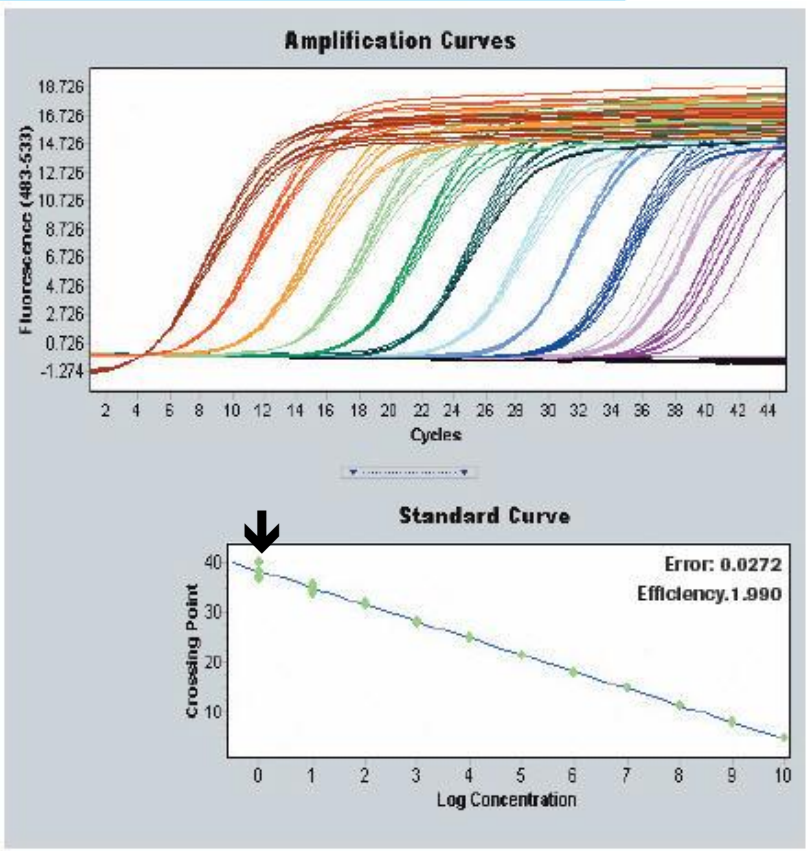
Applications of the PCR refernce curve

- Qualitative identification of the sample
- To identify the ‘analytical range’, LLOD, LOD and LOQ of a (RT)-qPCR analysis
- To compare the curves e.g. for the optimization of single- and multiplex (RT)-qPCR using the slope of the curve
- TAKE CARE: quality requirements for reference curve

PCR reference curve

– Inverse association between C_q and initial concentration (viral- or bacterial load)





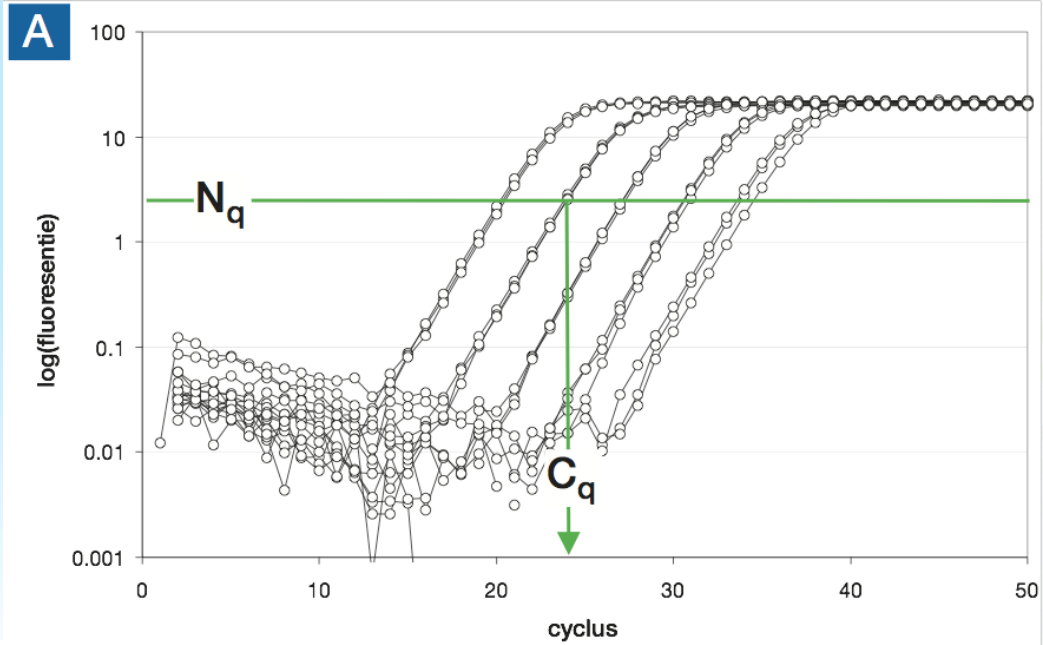
Q with respect to PCR reference curve

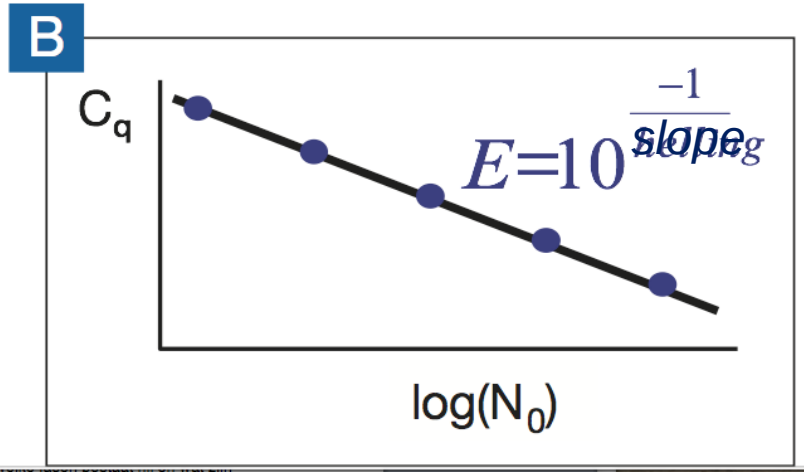
- The distance between the consecutive curves in a 10 Log dilution series of the sample is ideally 3.3 Cq-units
- Watwhat is happening when the distance becomes 4?
- In that case, what happens with the slope? Steeper or flatter?
- What is the ideal slope?

