

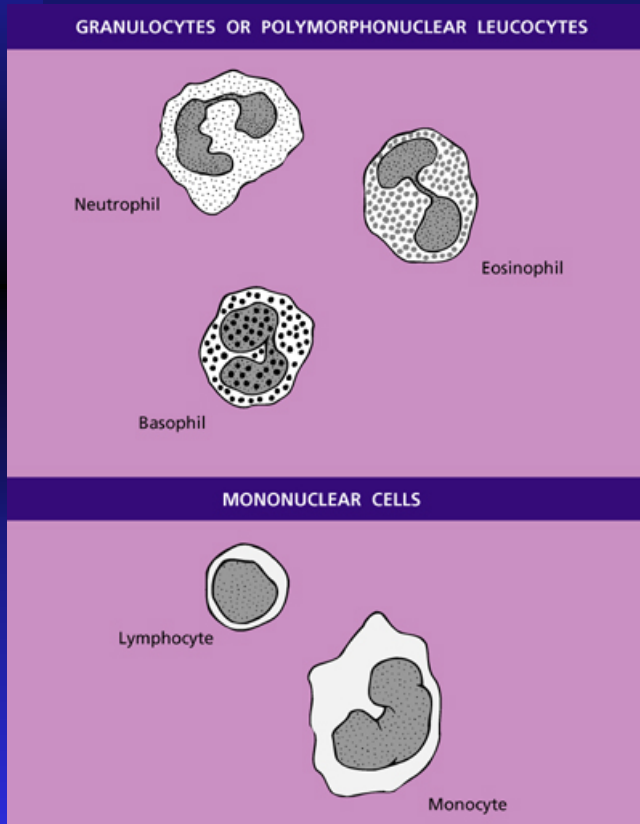
# Real-time PCR in Hematology

Marleen Bakkus

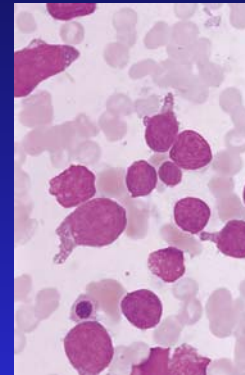
BVAC/ABCA Workshop

18/11/04

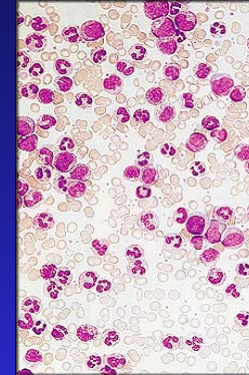
# Leukemia



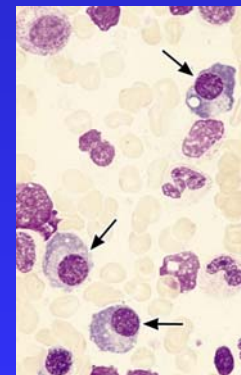
AML



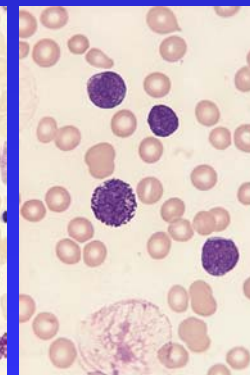
CML



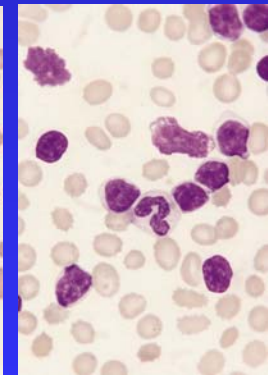
MM



B-ALL



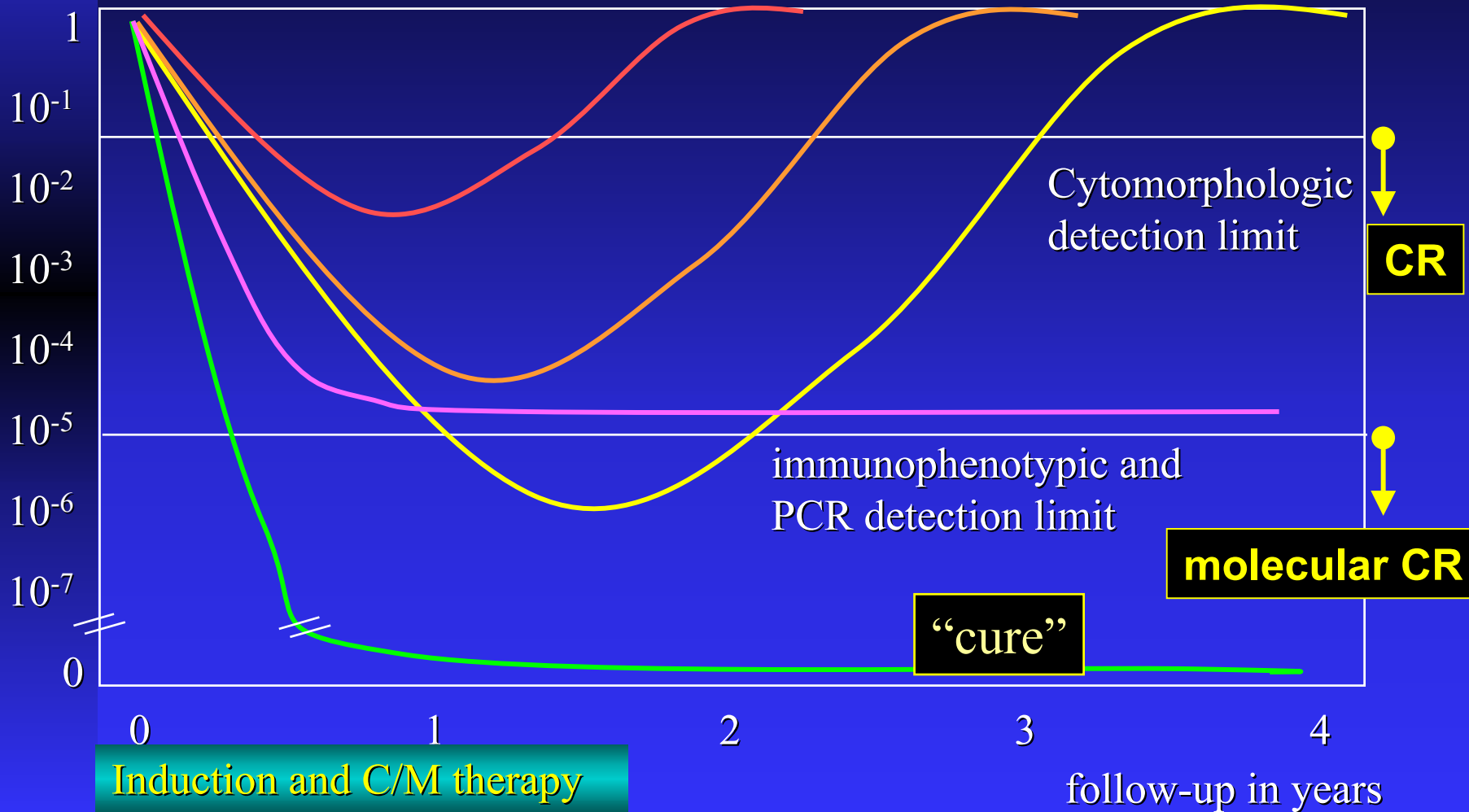
CLL



# Introduction

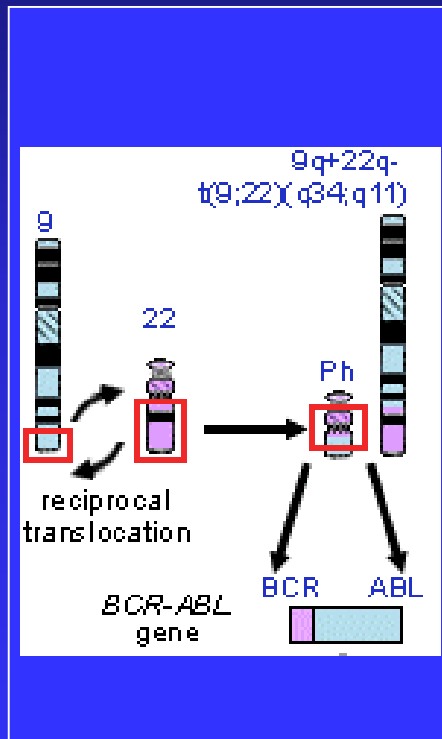
- Molecular biological techniques : radical improvement in the evaluation of hematological disorders.
- Current treatment protocols for acute or chronic leukemia are based on prognostic factors, like aberrant fusion transcripts.
- MRD or minimal residual disease information is important for prediction of relapse and early therapeutic intervention.

