A method to determine LOD and Cco in real time PCR (for regulated plant pathogens)

Chandelier Anne[1], Planchon Viviane[2]

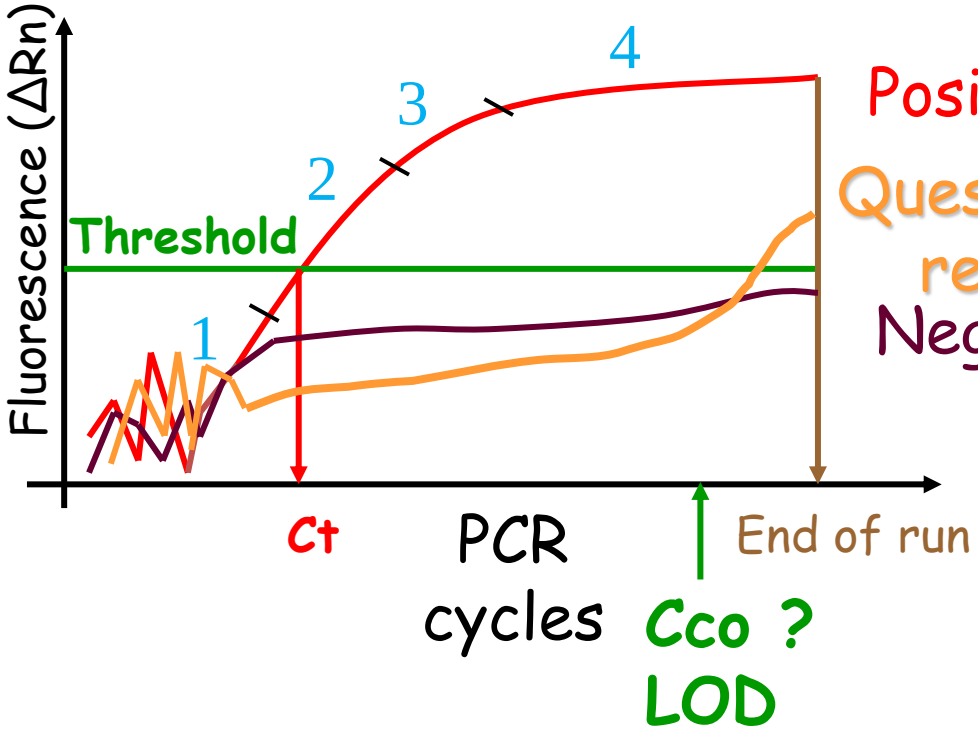
Walloon Agricultural Research Centre (Belgium)

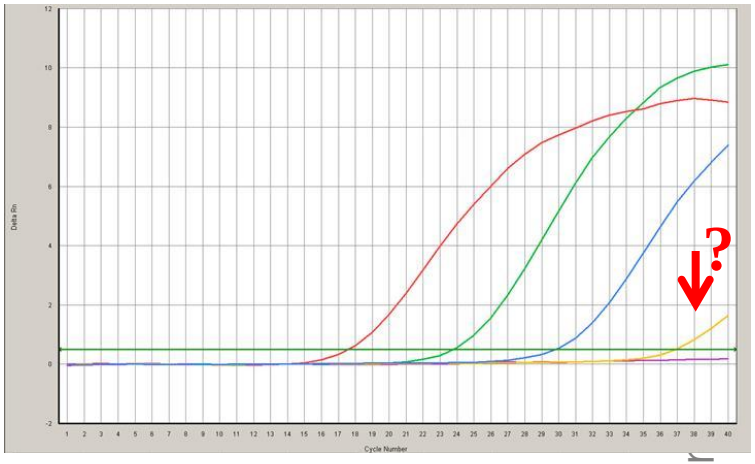
[1] Pest Biology and Monitoring Unit, Mycology laboratory
[2] Farming Systems, Territories and Information technology Unit

Walloon Agricultural Research Centre

Wallonie

Limit of detection and Cycle cut off





The **limit of detection** (LOD) corresponds to the point where, with a stated probability, one can be confident that the signal due to the target can be distinguished from the background signal. The LOD is associated with a **cycle cut off** (Cco).

Two kinds of risks :

- **Consumers' risk** (Plant Protection Service)
  Risk of **false negatives** (The probability to accept a contaminated sample)

  - sample with very low pathogen titer

  - sample with PCR inhibitors

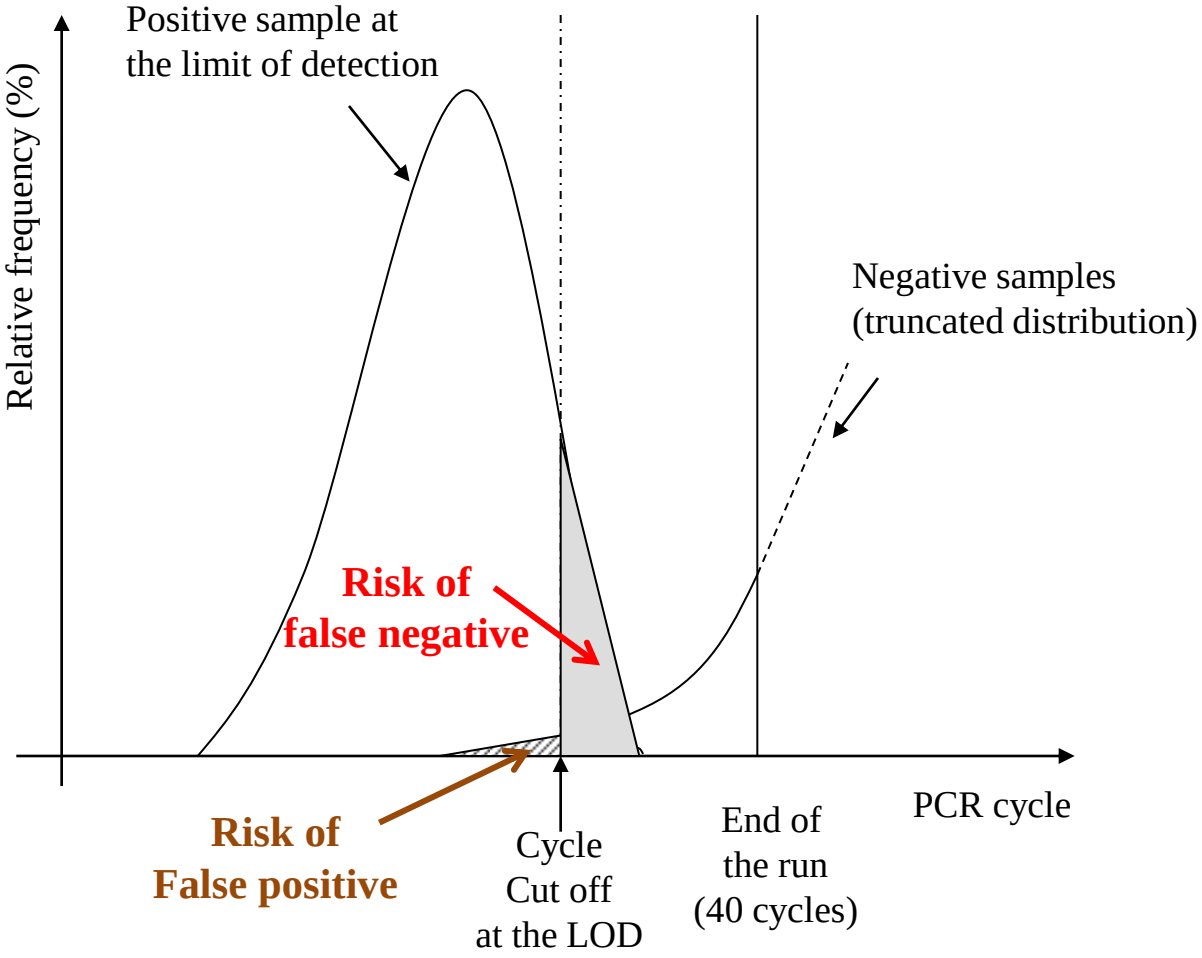
- **Producers' risk** (Nurseryman, farmer, forest manager,…):
  Risk of **false positives** (The probability to reject an healthy sample)

  - primers design not optimal - Background in the PCR

  - sample with contaminations

Can (normally) be monitored by the use of experimental controls

The cycle cut off should take account of both types of risks



Risks of false positive and false negative results should be determined

Description of one the methods used at CRAW to define a Cco

- **STEP 1**: Set of **true** negative samples
  (verification by another method, preferentially 2 methods)

- **STEP 2**: Calculation of the producers' risk cycle cut off (**PRCt**)
  - **case I**: important "background"
  - **case II**: low or null "background" (no signal for uninfected plants)

- **STEP 3**: Set of **true** positive samples (with known concentrations): spiked samples