- What happens with the Cq value when by-products appear using an:
  - Intercalating dye?
  - Probe?

Derivative of PCR reference curve

Y= αX+b
α=slope





C

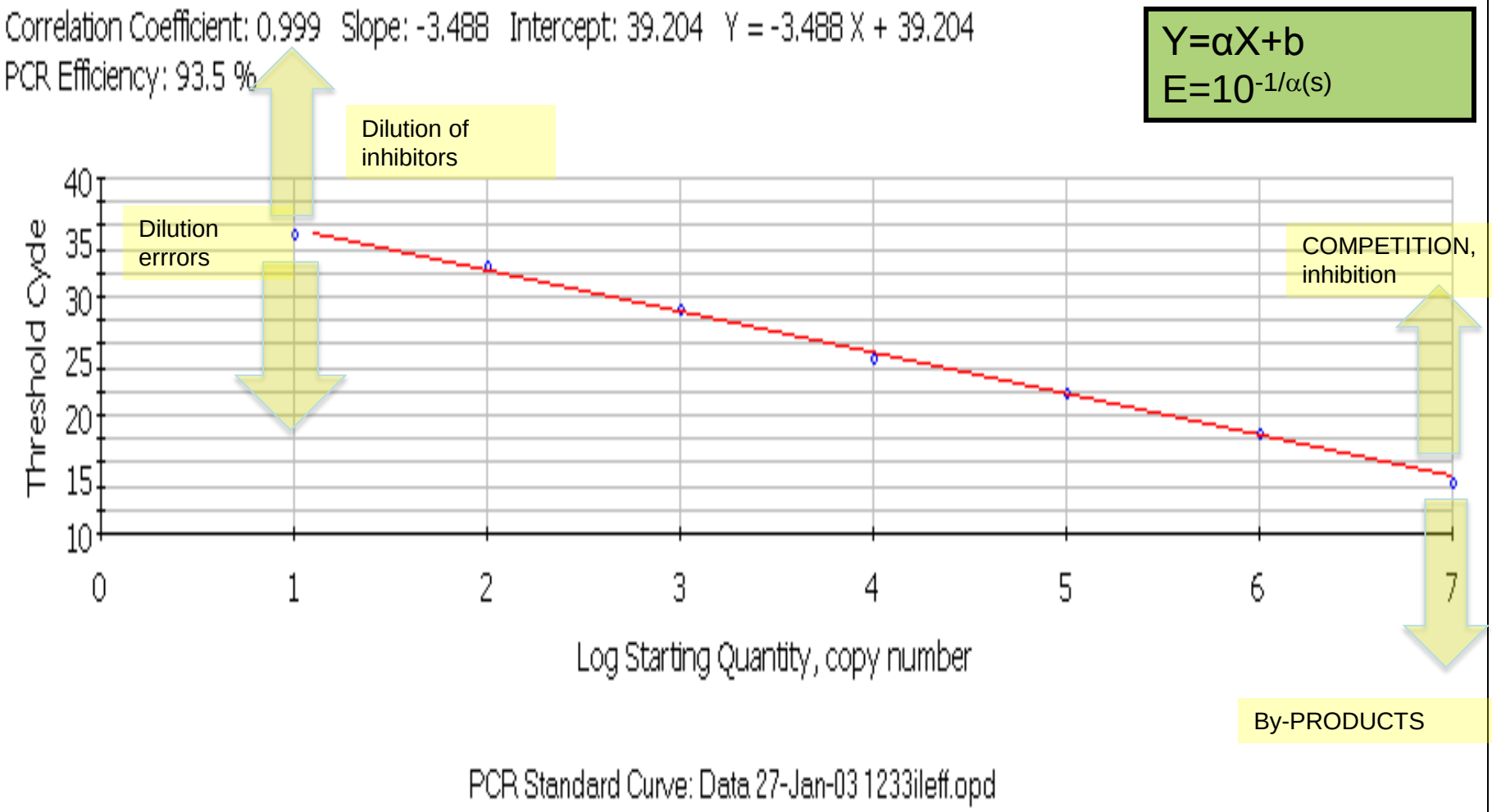
slope	efficiency
helling	efficiëntie
-3.10	2.1
-3.32	2.0
-3.59	1.9
-3.92	1.8

Requirements for the PCR reference curve (validation)

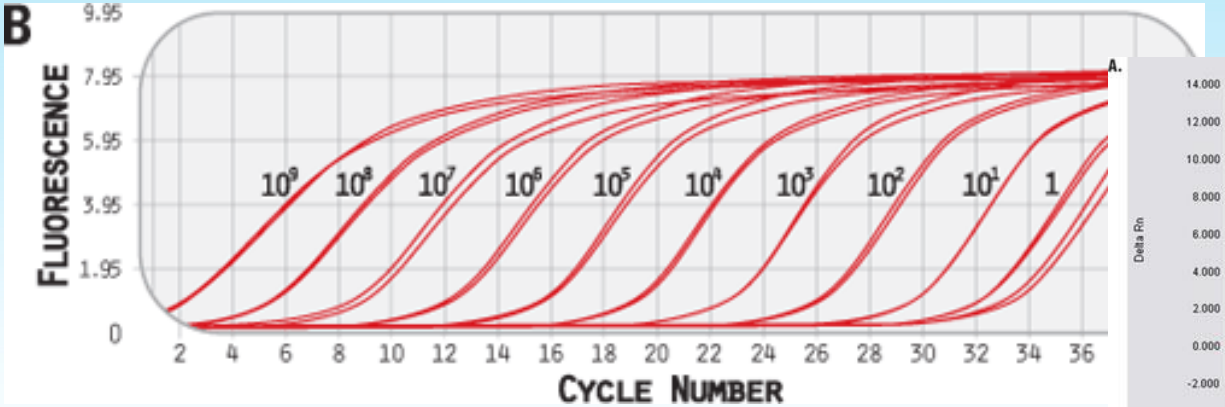
- Dilution series (10X): at least 5 log scales
- Datapoint: 3-5 times in separate tests (for SD calculation)
  - SD max <5 % per datapoint
- Reference curve:
  - Linear correlation R ≥0.985 (or R^2 ≥0,95)
  - Slope of linear line: -3,1 tot -3,6
  - Usable area (analytical range)
    - All points with SD <5%
    - At least 10xSD difference with negative sample
  - Run to run variation <5%