# Effect of therapy on tumor cell number



# Recognition I

- Unique DNA/RNA markers due to **translocation**



t(9;22) CML 100% **unfavourable**  
ALL <40%

t(8;21) AML 5-10% **favourable**

t(15;17) AML 5-10% **favourable**

inv16 AML 5-10% **favourable**

t(14;18) FL >90%  
DLBCL 10-20%

t(11;14) MCL >90%

t(11;18) MALT 30%

t(2;5) ALTL

**classification**

# Translocations: fusiontranscripts

- **t(1;19):E2A/PBX1**
- t(3;5):NPM/MLF1
- t(3;21):AML1/MDS1
- **t(5;12):TEL/PDGFRb**
- t(5;17):NPM/RARA
- t(6;9):DEK/CAN
- **t(8;21):AML1/MGT8**
- t(9;9):SET/CAN
- t(9;12):TEL/ABL
- **t(9;22):BCR/ABL**
- t(11;17):PLFZ/RARA
- **t(12;21):TEL/AML1**
- t(12;22):TEL/MN1
- **t(15;17):PML/RARA**
- t(16;21):TLS/ERG

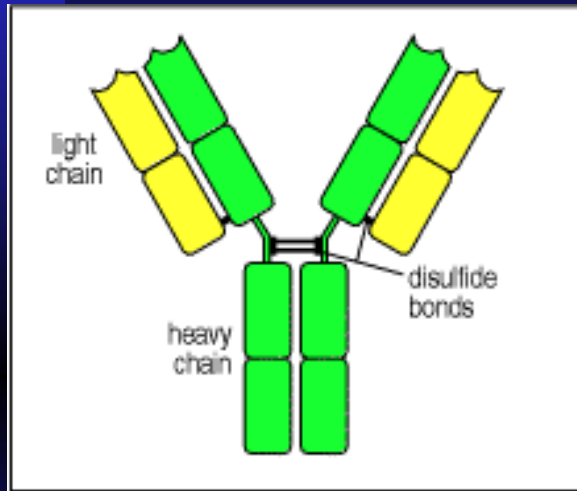
- **Inv16:CBFb/MYH11**
- t(17;19):E2A/HLF
- TAL1D:SIL1/TAL1

## MLL1 translocaties

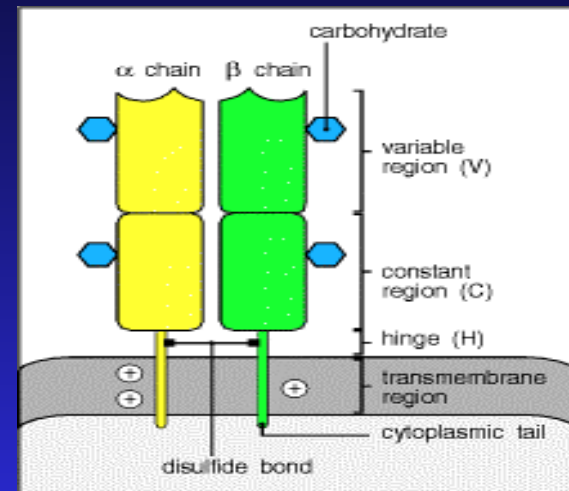
- t(1;11):MLL1/AF1p
- t(1;11):MLL1/AF1q
- **t(4;11):MLL1/AF4**
- t(6;11):MLL1/AF6
- t(9;11):MLL1/AF9
- t(10;11):MLL1/AF10
- t(11;17):MLL1/AF17
- t(11;19):MLL1/ENL
- t(X;11):MLL1/AFX)

# Recognition II

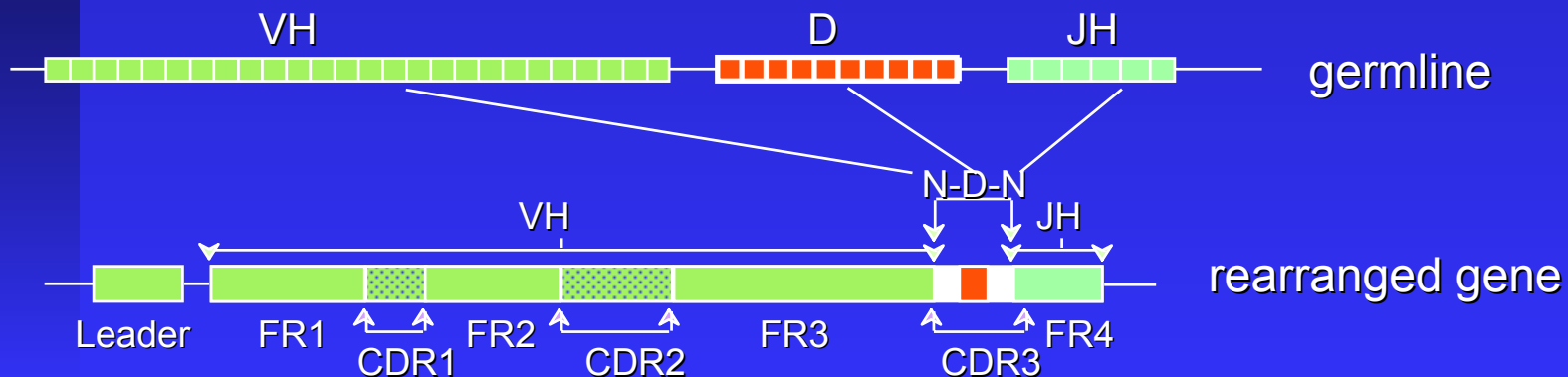
- Unique DNA due to **gene rearrangement**



Immunoglobulin (Ig)



T-cell receptor (TCR)





# Recognition III

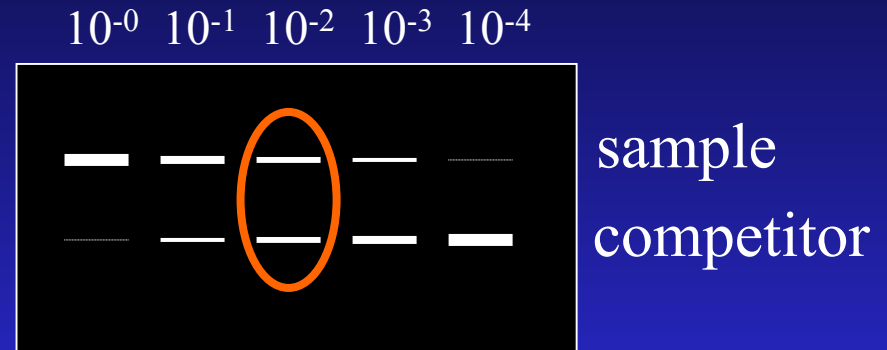
## ■ Overexpression

- ◆ WT1: Wilms' tumor gene 1 (AML, CML, ALL, MDS)
- ◆ Cyclin D1: due to t(11;14) (MCL, CLL, MM)
- ◆ PRV-1: polycythemia rubra vera-1, (polycythemia vera vs secondary erythrocytosis)

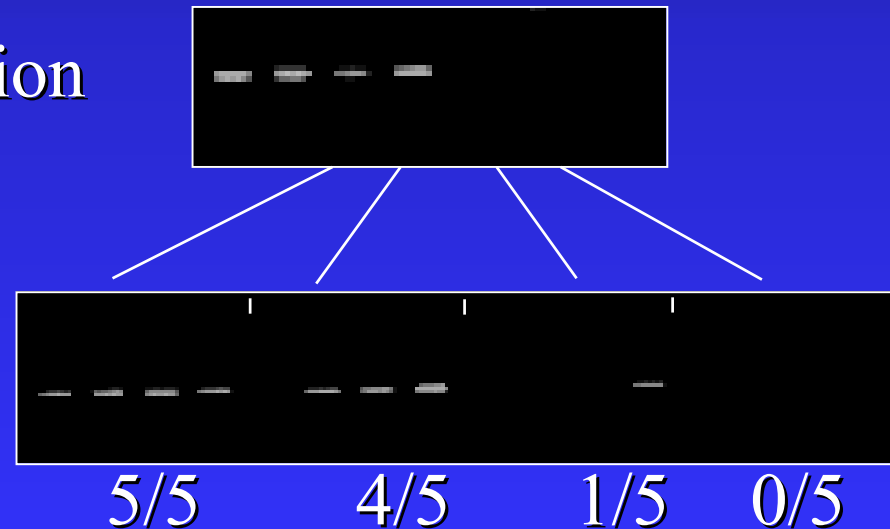
# Quantification I

## ■ History

### ◆ Competitive PCR



### ◆ Limiting dilution



# Quantification II

## ■ Real-time quantitative PCR: RQ-PCR

### ◆ Since 1996 TaqMan probes

Real time quantitative PCR. Genome Res. 1996 Oct;6(10):986-94

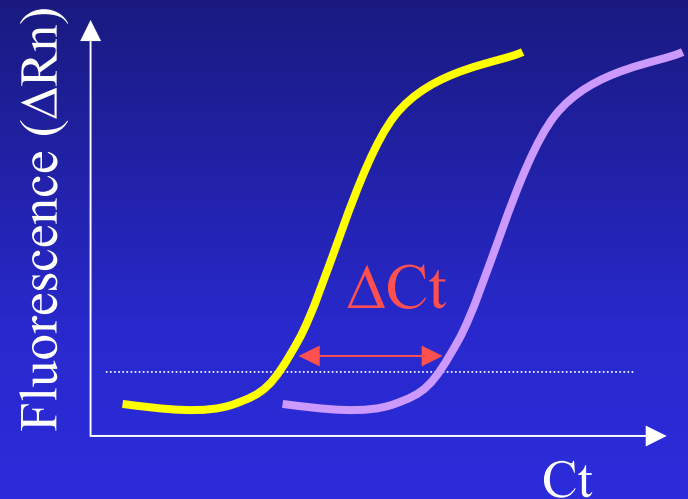
Heid CA, Stevens J, Livak KJ, Williams PM.