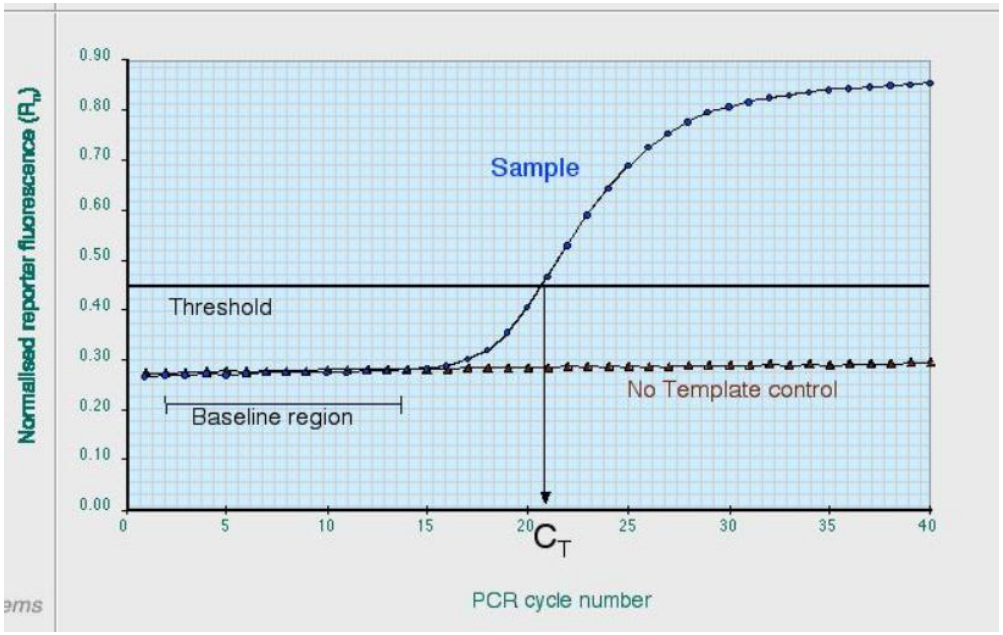
- **STEP 4**: Calculation of the **LOD** of the test (based on PRCt) and the corresponding cycle cut off (**Cco**)

Prerequisites for the calculation of LOD/Cco

- Specific consumables (DNA extraction kit, PCR reagents, PCR tubes,…)

- Specific thermocycler (ABI7000)

- Analysis settings
  - Threshold (ΔRn=0,5)
  - Baseline region (3-15)



- Levels of risk
  - risk of false positives: 5% (risk for the Producer)
  - risk of false negatives: 5% (risk for the Plant Protection Service)

Examples which will be described:

Case I: high background

- *Phytophthora ramorum* (oomycete)
- Primers of Hayden et al. (EPPO)
- Internal Transcribed Spacer (multi-copy gene)
- Dual-labelled probe (FAM-BHQ1)

- *Chalara fraxinea* (fungus)
- Primers of Chandelier et al. (EPPO, December 2013)
- Internal Transcribed Spacer (muti-copy gene)
- Dual-labelled probe (FAM-BHQ1)

Case II: low background

- *Phytophthora kernoviae* (oomycete)
- Primers of Schena et al. (EPPO)
- Ras-related protein (single copy gene)
- Dual-labelled probe (FAM-BHQ1)

Case I: *Phytophthora ramorum*, ITS

- Regulated pathogen (oomycete)
- Mainly on Rhododendron in nurseries (except in UK, in forest, larch)
- First detected in Belgium in 2002
- Survey organized by the Belgian Plant Protection Service

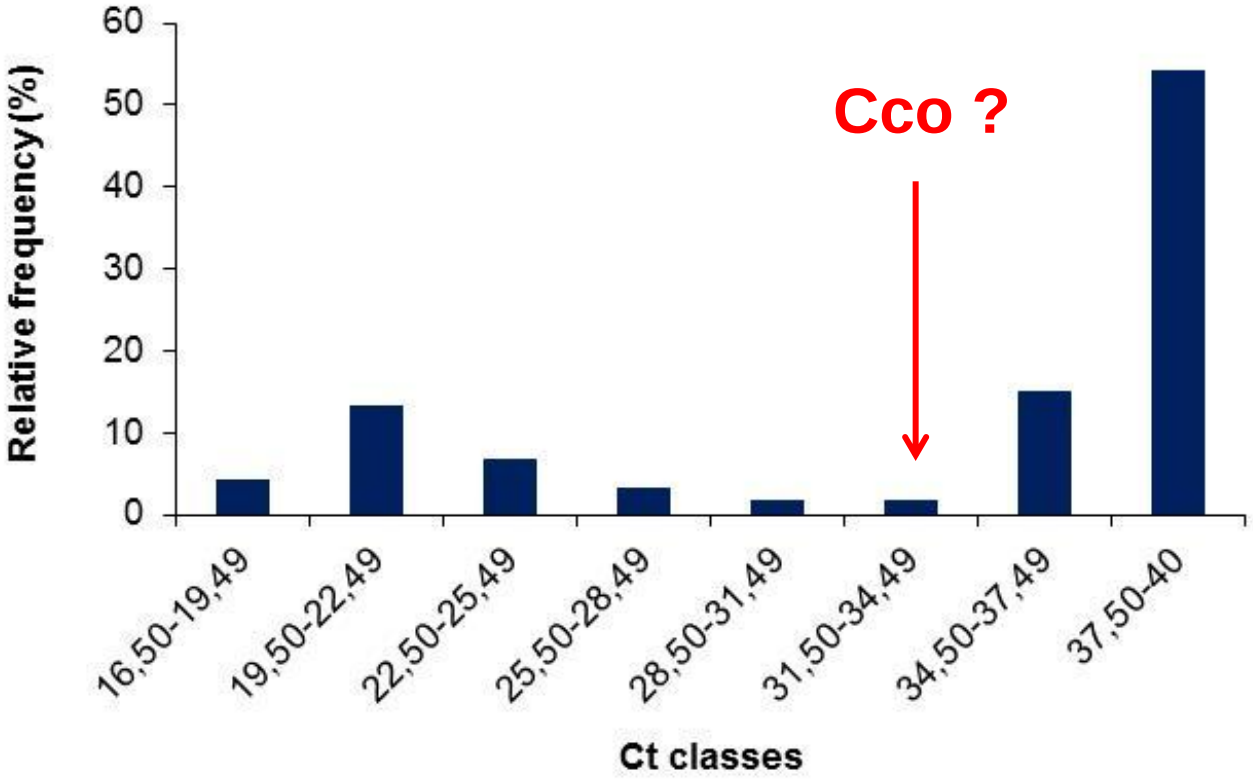
2009	2010	2011	2012
24,1	2,7	7,7	0,0

- BELAC accreditation (real-time PCR)

Case I *(Phytophthora ramorum)*

STEP 1 - Set of **true** negative samples

- 120 samples (from year 2009, Belgian Plant Protection Service)
- Analysis with the selected real-time PCR method



Provisional Cco established at 34

- Comparison with two other detection methods
  - isolation on PARP
  - another real time PCR (Schena et al., ras-related protein)

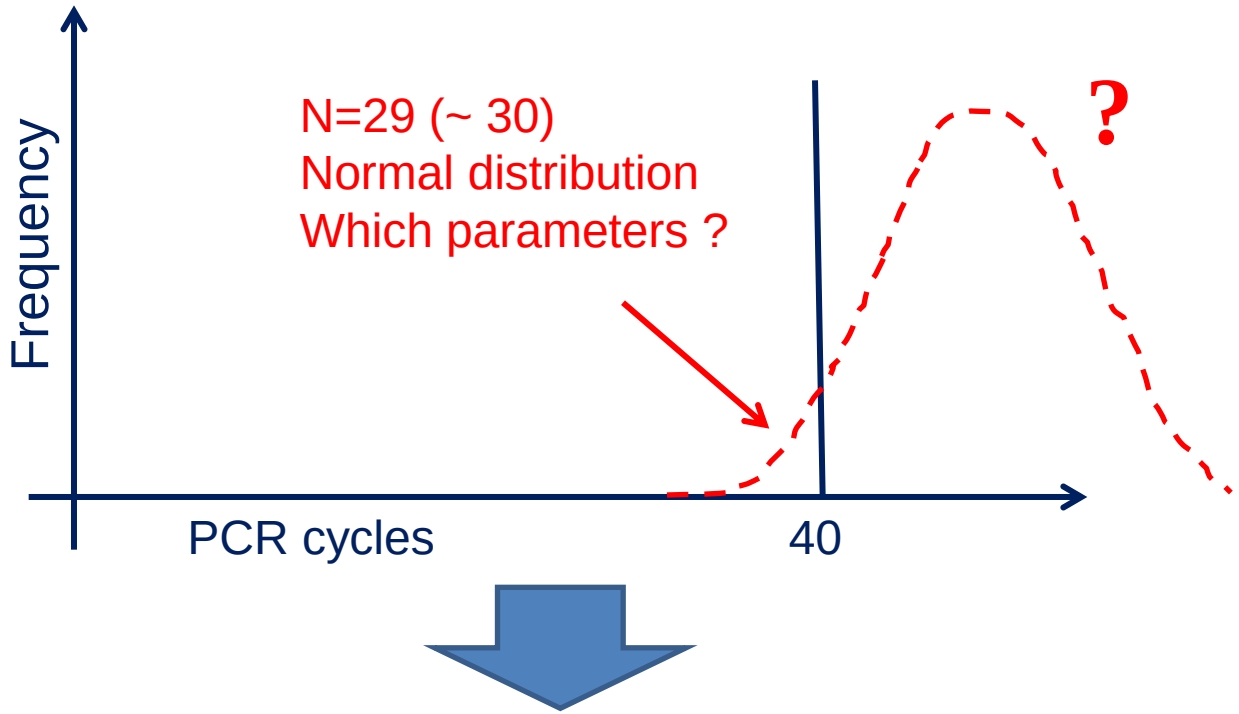
- Selection of samples negative with the 3 methods
  (Cco $_{Hayden}$ at 34; Cco $_{Schena}$ at 36 – based on literature)

		Hayden et al		
		+	-	total
Isolation	+	21	0	21
	-	13	86	99
	Total	34	86	120
Schena et al	+	29	0	29
	-	5	86	91
	Total	34	86	120

86 samples (among which 29 samples with Ct <40 with the test of Hayden et al.)