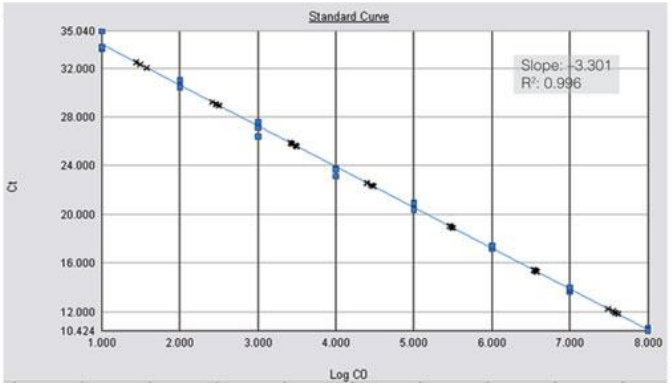
- TAKE CARE: dilution reagent (saline, water, matrix) is not always stable!

Interpretation of the PCR ref curve



What is striking in the 10-fold dilution series (2)?





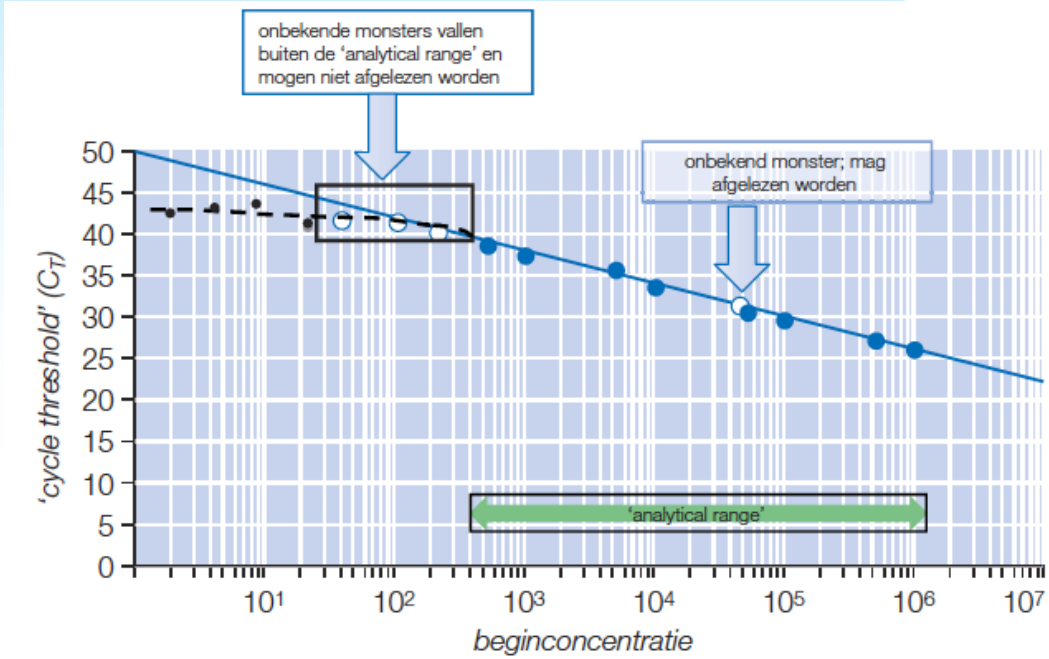
Determine the analytical range

- Using the refernce curve (used as a calibrationcurve)
  - SD criterium (5%), datapoints (upper- and lower level) and known concentrations
    - Upper level:dilute sample
    - Lower level: more complex, for reasons of false negativity, probability of detection and proper cut-off value

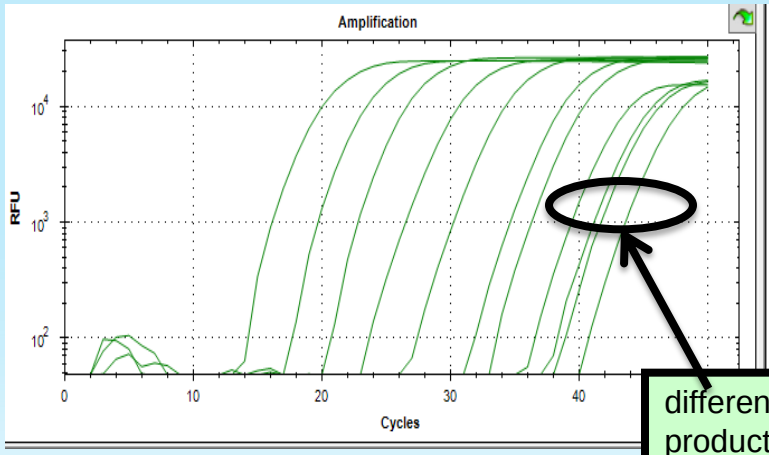
Refernce curve: ‘analytical range’