BioAnalytical Technology Department, Genentech, Inc., South San Francisco, California 94080, USA.

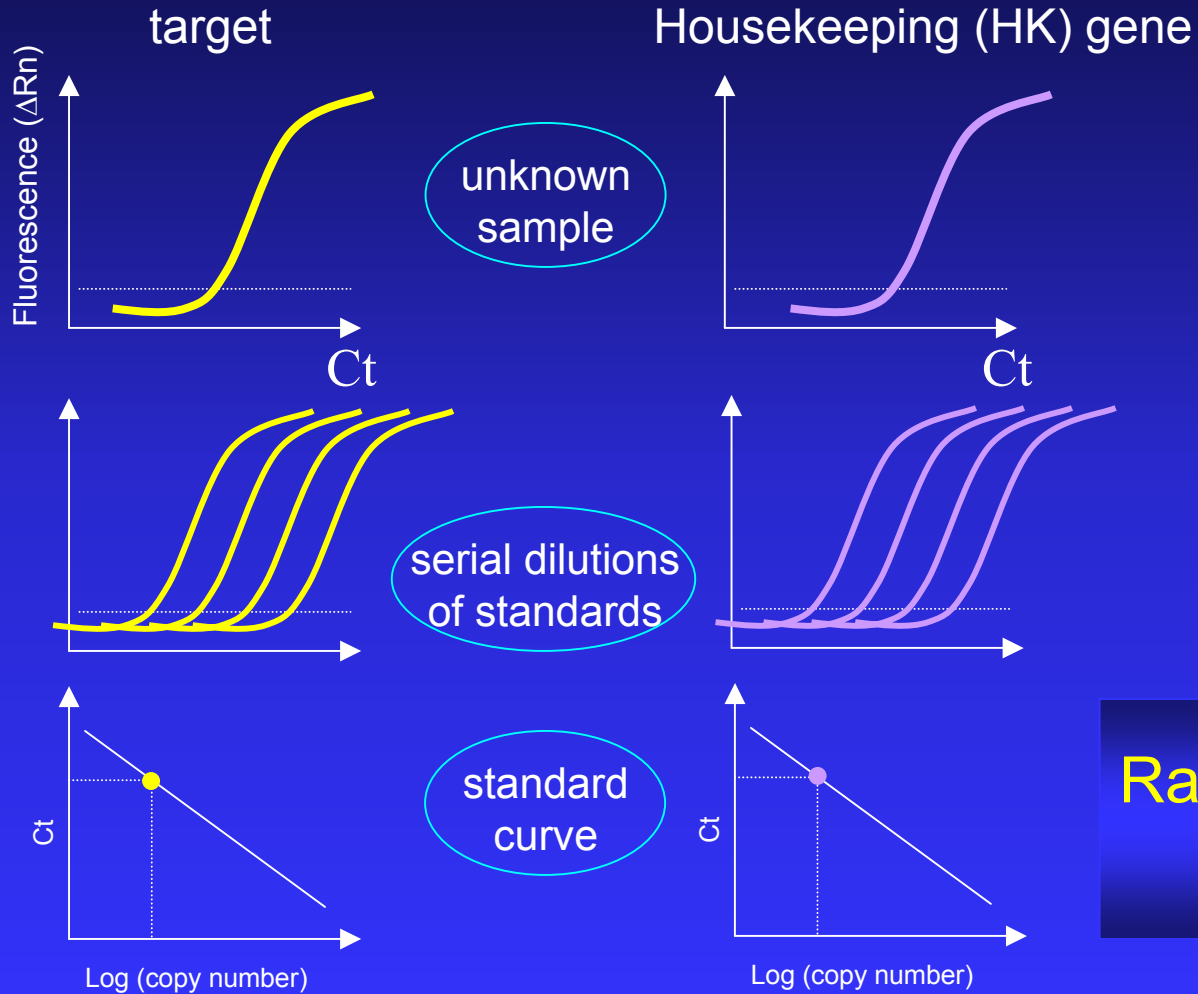
We have developed a novel "real time" quantitative PCR method. The method measures PCR product accumulation through a dual-labeled fluorogenic probe (i.e., **TaqMan Probe**). This method provides very **accurate and reproducible quantitation of gene copies**. Unlike other quantitative PCR methods, real-time PCR does **not require post-PCR sample handling**, preventing potential PCR product carry-over contamination and resulting in **much faster and higher throughput assays**. The real-time PCR method has a **very large dynamic range** of starting target molecule determination (at least **five orders of magnitude**). Real-time quantitative PCR is **extremely accurate and less labor-intensive** than current quantitative PCR methods.

# Comparative method

- $\Delta C_t$  method
- No standard-curve
- 2 PCR's of equal efficiency
- $\Delta C_t = C_t$  target gene –  $C_t$  housekeeping gene
- Ratio:  $2^{-\Delta C_t}$



# Standard-curve method



$$\text{Ratio: } \frac{\# \text{target}}{\# \text{HK}}$$

# Housekeeping genes I

- Stable expression
- Normalises for differences in amount, quality of starting material, RNA prep, cDNA synthesis
- Transcription level in same range as target gene

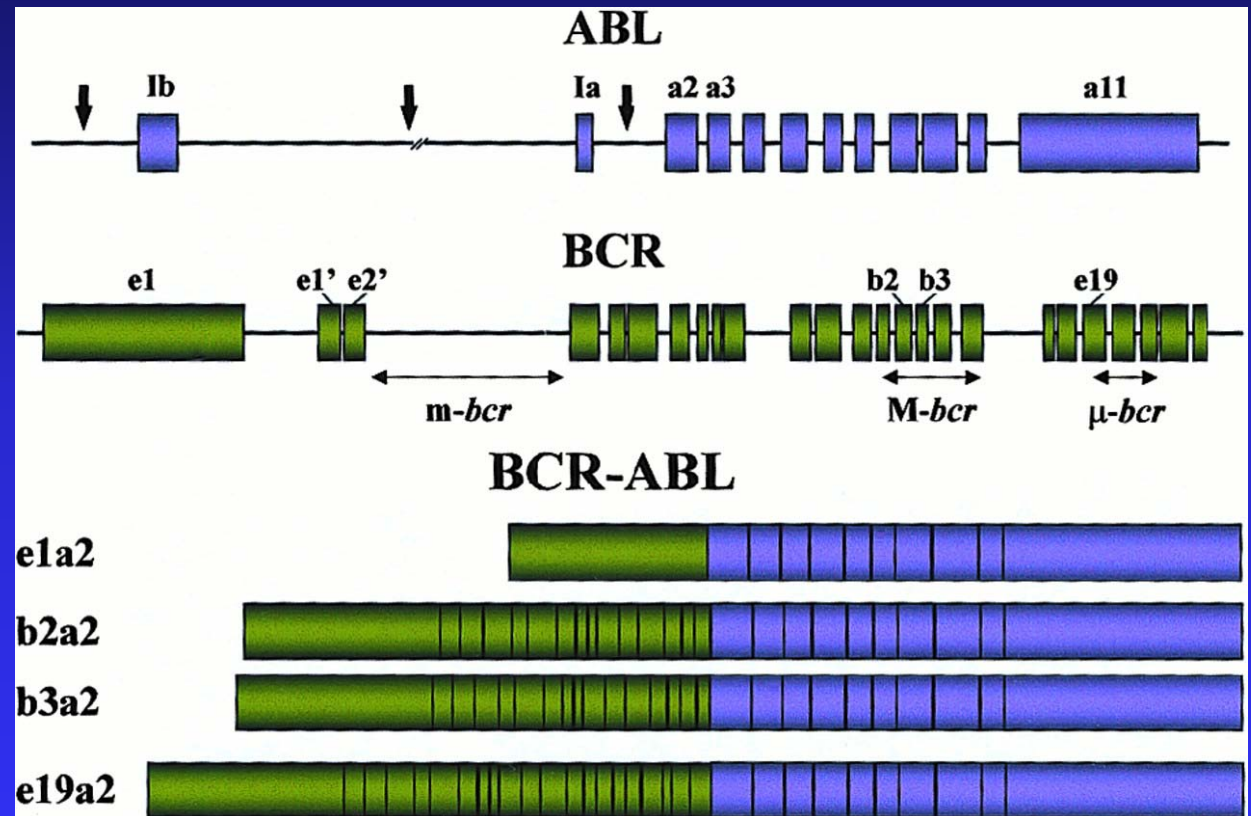
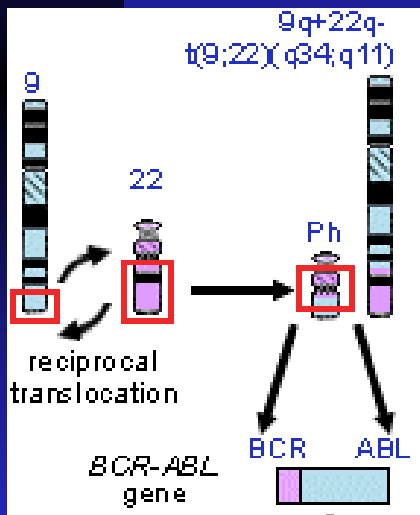
*Vandesompele J. et al: Genome Biology, 2002*