STEP 2 – Determination of PRCt

1. Use of Ct values from the true negative samples to determine the normal distribution which fits the best to the data (truncated distribution)



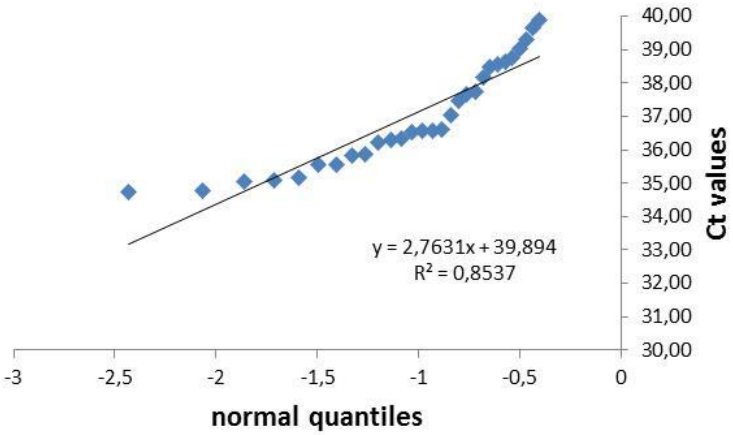
Estimated parameters of the normal distribution (**Excel sheet 1**)

Inserted data

a) Calculation of normal quantiles from observed data

n°	Ct	Z obs	Prob	qnorm	Z estimé	F(x)
1	**34,14**	-1,71232347	0,00750751	-2,4320167	-1,98699691	0,02346137
2	34,73	-1,46004264	0,01951952	-2,06377516	-1,78166506	0,03740193
3	34,76	-1,44075279	0,03153153	-1,85874661	-1,77122445	0,03826169
4	35,03	-1,26714407	0,04354354	-1,71096764	-1,67725903	0,04674592
5	35,07	-1,24142426	0,05555556	-1,59321882	-1,66333822	0,04812238
6	35,17	-1,17712474	0,06756757	-1,49415491	-1,62853621	0,05170562
7	35,56	-0,92635659	0,07957958	-1,40790515	-1,49280838	0,06774368
8	35,56	-0,92635659	0,09159159	-1,33101771	-1,49280838	0,06774368
9	35,83	-0,75274788	0,1036036	-1,26128216	-1,39884295	0,08093004
10	35,88	-0,72059812	0,11561562	-1,19719327	-1,38144195	0,08357156
11	36,21	-0,50840969	0,12762763	-1,1376773	-1,26659531	0,10265001
12	36,30	-0,45054011	0,13963964	-1,0819398	-1,23527351	0,10836437
13	36,35	-0,41839035	0,15165165	-1,0293754	-1,2178725	0,11163621
14	36,51	-0,31551111	0,16366366	-0,97951146	-1,16218929	0,12257929
15	36,55	-0,2897913	0,17567568	-0,93197131	-1,14826848	0,12542887
16	36,58	-0,27050145	0,18768769	-0,88644946	-1,13782788	0,12759618
17	36,62	-0,24478164	0,1996997	-0,84269437	-1,12390708	0,13052622
18	37,03	0,01884641	0,21171171	-0,8004961	-0,98121884	0,16324242
19	37,47	0,30176432	0,22372372	-0,75967739	-0,82808999	0,20380977
20	37,66	0,42393341	0,23573574	-0,72008693	-0,76196618	0,2230401
21	37,73	0,46894308	0,24774775	-0,68159434	-0,73760477	0,23037733
22	38,15	0,73900108	0,25975976	-0,64408623	-0,59143633	0,27711405
23	38,48	0,95118951	0,27177177	-0,60746322	-0,4765897	0,31682716
24	38,57	1,00905909	0,28378378	-0,57163753	-0,44526789	0,32806309
25	38,63	1,0476388	0,2957958	-0,53653104	-0,42438668	0,33564192
26	38,76	1,13122818	0,30780781	-0,50207379	-0,37914407	0,35229044
27	39,02	1,29840694	0,31981982	-0,4682027	-0,28865885	0,38642123
28	39,28	1,46558571	0,33183183	-0,43486053	-0,19817362	0,42145461
29	39,63	1,69063404	0,34384384	-0,40199502	-0,07636659	0,46956373
30	39,90	1,86424276	0,35585586	-0,36955819	0,01759884	0,50702056

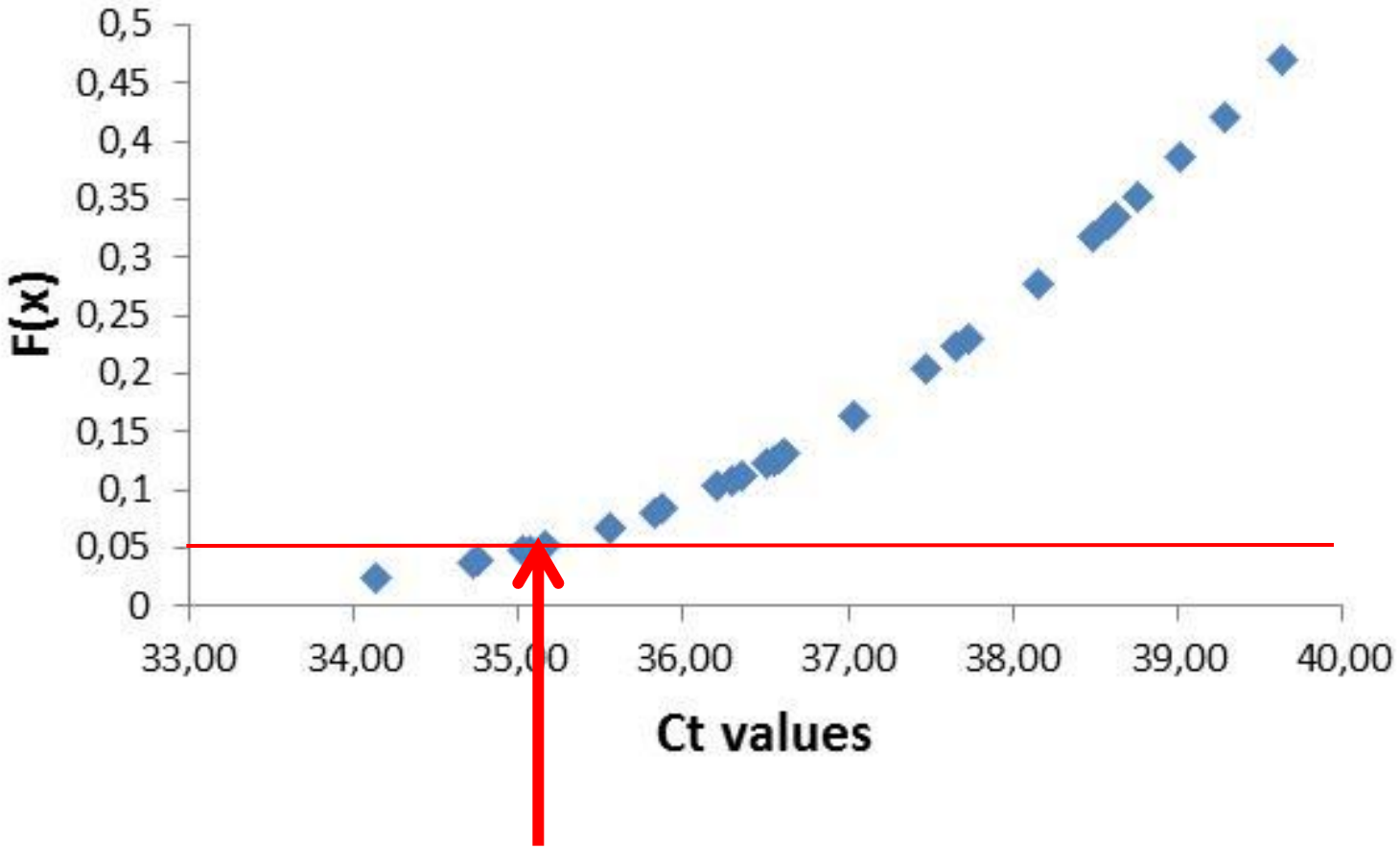
b) Linear regression
- Slope and intercept



c) risk of false positive = 5%

STEP 2 – Determination of PRCt

3. Determination of the PRCt for a fixed probability (Excel file 1)



PRCt = 35,12 cycles, with a **producers'risk** fixed at 5% (p=0,05)

STEP 3: Set of **true** positive samples

- **Great amount** of target DNA
  - accurate A_{260} value
  - different concentrations (3 to 5 levels)
  - 30 replicates / level (~ normal distribution)

- **Matrix effect** (interaction with plant DNA)