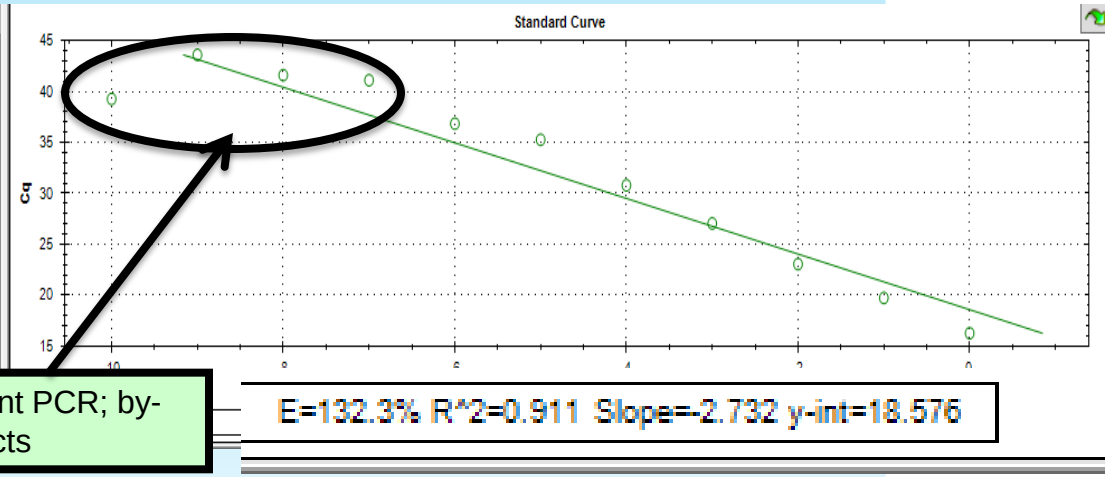
- Analytical range:
  - Concentration area in which test results are reliable (also useful area for quantification)

- Cq > LOD?
  - Results not reliable for diagnostics

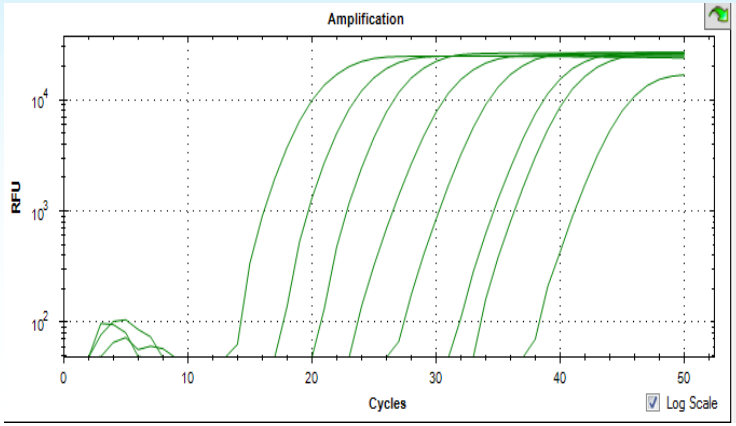


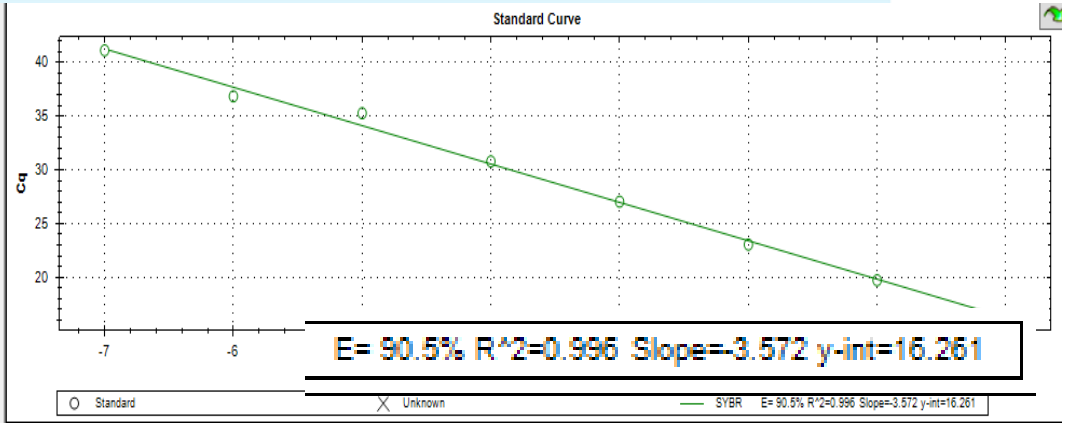
Optimal PCR results: same plateau, regardless the concentration