<http://medgen.ugent.be/~jvdesomp/genorm/>

# Houskeeping genes II

- Abl for peripheral blood and bone marrow FG analysis and overexpression (diagnosis and MRD), recommended by the EAC group
- Albumin for DNA (from the guidelines of the ESG-MRD-ALL)

# BCR/ABL gene

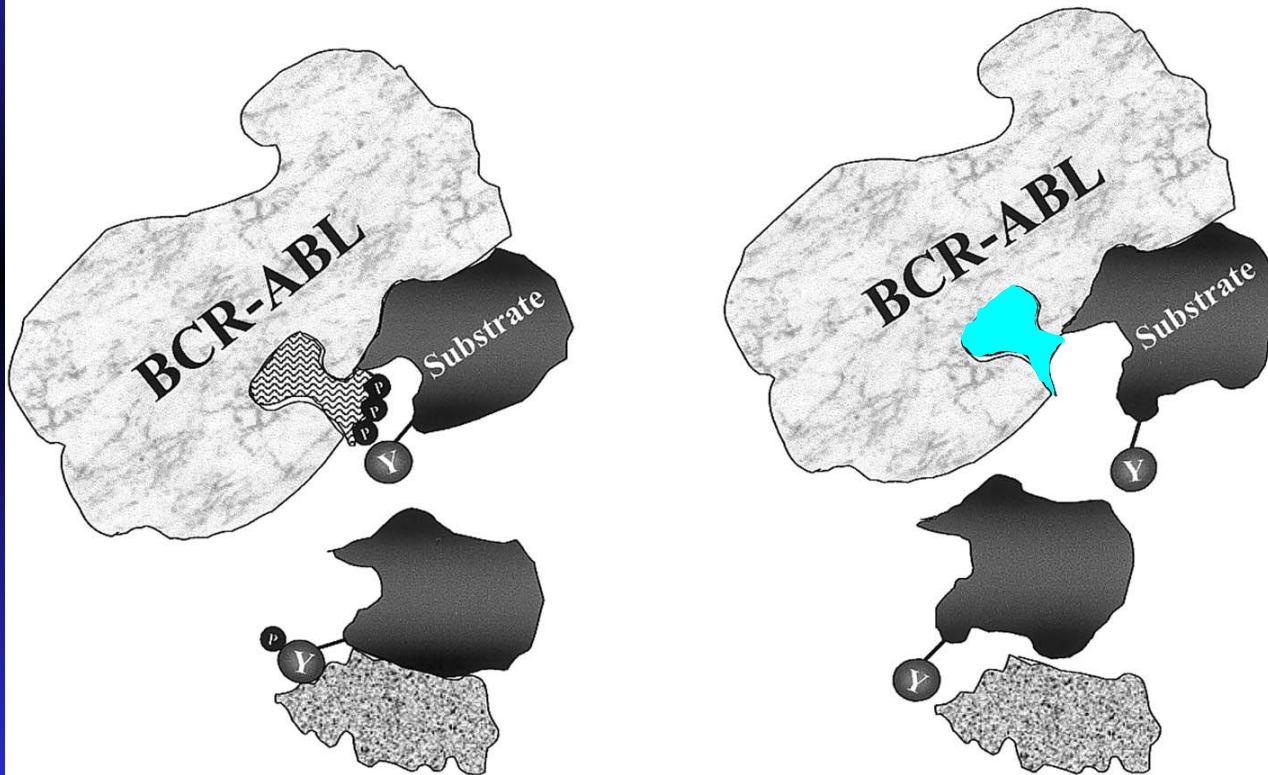


# Treatment options CML

- Chemotherapy
- Interferon
- BMT plus DLI (MRD monitoring is essential)
- Novel : STI571/Glivec/Imatinib

# Bcr-abl tyrosine kinase

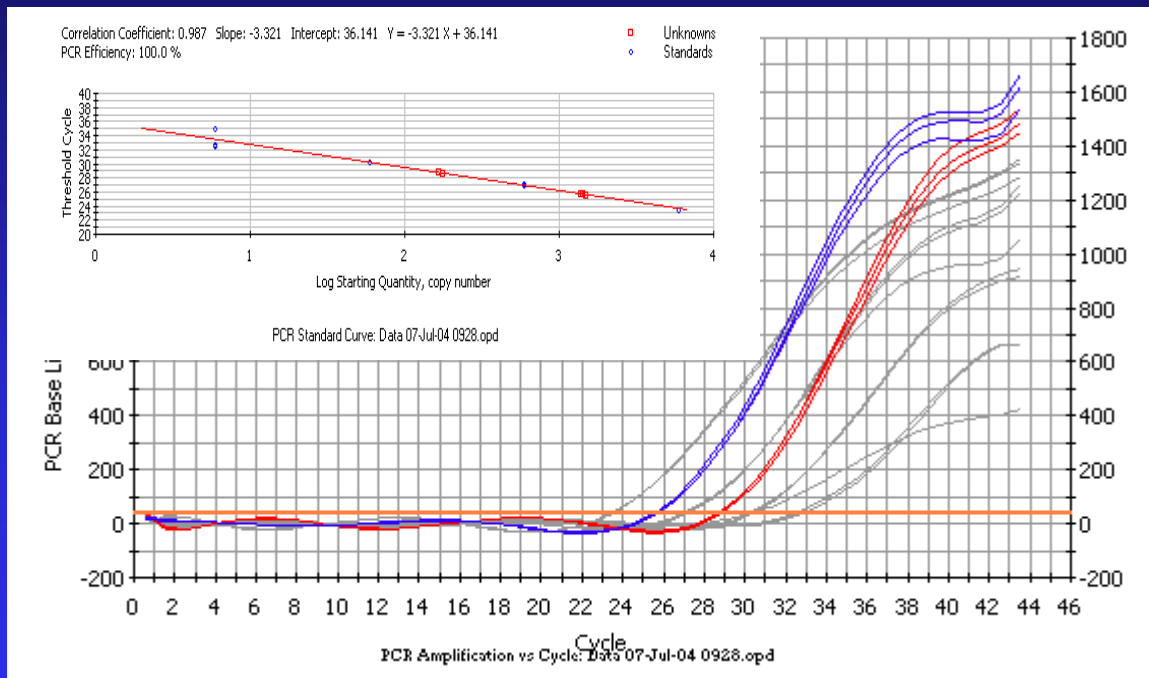
## ATP-binding competitors



# RQ-PCR for Bcr-Abl

- Primers and probes for M-bcr and m-bcr breakpoints
- TaqMan Probe: FAM-TAMRA
- Reference gene: Abl (TexasRed-BHQ)
- Standards: plasmid dilutions (10-100-1000-10,000 copies/5 $\mu$ l) (Ipsogen) or cDNA from K562 cell line diluted in cDNA from HL60 cell line.
- Platform: iCycler from Biorad