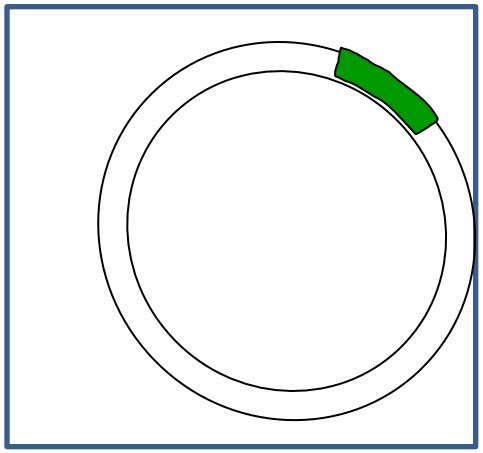
Infected plant

- Low yield
- No possibility of quantification

Mycelium

- Low yield

Plasmid DNA

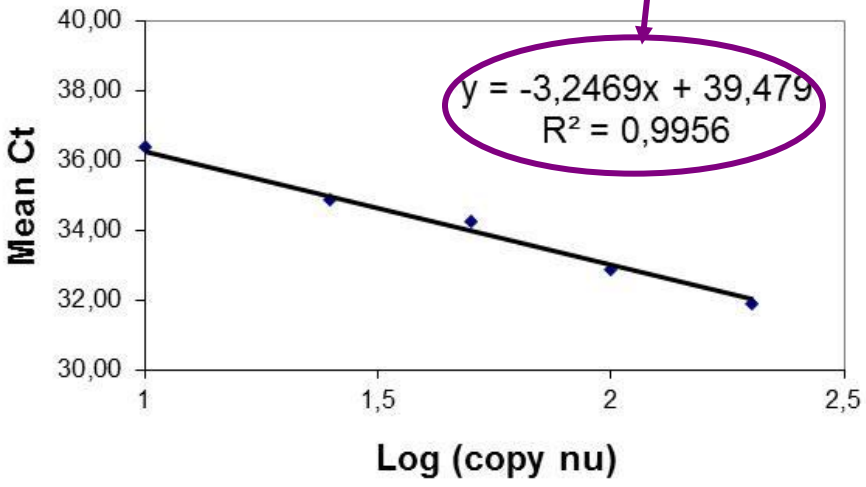


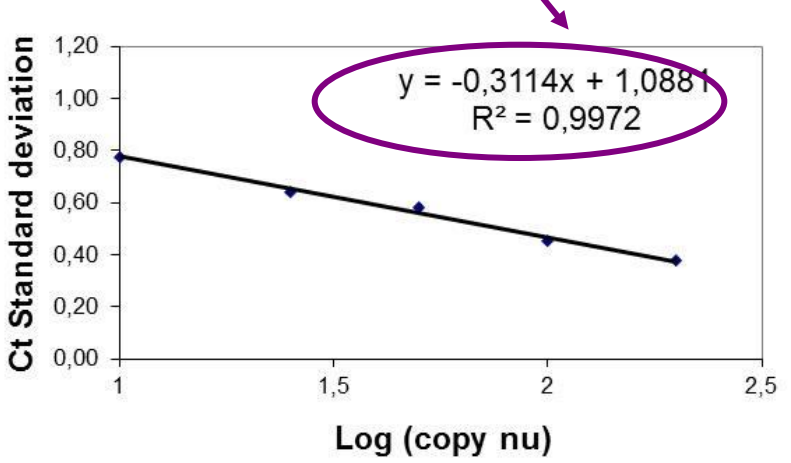
- High yield
- Stability over time
- Spiking in plant DNA
- Converted in copies

STEP 4: Determination of LOD (and Cco)

- **Sets of data for positive results**
  - Serial dilutions of plasmid DNA in plant DNA (spiked samples)
  - 5 concentration levels (200, 100, 50, 25, 10 copies / PCR)
  - 3 PCR runs (30 replicates / level)

1. Normality of the distributions (test of normality)
2. Calculation of mean and standard deviation for each concentration level
3. Establishment of the relationship between the log-transformed copy number and the mean Ct (1) and the standard deviation Ct (2)





Excel sheet 2

Excel sheet 2 (inserted data)

Equation of standard dev.

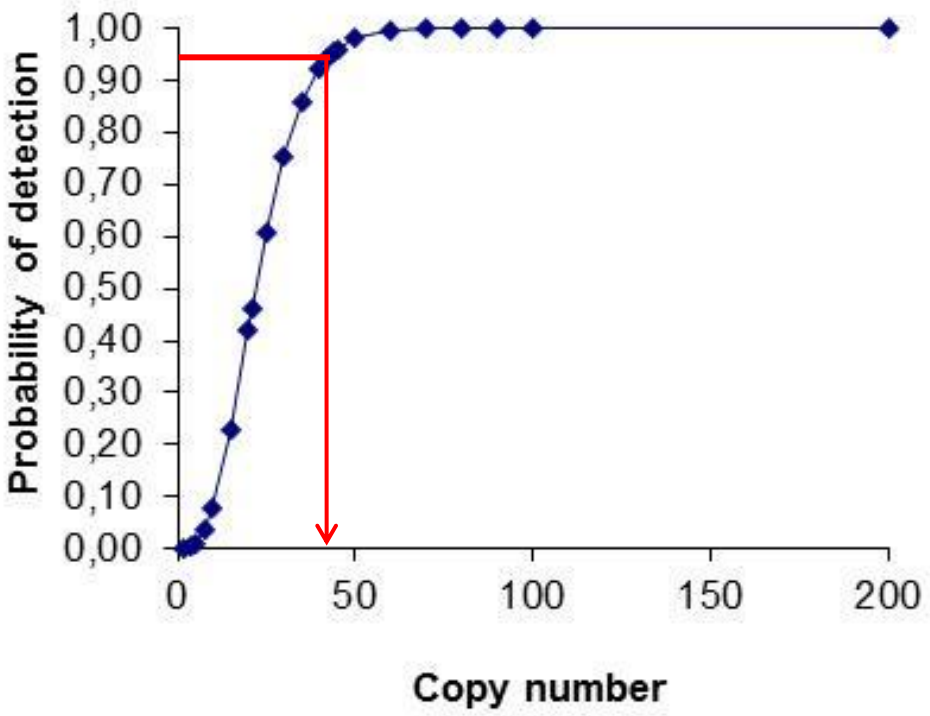
Equation of the mean

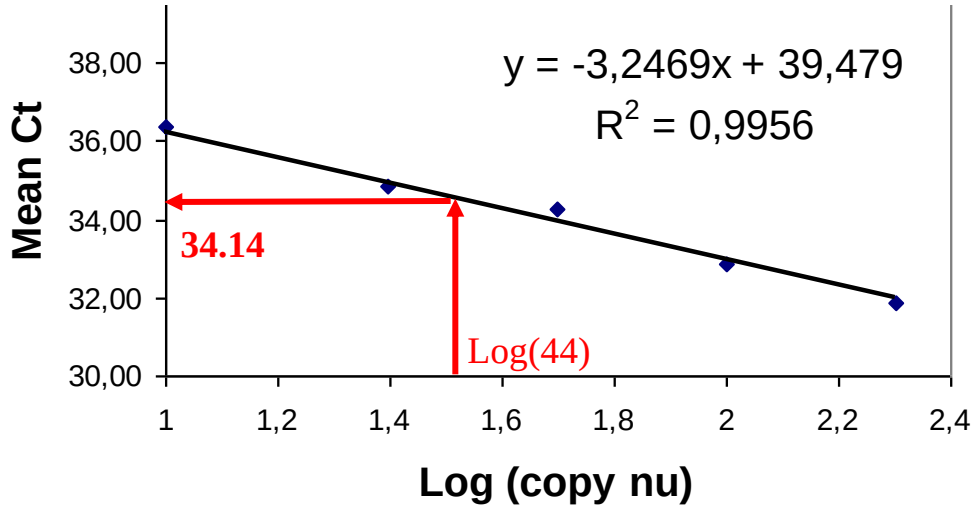
Normal standard Variable Z=(PRCt-mean/Std)

Probability for normal distribution

copies	log copies	Mean	std	Z2	P2
2	0,30	38,50	1,00	-3,40	0,00
4	0,60	37,52	0,90	-2,67	0,00
5	0,70	37,21	0,87	-2,40	0,01
8	0,90	36,55	0,81	-1,77	0,04
10	1,00	36,23	0,78	-1,43	0,08
15	1,18	35,66	0,72	-0,75	0,23
20	1,30	35,26	0,68	-0,20	0,42
21	1,32	35,19	0,68	-0,10	0,46
25	1,40	34,94	0,65	0,27	0,61
30	1,48	34,68	0,63	0,69	0,76
35	1,54	34,47	0,61	1,07	0,86
40	1,60	34,28	0,59	1,43	0,92
42	1,62	34,21	0,58	1,56	0,94
44	1,64	34,14	0,58	1,69	0,95
45	1,65	34,11	0,57	1,76	0,96
50	1,70	33,96	0,56	2,07	0,98
60	1,78	33,71	0,54	2,64	1,00
70	1,85	33,49	0,51	3,17	1,00
80	1,90	33,30	0,50	3,67	1,00
90	1,95	33,13	0,48	4,13	1,00
100	2,00	32,99	0,47	4,58	1,00
200	2,30	32,01	0,37	8,36	1,00

Consumers'risk = 5% (P=0,95)





Normal distribution

Z = (PRCt – MeanCt)/SdCt

P = Probability of detection (0,95)