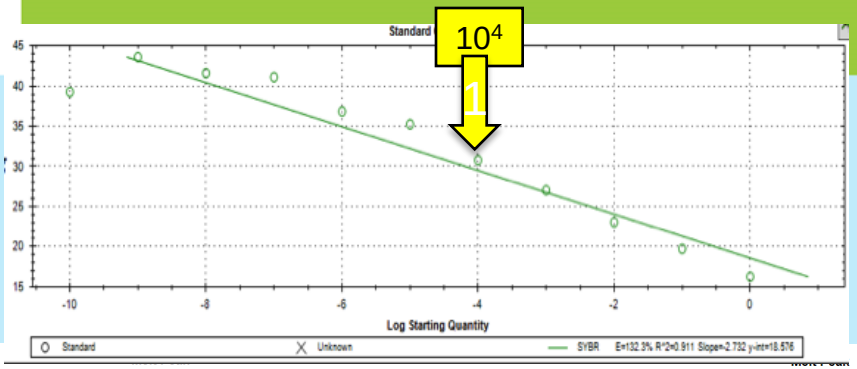


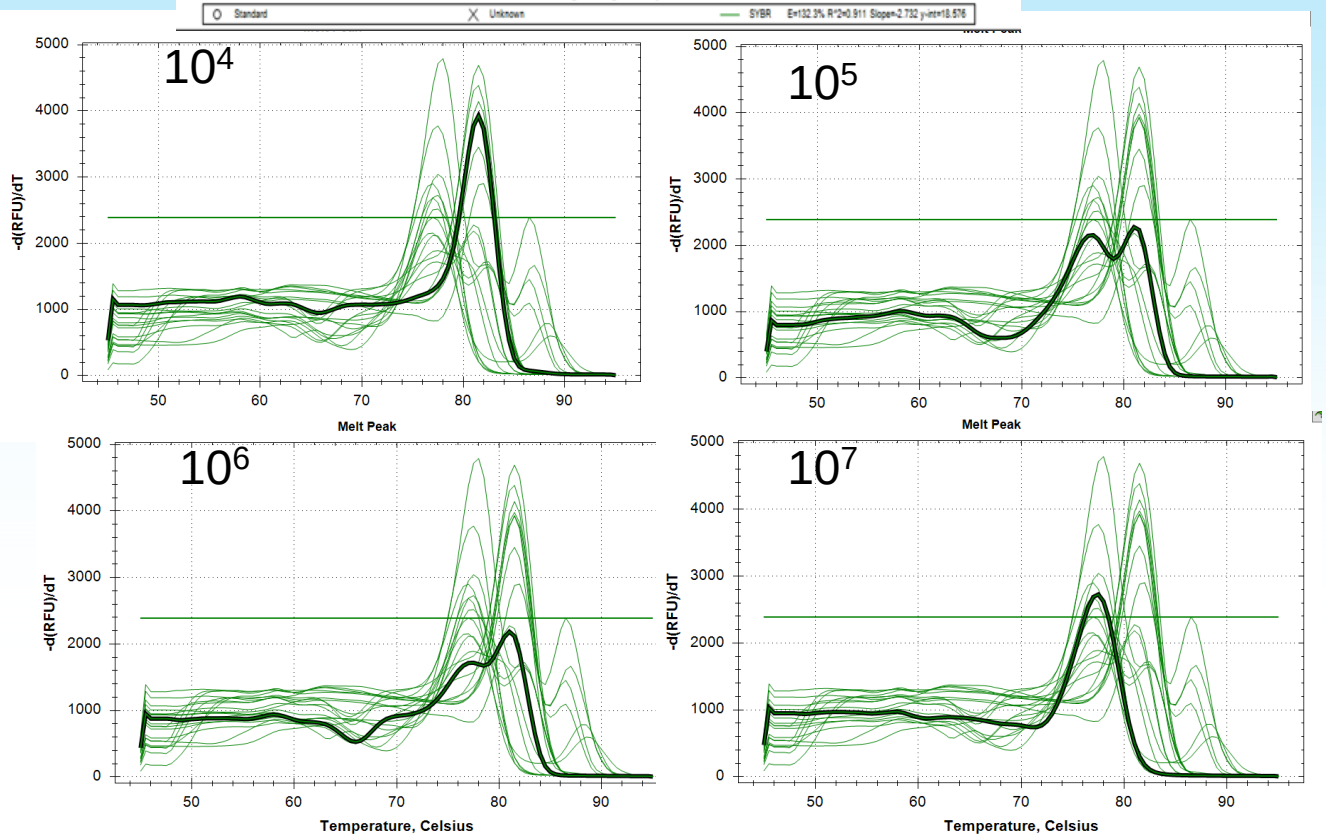
different PCR; by-products





Which datapoints are suitable?



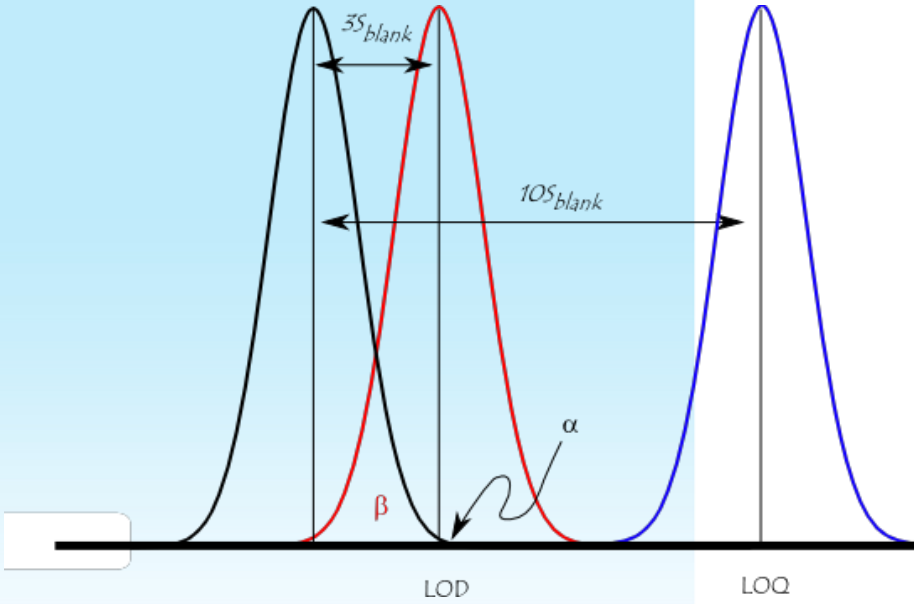


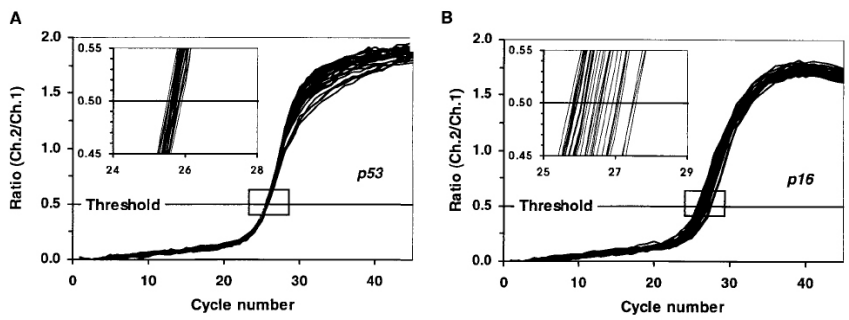
- Check with melting curve analysis!
- 10^5 should be excluded! Shape of curve is OK! watch ΔCq

- Can you extrapolate to define ‘cut off’ (interception Y-axis)?