# RQ-PCR Bcr-abl



— unknown 1  
#cop: 1420

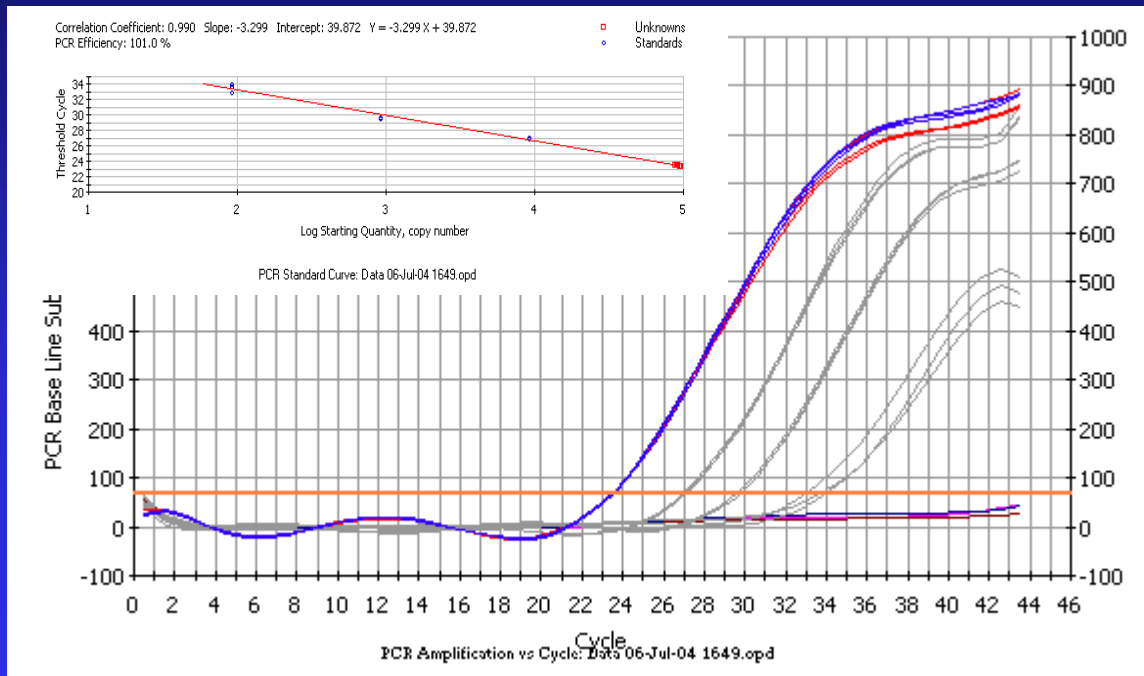
— unknown 2  
#cop: 169

TaqMan probe: FAM-TAMRA

Standards: K562 cDNA in HL60 cDNA

Sensitivity:  $10^{-5}$

# Control gene Abl



— unknown 1  
#cop: 92900

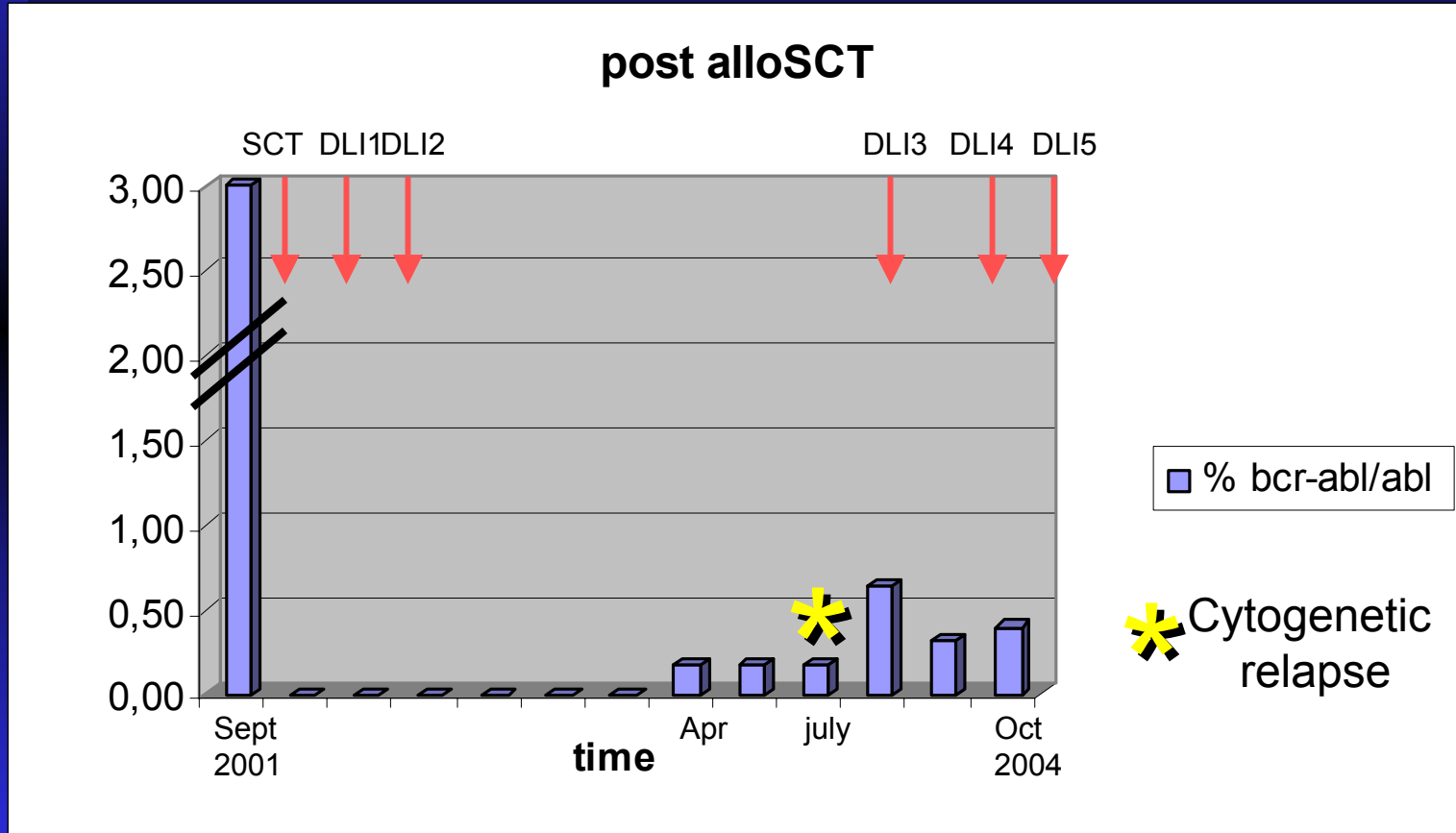
— unknown 2  
#cop: 94800

— Quality of cDNA/RNA sample is good, quantification possible

# Quantification

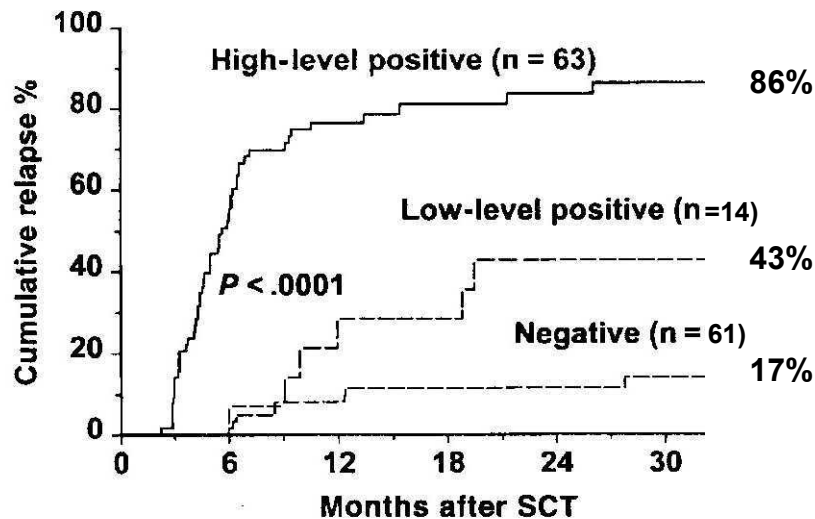
	Bcr-Abl copies	Abl copies	Bcr-Abl/Abl
UNK 1	1420	92,900	1.5%
UNK 2	169	94,800	0.18%