LOD = 44 copies

Cco=34,14

ABI7000
Treshold
Baseline
PCR Master Mix
Pipets…

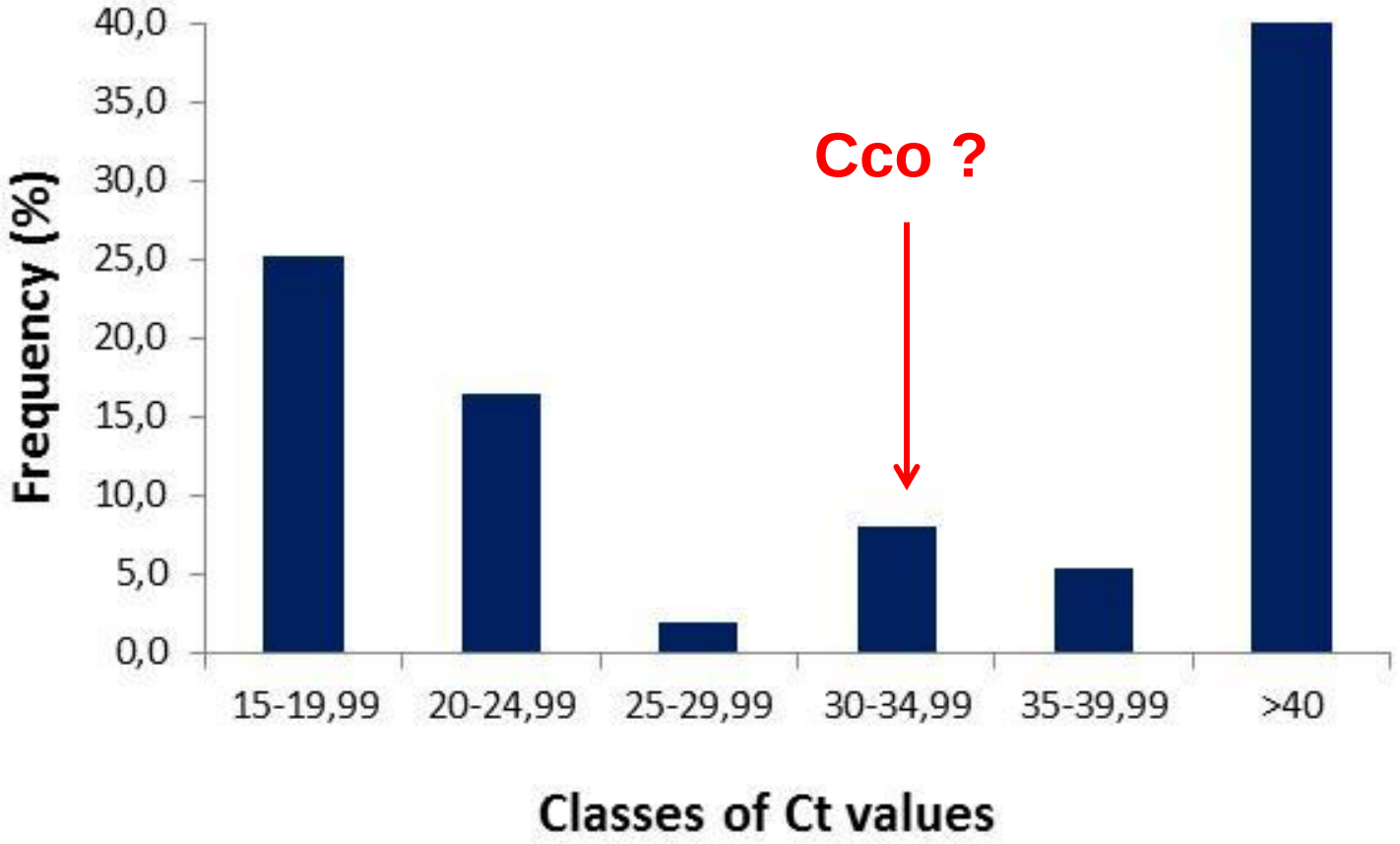
Case I - *Chalara fraxinea*, ITS

- Pathogen on EPPO alert list (fungus)
- On ash (*Fraxinus* sp.) in forest and nurseries
- First detected in Belgium in 2010
- Survey organized by CRAW / Walloon Forest Department since 2010
- No accreditation

Case I - *Chalara fraxinea*, ITS

- **Step 1**: Set of true negative samples

  - 387 samples (branches) – survey 2010, 2011, 2012



Provisional Cco at 34

- Comparison with conventional PCR (Johansson et al. 2010 (ITS))

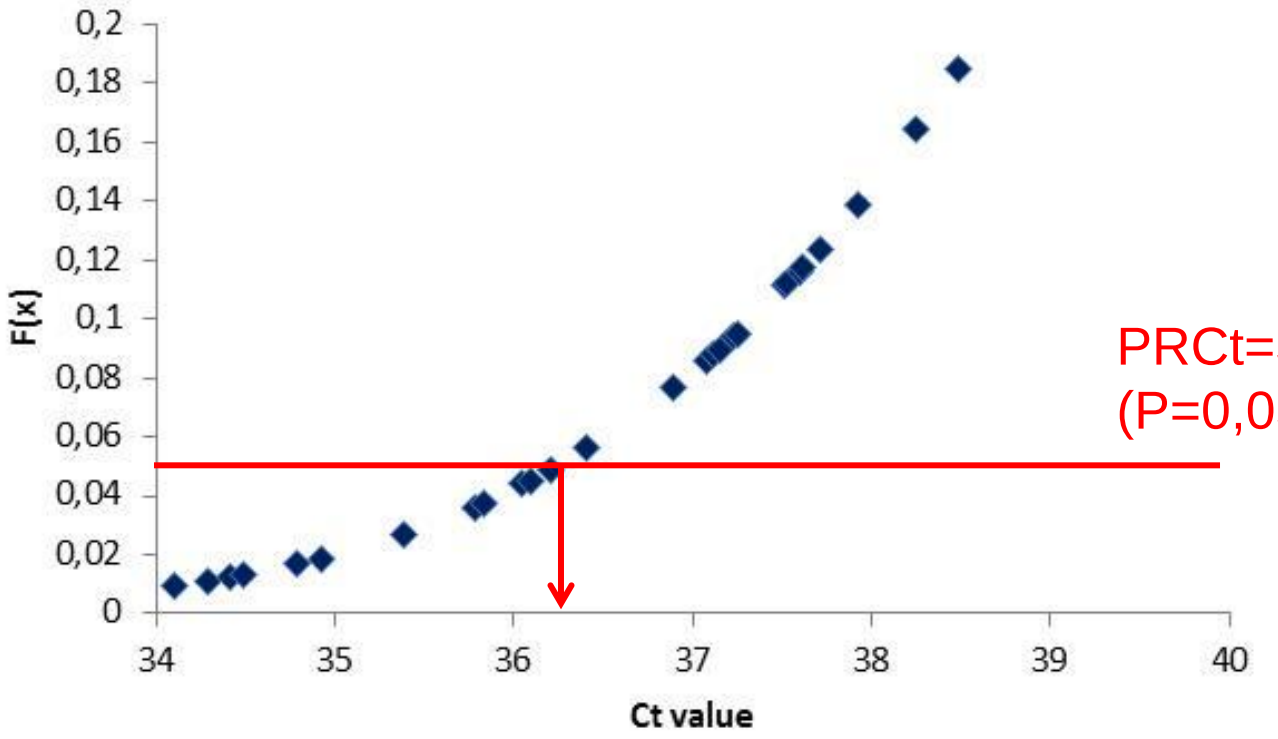
- 192 negative samples with both methods (**27 with Ct values < 40**)

		Chandelier et al. (real time)		
		+	-	total
Johansson	+	189	0	189
et al. (PCR)	-	6	192	198
	Total	195	192	387

The 6 samples displayed Ct values between 32,5 and 34

Case I - *Chalara fraxinea*, ITS

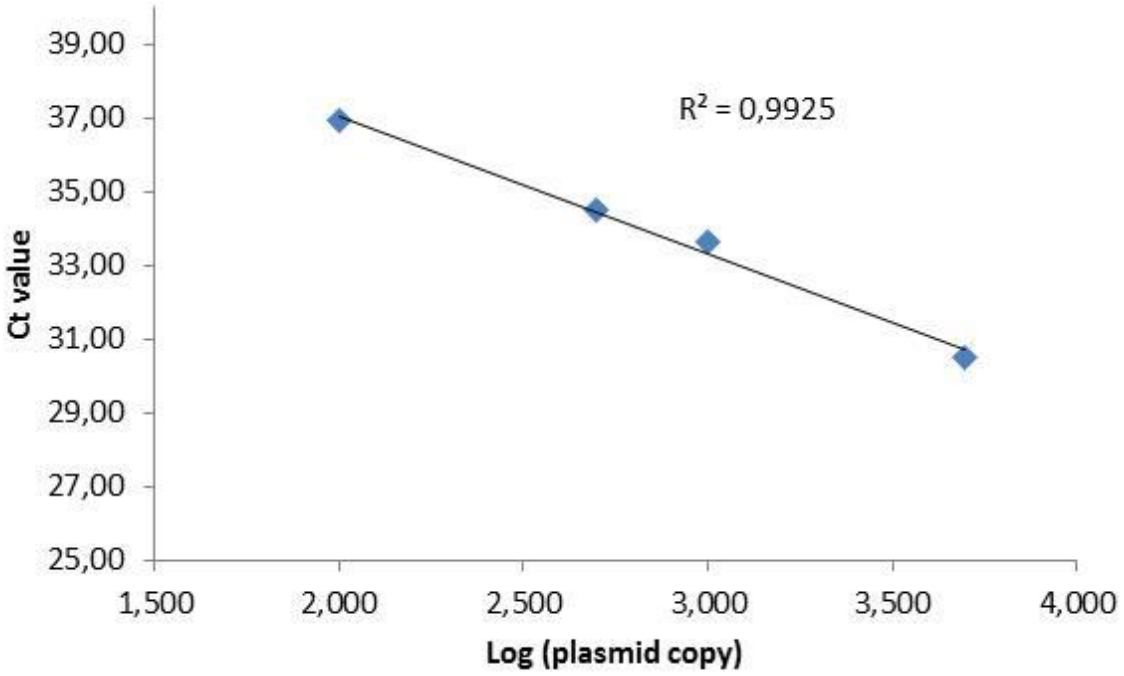
- **Step 2**: Calculation of PRCt (with **Excel sheet 1**)
  - 192 Ct values (27 with Ct values < 40)
  - Normal distribution based on the 27 Ct values (truncated distr.)
  - Probability of false positive values fixed at 5% (P=0,05)
  - Use of Excel sheet 1