LOD, LOQ

- LOD: lowest concentration of sample where >95% of the testruns give positive results (stability of the test)
- LOQ: highest and lowest concentration of a sample that can be detected with acceptable precision and accuracy





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Quality assurance PCR design

technical validation part 2

TopLab

PCR specificity on different levels

- Primer specificity
- Amplicon specificity
- Specificity method to determine
  - Understanding FP; FN results
  - “fit” analytical range with sample?
- Diagnostic specificity
  - Differentiation between “health/disease”?

Validation: IQA

1. Start with clean target DNA (culture) in clean environment (water, buffer) – ref curve
2. Start with known POS/NEG samples, select strong pos, weak but consistent pos and negative samples + NTC (develop IC)
- Proper results with standards and controls
3. Same in matrix (check again analytical range)

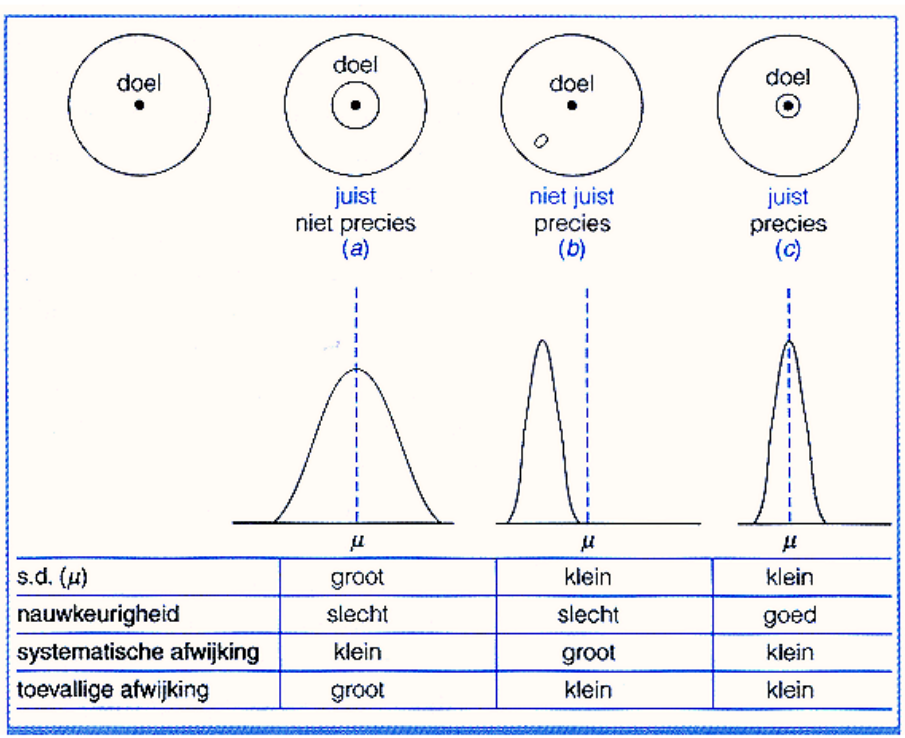
Technical validation: quality criteria

- sensitivity
- Analytical sensitivity/specificity
  - using ref curve

- Accurate
  - Correctness and precision
- Reproducibility

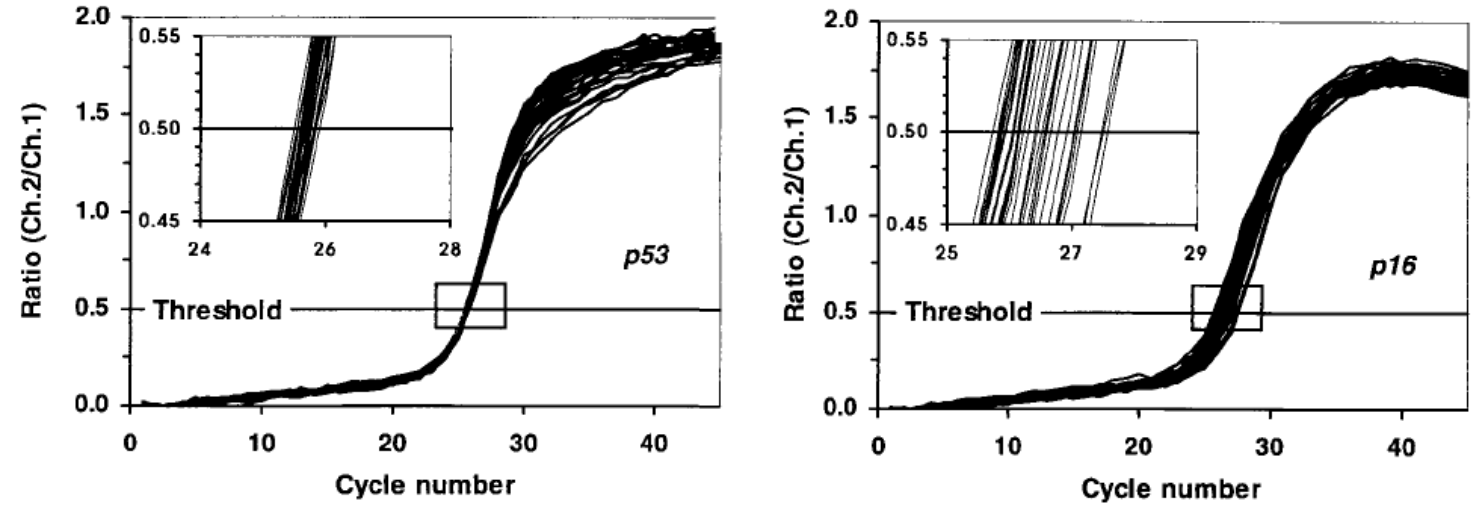
Quality criteria Accuracy; balansce between correctness and precision