# Example RQ-PCR bcr-abl





# MRD post SCT



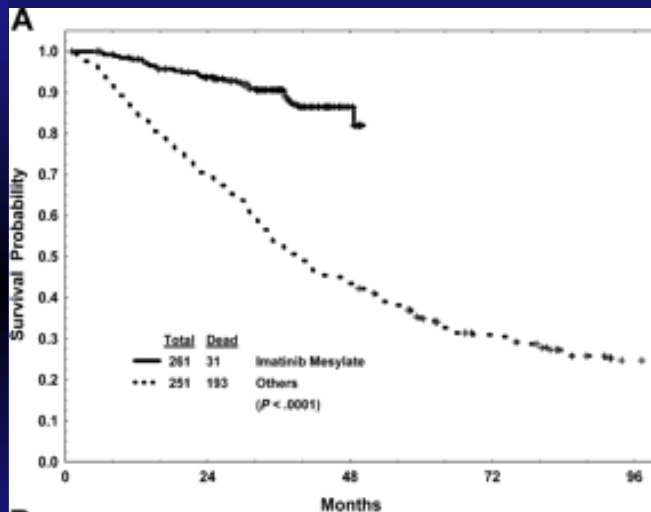
Test: 3-5 mnd post SCT

High-level:  $\geq 0.02\%$  bcr-abl/abl

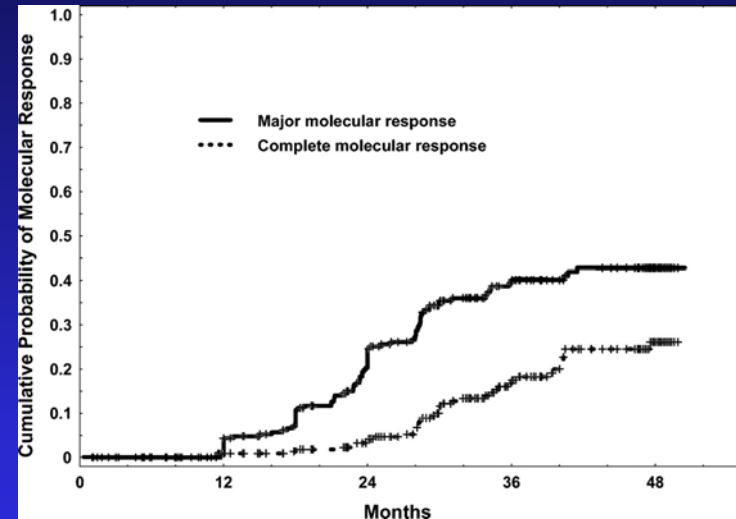
Low-level:  $< 0.02\%$  bcr-abl/abl

*Ref: Olavarria E., Blood, 2001, 97, 1560.*

# Imatinib treatment



Survival of 261 pt after failed response to IFN- $\alpha$  and 251 historical pt treated with other regimens.



Cumulative rates of major (BCR-ABL/ABL less than 0.05%) and complete (undetectable) molecular responses.

*Kantarjian HM et al, Blood, 2004*

# Most frequently used FG in RQ-PCR

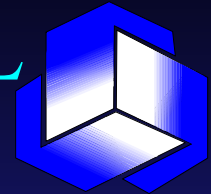
aberration	FG	Malignancy	MRD quantification useful?
t(8;21)	ETO/AML1	AML-M2	?
Inv(16)	CBFB/MYH11	AML-M4neo	?
t(9;22)	BCR/ABL	CML/ALL/AML	++ 0.02-0.05%
t(15;17)	PML/RARA	AML-M3	- 0%
t(4;11)	MLL/AF4	AML/ALL	?
t(12;21)	TEL/AML	ALL	?
t(1;19)	E2A/PBX1	ALL	?

# Ig/TCR RQ-PCR

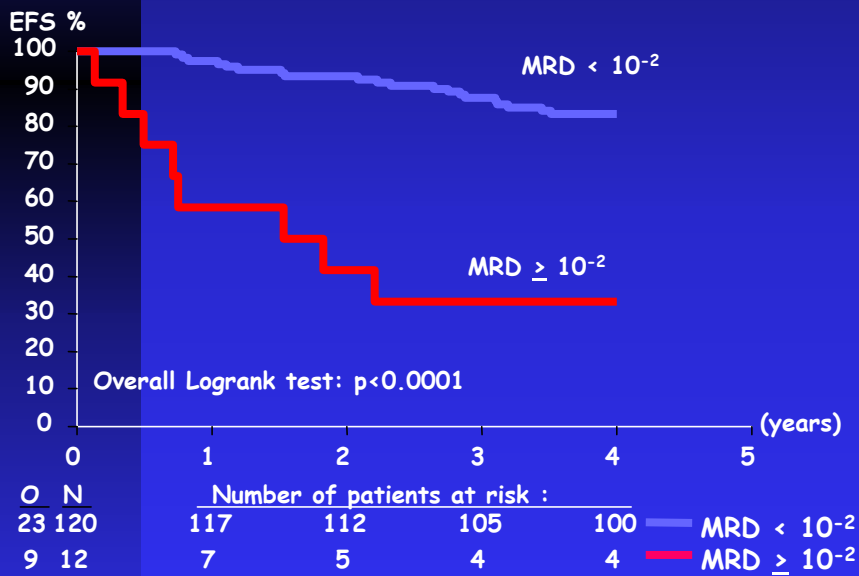
- MRD detection in childhood-ALL
- High sensitivity and specificity
- allele-specific oligo (ASO) required for each patient

# MRD studies in childhood-ALL

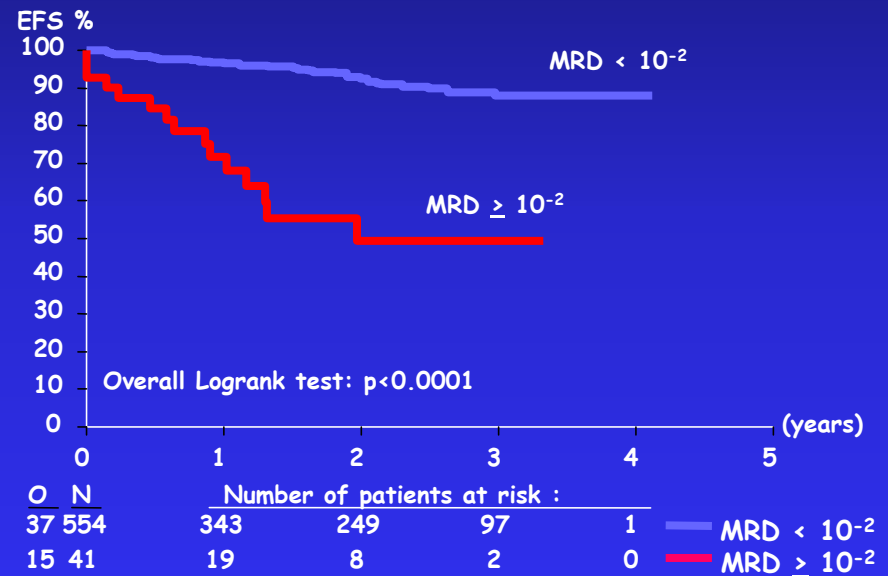
## EORTC



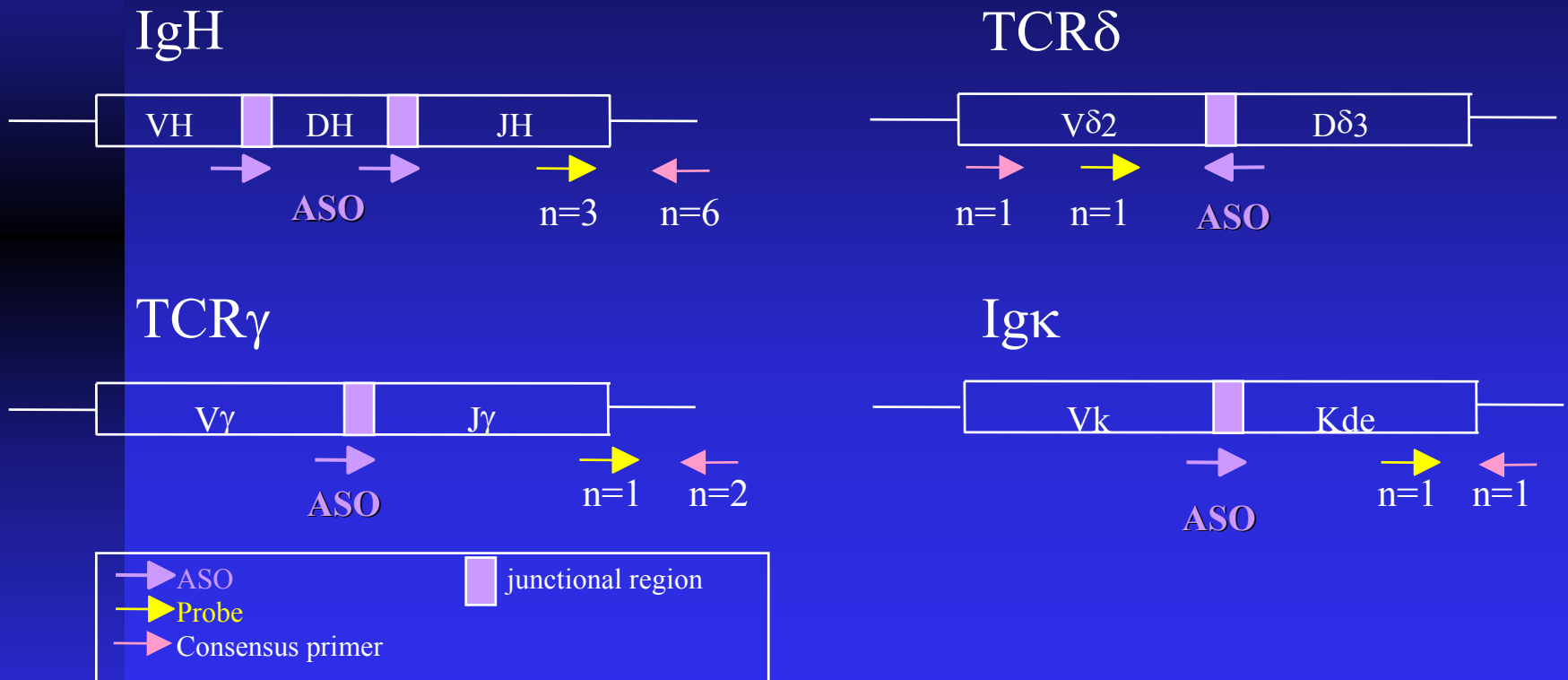
**EORTC 58 881**  
(median follow up >5 years)