Case I - *Chalara fraxinea*, ITS

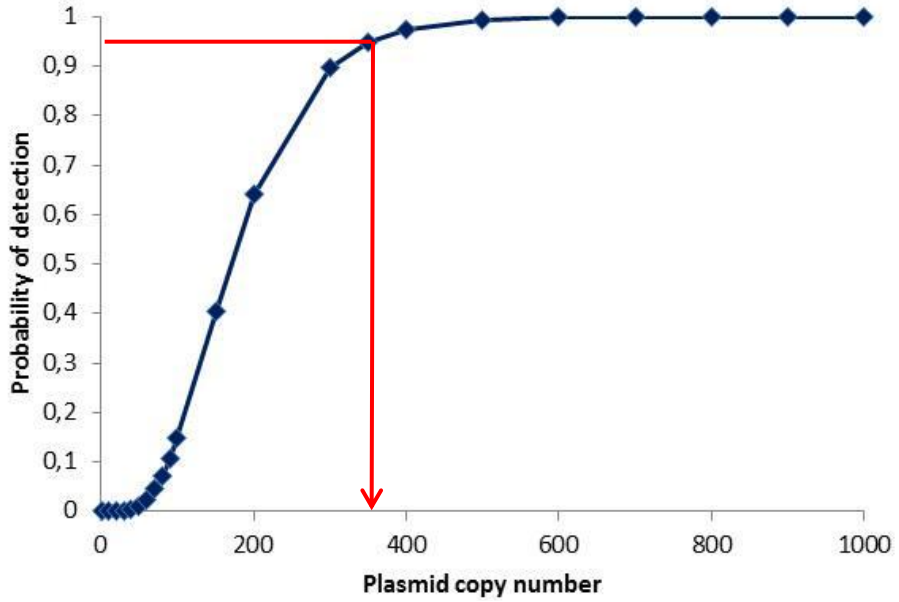
- **Step 3**: set of true positive samples

  - Plasmid DNA in DNA from healthy plant (ash twigs)
  - 4 concentration levels (5000, 1000, 500 and 100 plasmid copies)
  - 15 repetitions/level

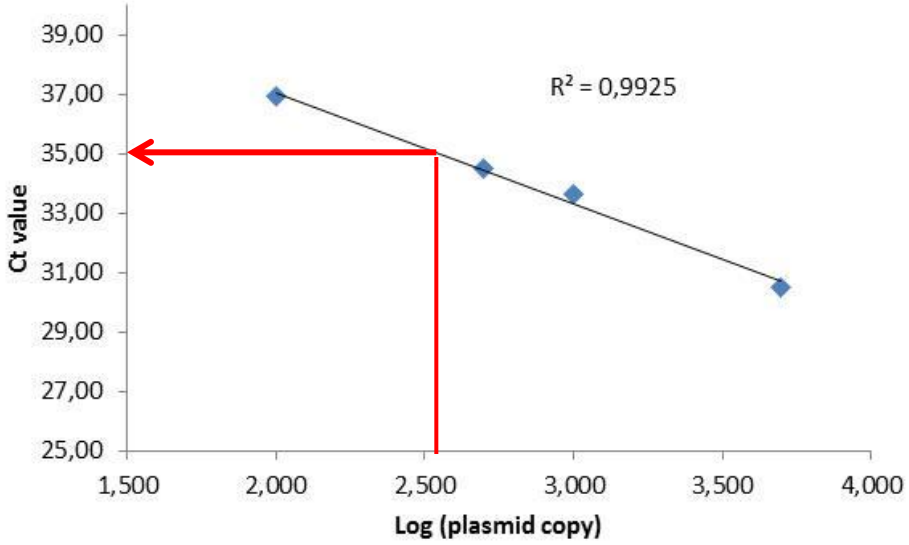


Case I - *Chalara fraxinea*, ITS

- **Step 4**: calculation of LOD and Cco (**Excel sheet 2**)



Normal distribution
Z = (PRCt – MeanCt)/SdCt
P = Probability of detection
LOD= 350 copies



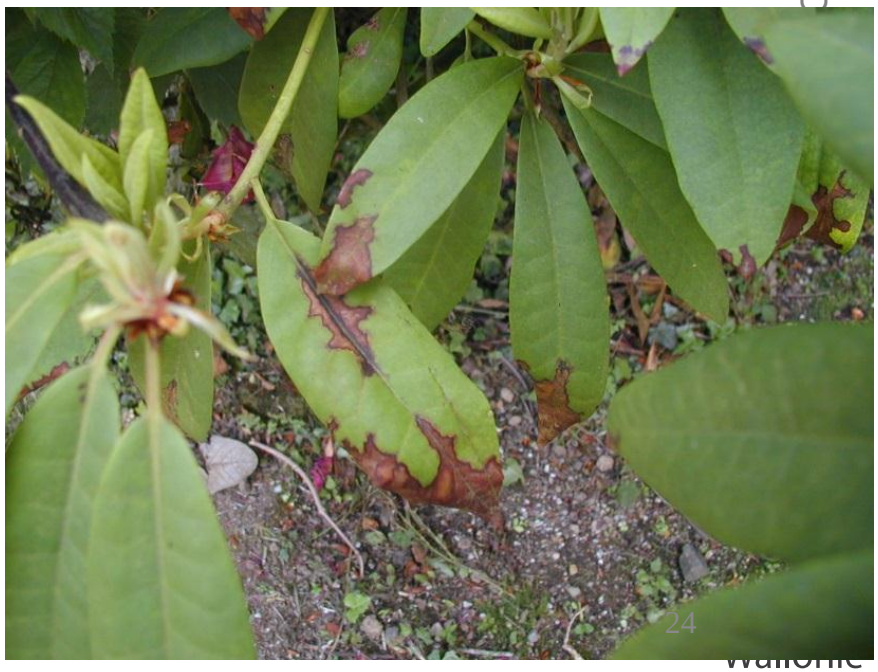
Cco=35,02

Walloon Agricultural Research Centre

Wallonie

Case II – Low background
Phytophthora kernoviae

- EPPO A2 list (oomycete)
- Mainly on Rhododendron and beech in forests (UK)
- Never detected in Belgium
- Survey organized by the BPPS since 2008
- Accreditation (real-time PCR)



Case II - *Phytophthora kernoviae*

Step 1: Set of true negative samples

- 270 samples (260 from BPPS, 2008-2009; 10 from a RT)
- 2 positive samples (from the RT)
- 268 negative samples
- All negative samples but one with Ct value < 40 [Ct=39,62]

Step 2 : Calculation of PRCt

→ No possibility to calculate a PRCt based on a truncated distribution (minimum ~ 30 values)

→ Risk of false positive results = 0

→ PRCt fixed at the end of the run (**PRCt=40**)

By calculation, using the **Excel sheet 1** …same conclusion

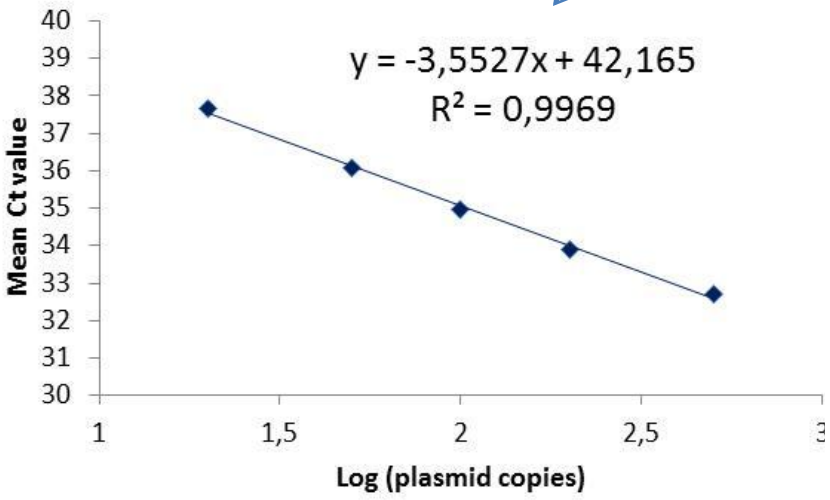
n°	Ct	Z obs	Prob	qnorm	Z estimé	F(x)
1	**40,00**	0	0,00232992	-2,82965431	-1,99965541	0,02276874
2	**40,00**	0	0,00605778	-2,50876038	-1,99965541	0,02276874
3	**40,00**	0	0,00978565	-2,33446674	-1,99965541	0,02276874
4	**40,00**	0	0,01351351	-2,21112724	-1,99965541	0,02276874
5	**40,00**	0	0,01724138	-2,11438077	-1,99965541	0,02276874
6	**40,00**	0	0,02096925	-2,03413	-1,99965541	0,02276874
7	**40,00**	0	0,02469711	-1,96517297	-1,99965541	0,02276874
8	**40,00**	0	0,02842498	-1,90446267	-1,99965541	0,02276874
9	**40,00**	0	0,03215284	-1,85005457	-1,99965541	0,02276874
10	**40,00**	0	0,03588071	-1,80062874	-1,99965541	0,02276874
11	**40,00**	0	0,03960857	-1,75524653	-1,99965541	0,02276874
12	**40,00**	0	0,04333644	-1,71321562	-1,99965541	0,02276874
13	**40,00**	0	0,04706431	-1,67401012	-1,99965541	0,02276874
14	**40,00**	0	0,05079217	-1,6372208	-1,99965541	0,02276874
15	**40,00**	0	0,05452004	-1,60252269	-1,99965541	0,02276874
16	**40,00**	0	0,0582479	-1,56965324	-1,99965541	0,02276874
17	**40,00**	0	0,06197577	-1,53839715	-1,99965541	0,02276874
18	**40,00**	0	0,06570363	-1,50857562	-1,99965541	0,02276874
19	**40,00**	0	0,0694315	-1,48003839	-1,99965541	0,02276874
20	**40,00**	0	0,07315937	-1,452658	-1,99965541	0,02276874
21	**40,00**	0	0,07688723	-1,4263253	-1,99965541	0,02276874
22	**40,00**	0	0,0806151	-1,40094609	-1,99965541	0,02276874
23	**40,00**	0	0,08434296	-1,37643846	-1,99965541	0,02276874
24	**40,00**	0	0,08807083	-1,35273076	-1,99965541	0,02276874
25	**40,00**	0	0,0917987	-1,3297599	-1,99965541	0,02276874
26	**40,00**	0	0,09552656	-1,30747004	-1,99965541	0,02276874
27	**40,00**	0	0,09925443	-1,28581151	-1,99965541	0,02276874
28	**40,00**	0	0,10298229	-1,26473989	-1,99965541	0,02276874
29	**40,00**	0	0,10671016	-1,24421535	-1,99965541	0,02276874
30	**40,00**	0	0,11043802	-1,22420196	-1,99965541	0,02276874
31	**40,00**	0	0,11416589	-1,20466726	-1,99965541	0,02276874
32	39,62	-19	0,11789376	-1,18558175	8,90205297	1