- Amplicon
  - correct:
    - Length
    - sequence
  - incorrect:
    - Mispriming
    - FN
  - Not precise:
    - Accidental and systematic mistakes
    - [Assessment runs]



s.d. (μ)	groot	klein	klein
nauwkeurigheid	slecht	slecht	goed
systematische afwijking	klein	groot	klein
toevallige afwijking	groot	klein	klein

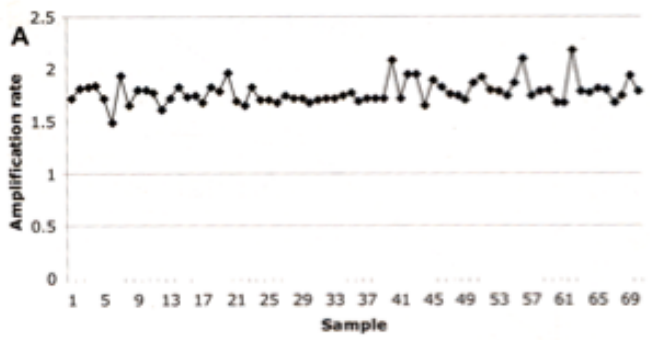
Quality criteria: precision, difference between PCRs



- Two targets
  - correctness same
  - Precision differs
    - Important in reaction control and monitoring

quality criteria: reproducibility

- Int quality controles
  - Run to run (reproducibility)
  - Certification of reagents
    - parallel assays
    - Helps with identification issues
      - loss of quality in current batch
      - Deviations in new batch
    - Use for Taq DNApol, primers, probes, controls, standards
- Monitoring of controls (plot)



Validation of a new test; concordance

- determine:
  - Accuracy
    - Control samples; known pos/neg samples
  - Method to calculate specificity/sensitivity
  - Diagnostic specificity/sensitivity
    - Influence of FP/FN on convenience of test
    - Define a reference method (objective/indepent)
      - Culture, microscopy, ELISA
      - Ref methods is existing diagnostics: gold standard
    - Determine FN; FP

Concordance test (= association between tests)

- observed concordance
  - test A
    - PCR
    - conventional
    - Single plex
  - test B
    - Real Time
    - multiplex

	methode A	
methode B	POS	NEG
POS	a	b
NEG	c	d

$$\text{Observed concordance} = \text{Obs} = \frac{a + d}{a + b + c + d}$$

Concordance test; using known POS/NEG samples

- 100%
  - c and b zero
- <100%
  - Different results
  - method B new
    - more POS (b): test better; precision ↑
    - more POS (c) test worse; preciesion □

	methode A	
methode B	POS	NEG
POS	a	b
NEG	c	d

$$\text{Observed} = \text{Obs} = \frac{a + d}{a + b + c + d}$$

concordance

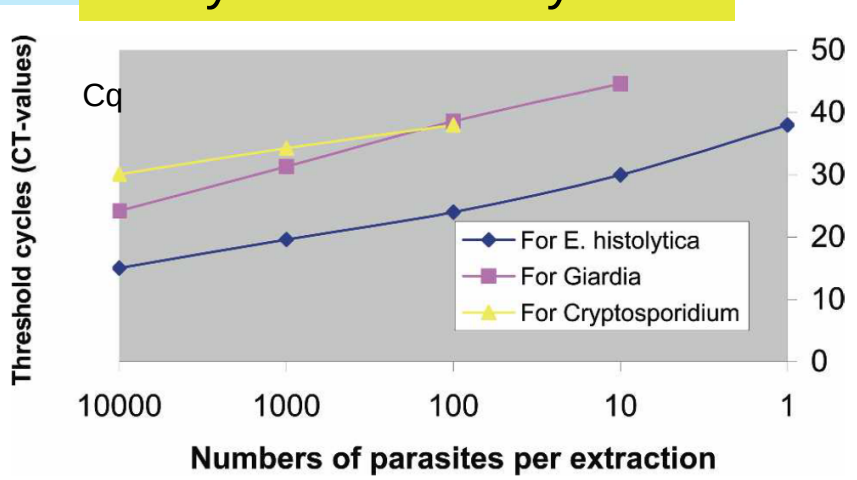
Clinical samples: Concordance test (C3)

TABLE 2

Stool samples diagnosed according to "gold standard" diagnosis and singleplex and multiplex real-time PCR assays

Organisms	"Gold standard" diagnosis	Singleplex real-time PCR	Multiplex real-time PCR
E. histolytica only	19	20	19
G. intestinalis only	29	27	28
Cryptosporidium spp.	25	22	21
E. histolytica and *G. intestinalis*	21	19	17
E. histolytica and *Cryptosporidium* spp.	2	3	2
Cryptosporidium spp. and *G. intestinalis*	4	4	5
Negative for all three parasites	29	34	37
Total	129	129	129

Analytical sensitivity



E. histolytica

"Gold standard diagnosis"	Multiplex real-time PCR assay		Singleplex real-time PCR assay	
	Positive	Negative	Positive	Negative
Positive	36	6	40	2
Negative	2	88	1	86
	38	91	41	88

ELISA versus single/multiplex PCR

HAQUE Am. J. Trop. Med. Hyg., 76, 2007,713

Endphase implementation test

- make a SOP
- define
  - 1e, 2e, line controls (IQA)
  - Planning 3e line controls (EQA)
- Monitor controls

Surveillance of PCR quality; diagnostic validation

validation part 3

Kwaliteitsborging; 3 niveau's

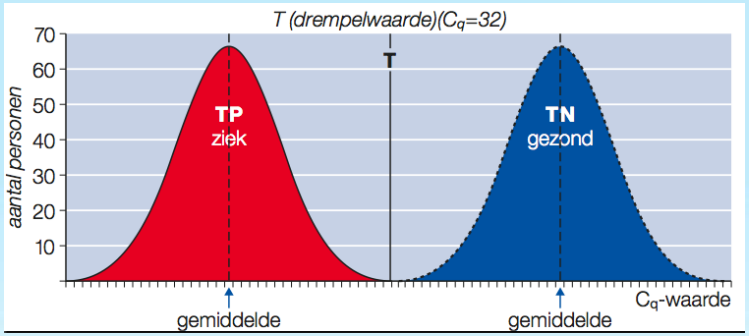
- Lab organisation/-management 
- Technical PCR test 
- Special requirements test for clinics
  - Healthy and disease correctly classified with test
    - disease: test POS
    - healthy: test NEG