**EORTC 58 951**  
(median follow up 2 years)

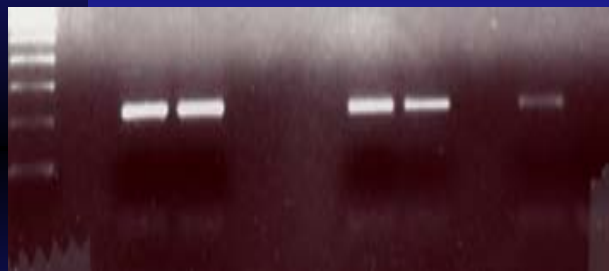


# ASO-primers in RQ-PCR



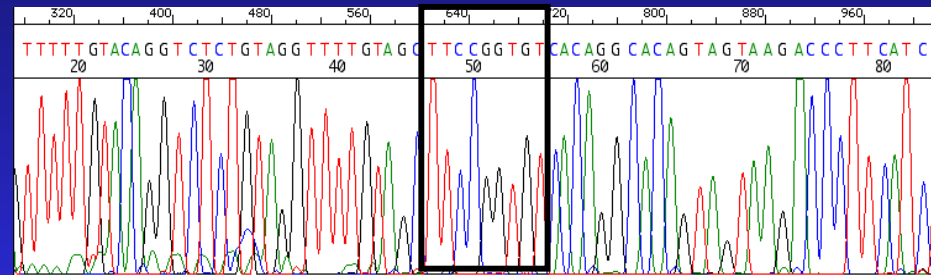
# Sequencing

a. PCR-products on agarose gel



Sample 88    Sample 56    control

b. Sequence on ABI-sequencer



Junction region

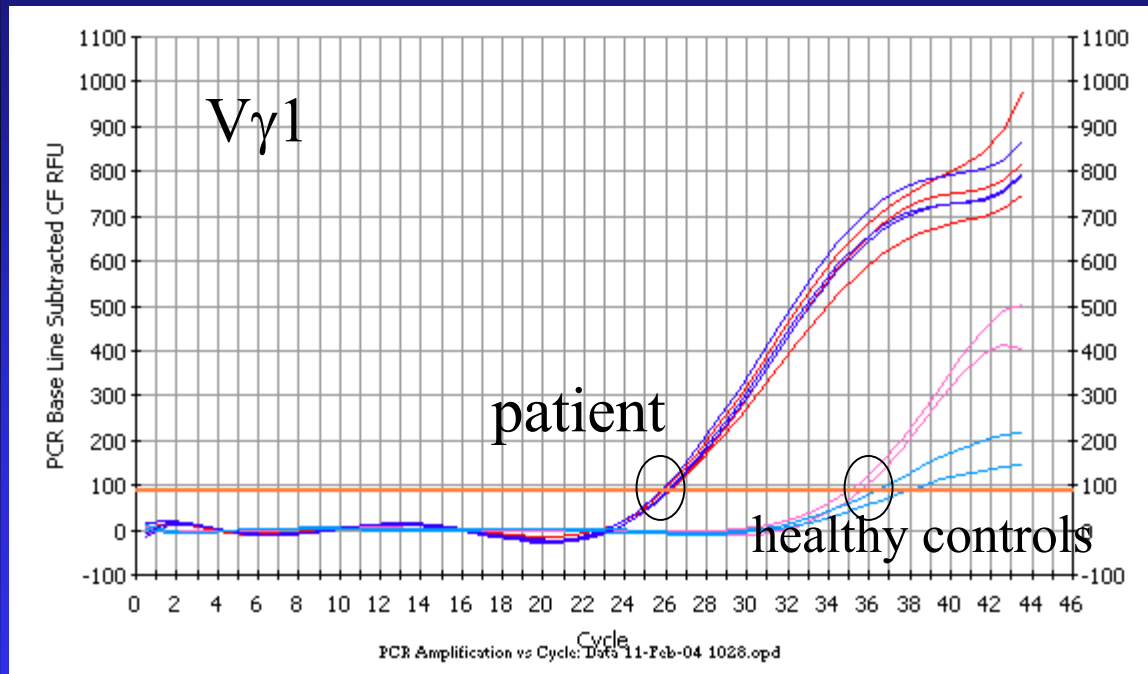
design ASO



# ASO-RQ-PCR

- Temperature gradient test of diagnostic sample 1/100 and healthy control sample (pool of at least 5 healthy volunteers)
- $\text{MgCl}_2$  optimization
- Standard curve test (diagnostic sample in healthy control  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $5 \cdot 10^{-4}$ ,  $10^{-4}$ ,  $5 \cdot 10^{-5}$ ,  $10^{-5}$ ) : sensitivity and quantifiable range
- Quantify unknown by standard curve method, control gene is Albumin.