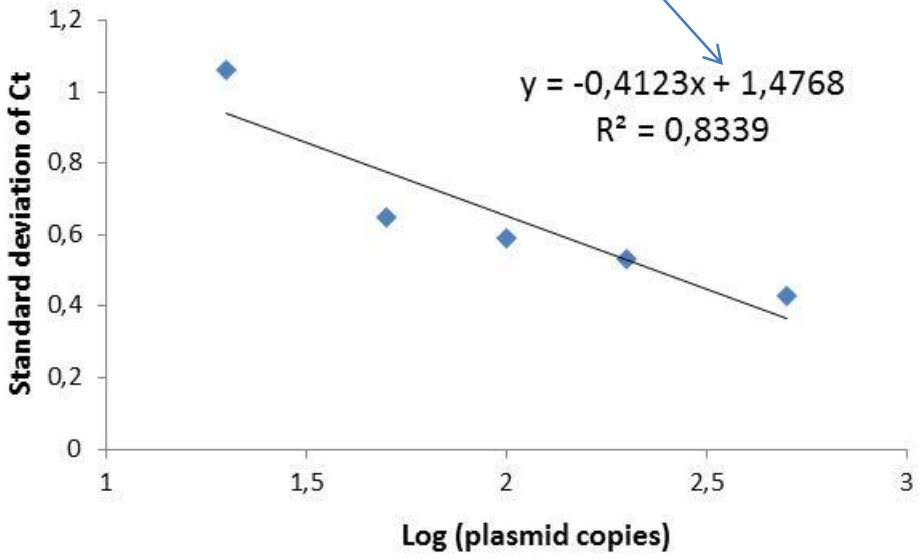
PRCt=39,9999

Case II - *Phytophthora kernoviae*

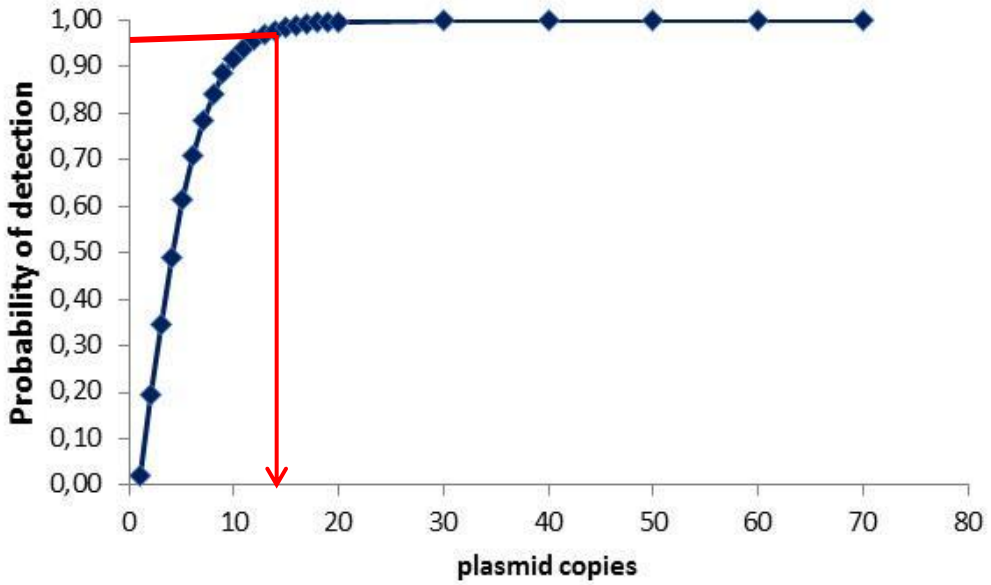
Step 3: set of true positive samples

- Plant DNA (Rhododendron leaves) spiked with plasmid DNA
- 5 concentration levels (500, 200, 100, 50 and 20 copies/PCR)
- Equation of the mean (1) and of the standard deviation (2)

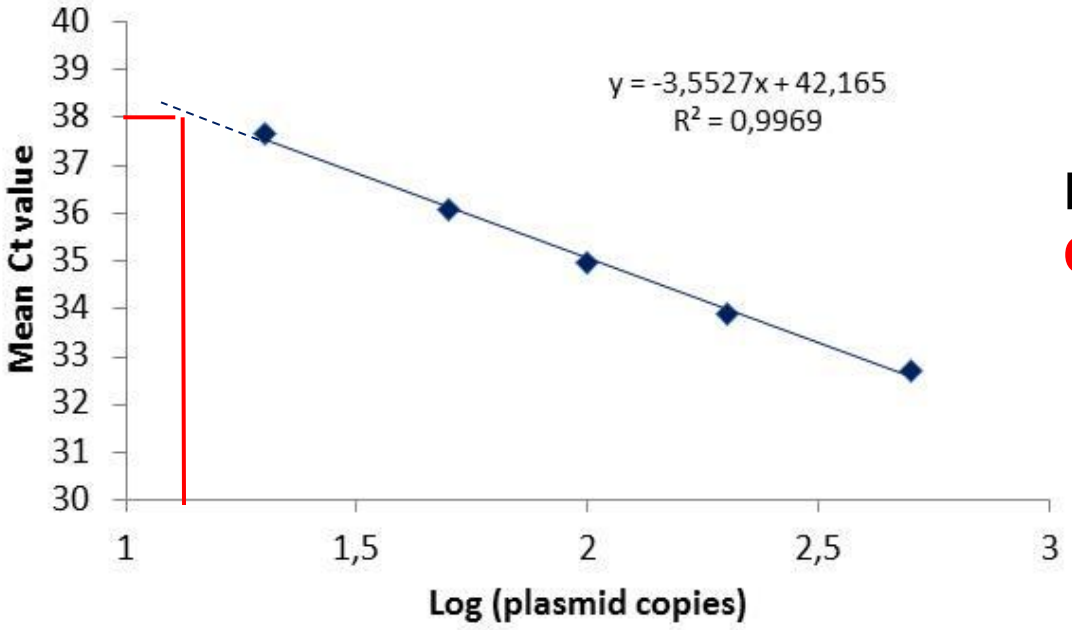




Step 4: Calculation of LOD and Cco (**Excel sheet 2**)



P=95% ,
LOD = 12 copies



LOD=12 copies,
Cco= 38,3 cycles

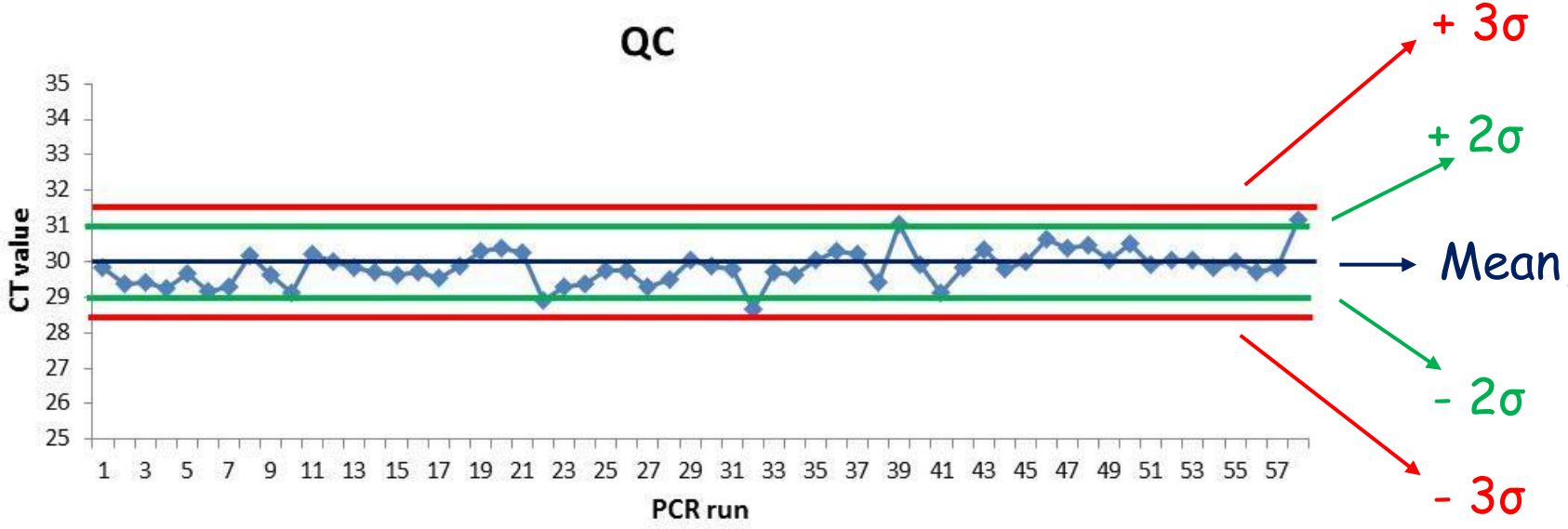
Interpretation of the data

Type	Ct
Negative DNA extraction control (water) **NIC**	No signal
Positive DNA extraction control (infected mat.) **PIC**	< Cycle Cut off
Negative PCR control (water) **NAC**	No signal
Positive PCR control (5000 copies/PCR) **PAC**	Control Chart
PCR Inhibition control (5000 copies/PCR) **IC**	$[Ct_{PIC} - Ct_{PPC} \leq 2]$
Sample (in duplicate)	< Cycle Cut off => + > Cycle Cut off => -