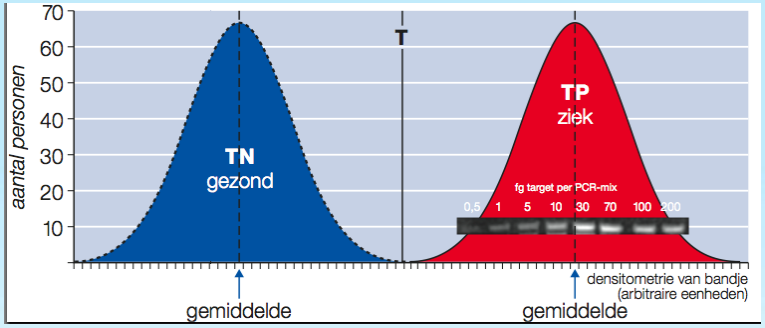
Specificity of PCR; in diagnostics

- Clinician:
  - yes/no answer
- Differential diagnose
  - yes/no not “sharp” defined
  - Additional info is needed (other tests; clinical anamnesis)
  - Important: risk on……. / probability

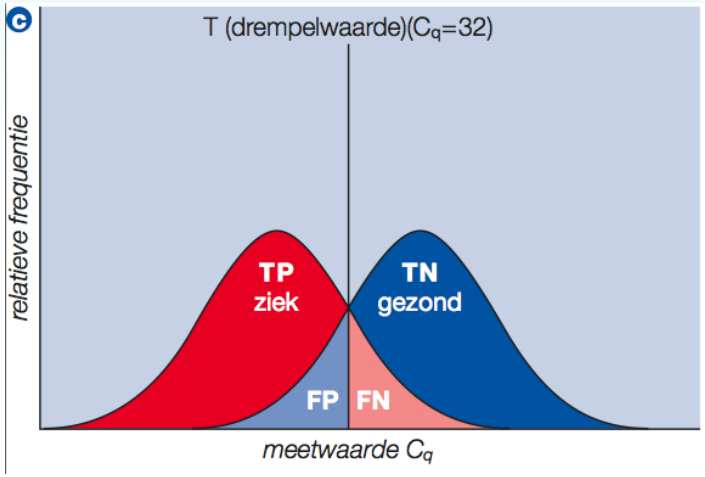
Specificity of PCR; in diagnostics

Populations separated by threshold (T); yes/no answer 100% certain





- Dynamic discrimination of populations

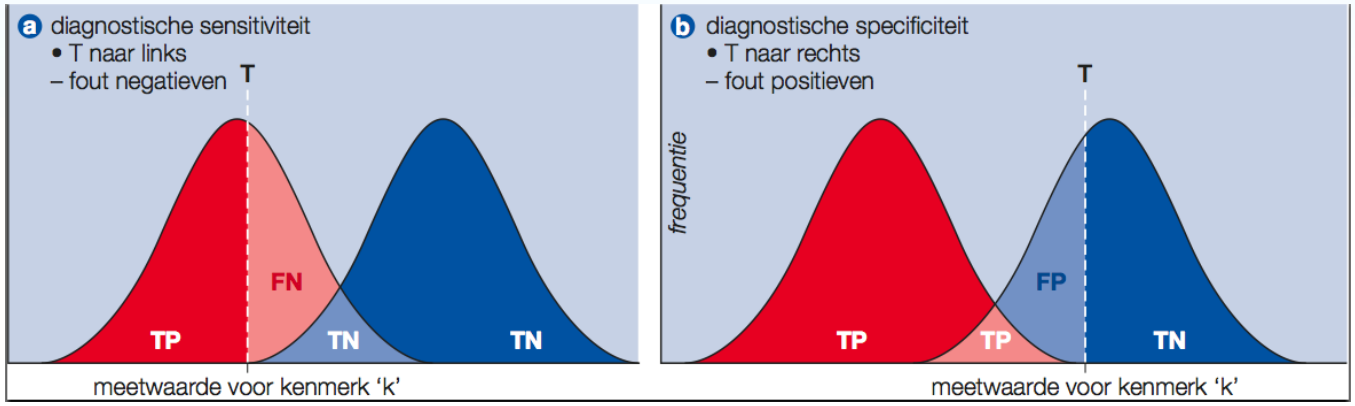


Populations overlap → FP/FN arises at threshold

Conclusion: there will be false diagnoses

Specificity of PCR; in diagnostics

- Fals positivity not acceptable
  - Invasive treatment as result of diagnose
    - Patient not ill→ therapy unacceptable
    - T-lijn shifts to the right on X-axis
    - Only “true positive” fals within the criterion
- False negativity not acceptable
  - Risk for FN too high → always treatment



Specificity of PCR; in diagnostics

Cases	No of test with Positive result	No of tests with Negative results	total
Number of illness	TP	FN	TP+FN
Number of Non illness	FP	TN	FP+TN
total	TP+FP	FN+TN	TP+FN+FP+TN

TP= "true positive"; ziektegevallen; correct vastgesteld met test
FN= "false negative"; ziektegevallen; foutief als ziektevrij bestempeld
FP= "false positive; ziektevrij, foutief als ziektegeval bestempeld
TN= "true negative"; ziektevrij, correct vastgesteld met test

Diagnostic sensitivity and -specificity

- Percentage diagnostic sensitivity
  - Level in which all “ill people” can be addressed correctly by the test

    TP/(TP +FN)X100

- Percentage diagnostic specificity
  - Level in which all “helathy people” can be addressed correctly by the test

    TN/(TN+FP)X100