Samples interpretation **IF** reaction controls give expected results

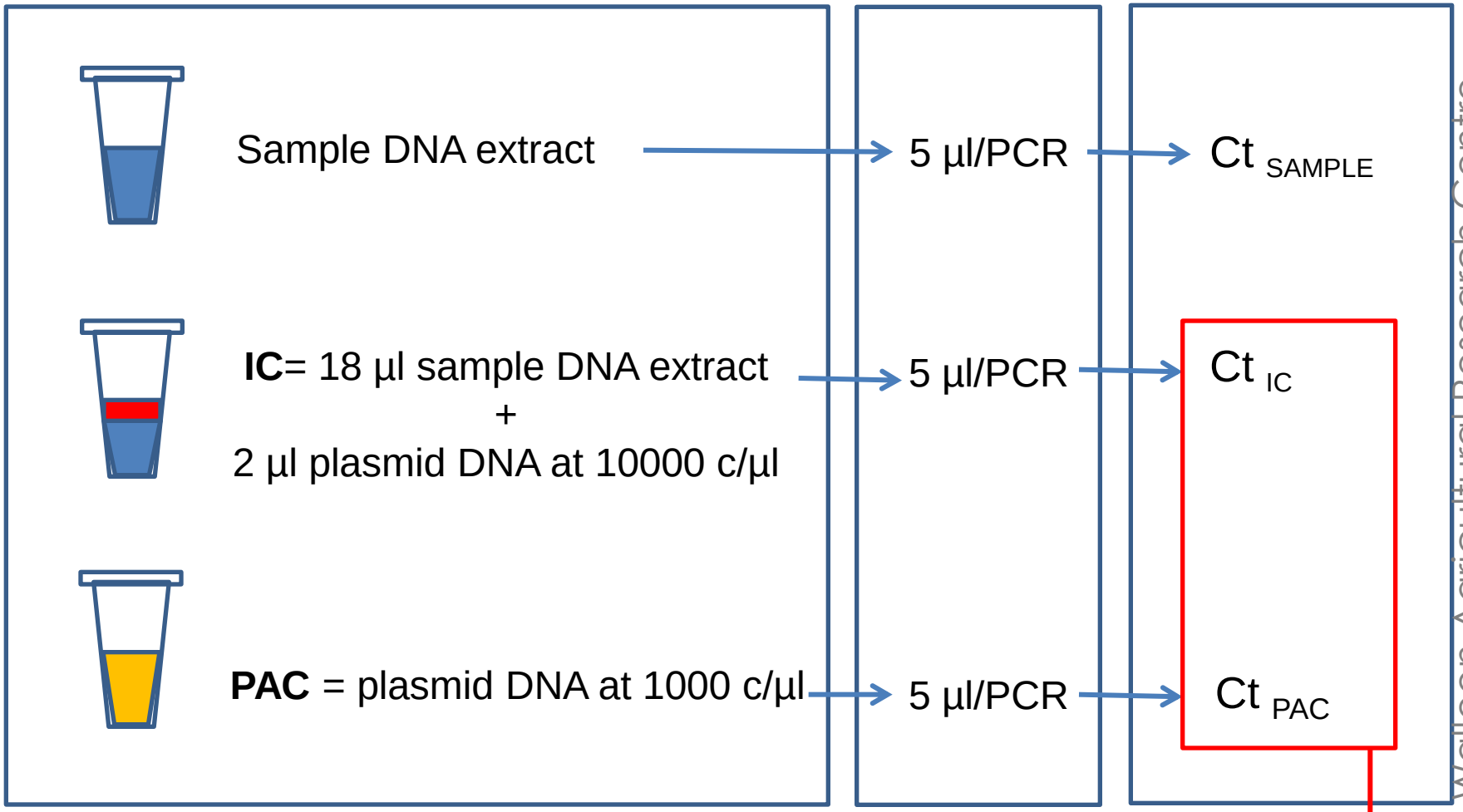
Control of Ct value deviations over time

- Plasmid DNA at 5000 copies/PCR
- Use of data from a **Control Chart (Excel sheet 3)**
- Ct value within limits - rejection if
  - PAC Ct value > mean +/- 3σ (1 x)
  - PAC Ct value > mean +/- 2 σ (2 x)



<u>Stability of the PAC</u>: Important to avoid multiple freezes /thaws (max. 3) !

Interpretation of the inhibition control (IC)



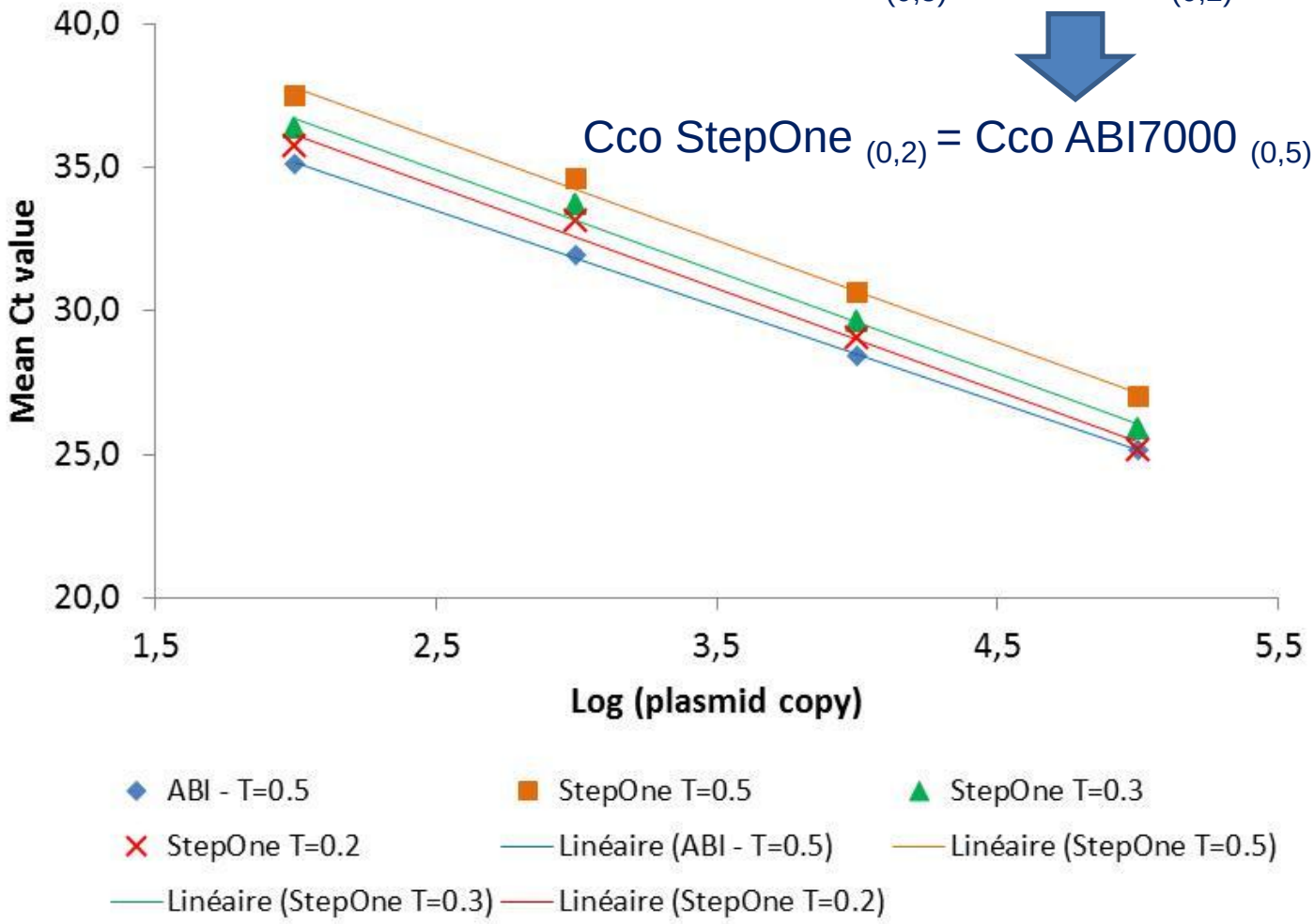
PAC and IC= same sequence, same concentration
=> same PCR Efficiency => if $Ct_{IC} > Ct_{PAC}$ => inhibition

Influence of the thermocycler: same LOD, ≠ Cco

- ABI 7000 – Treshold at 0,5 (accreditation)
- StepOne Plus - Treshold at 0,5 – 0,3 – 0,2

Delta [$ABI7000_{(0,5)}$ – $StepOne_{(0,2)}$] = 0,62

Cco StepOne $_{(0,2)}$ = Cco ABI7000 $_{(0,5)}$ + 0,62



Conclusions

- The arbitrary setting of a Cco can be very subjective, as disregarding data above this point can result in **erroneous interpretation of data** !

- A Cco should be based on intra-laboratory validation data (**not identical in each lab, even within lab** !!!)

- The method used should take account of **both types of risks**:
  - It is important to know the purpose of the test (detection of quarantine fungi, R&D project…)
  - These risks should be known by the customer (zero-tolerance ?)

- The chosen method should be **easy to use** in diagnostic labs (time, money, statistical expertise, Microsoft Excel)

- For comparison between labs, it would be better to talk about **LOD** (on reference material) rather than **Cco**

…the method was accepted by the Belgian Accreditation body (BELAC)

Thank you for your attention !

Acknowledgements
Dr R. Oger (CRAW, Statistical analyses of data)
Dr G. Berben (CRAW, EURL animal proteins in feedingstuffs)
Dr O. Fumière (CRAW, EURL animal proteins in feedingstuffs)