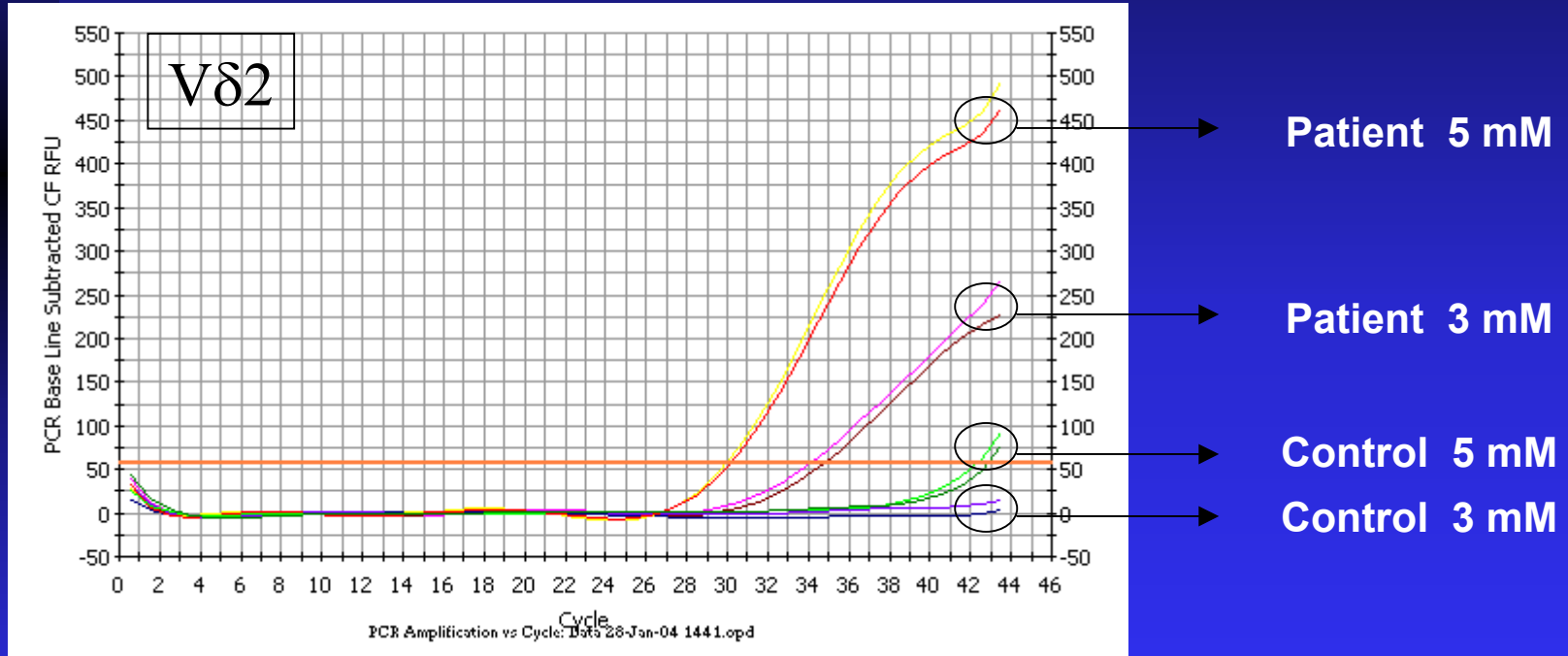
# Temperature gradient



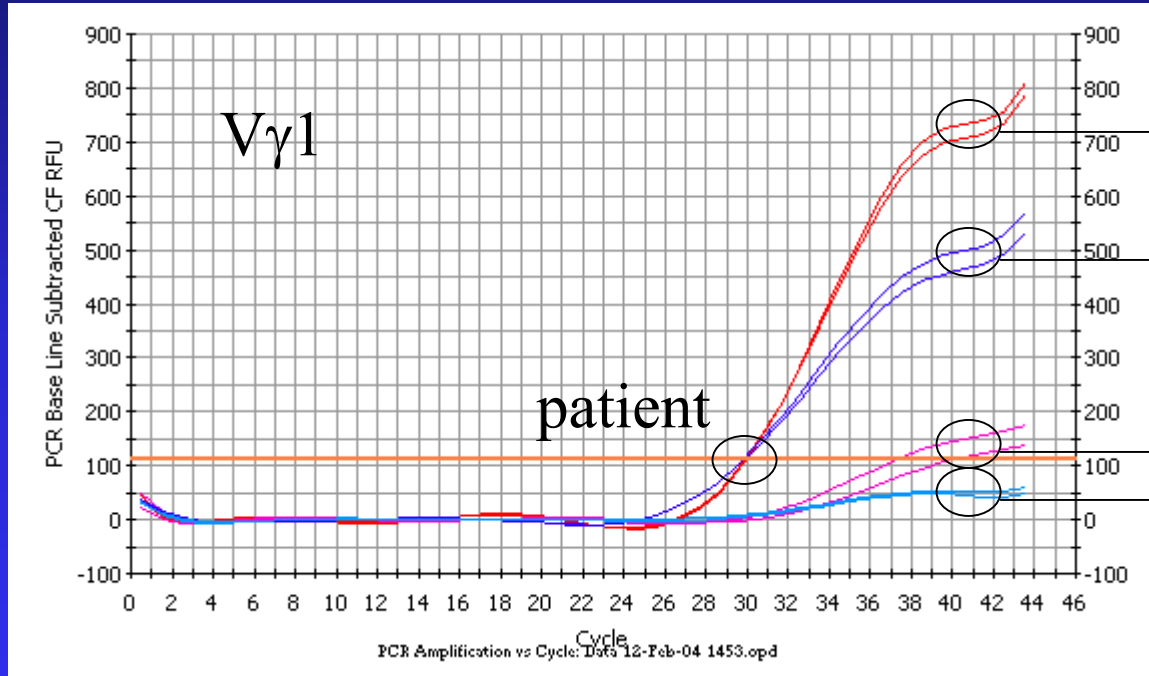
60°C

63°C

# Optimisation MgCl<sub>2</sub> I



# Optimalisation MgCl<sub>2</sub> II



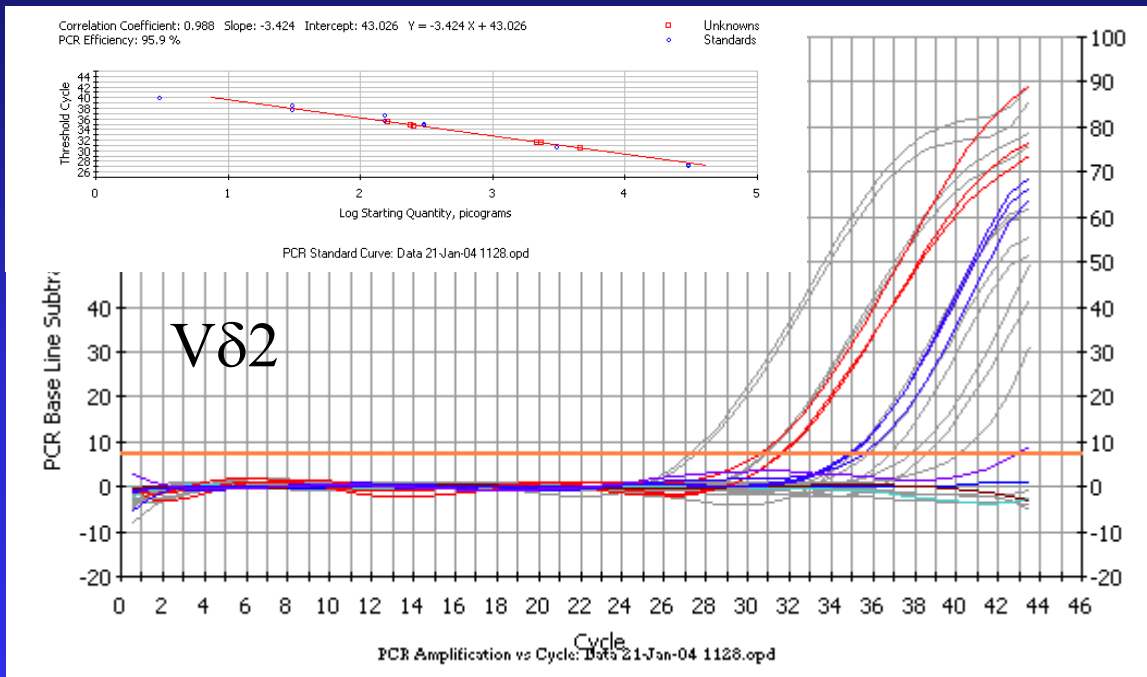
3 mM

5 mM

3 mM

5 mM

# d35 RQ-ASO PCR



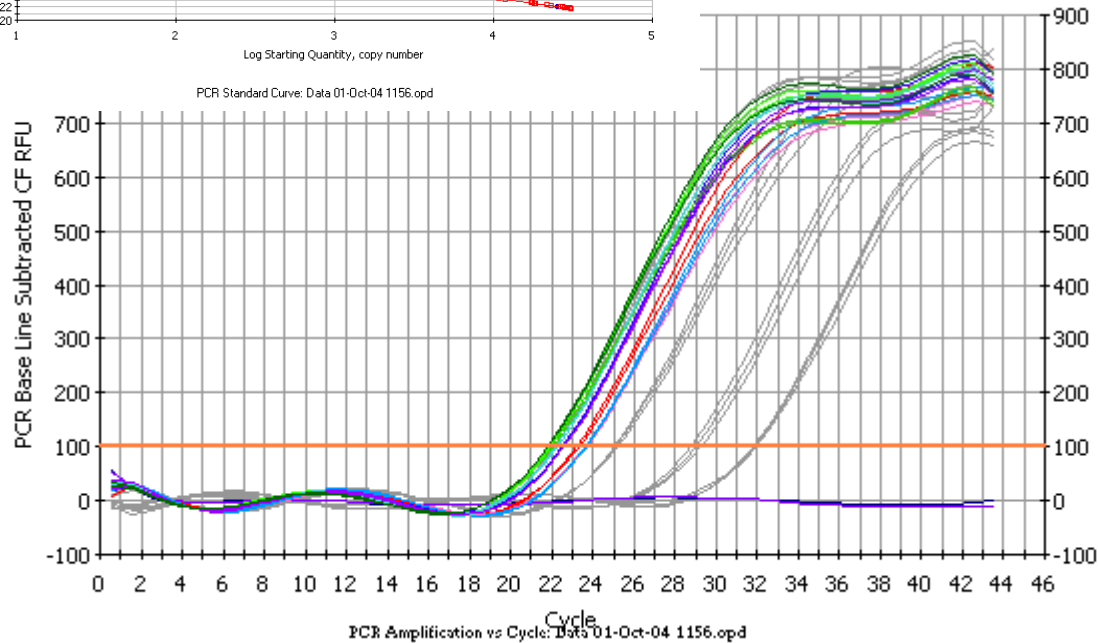
- d35 undiluted 1.14%
- d35 1/10 0.07%

Sensitivity:  $1.10^{-5}$

# Control gene Albumin

Correlation Coefficient: 0.998 Slope: -3.294 Intercept: 36.558  $Y = -3.294 X + 36.558$   
PCR Efficiency: 101.2 %

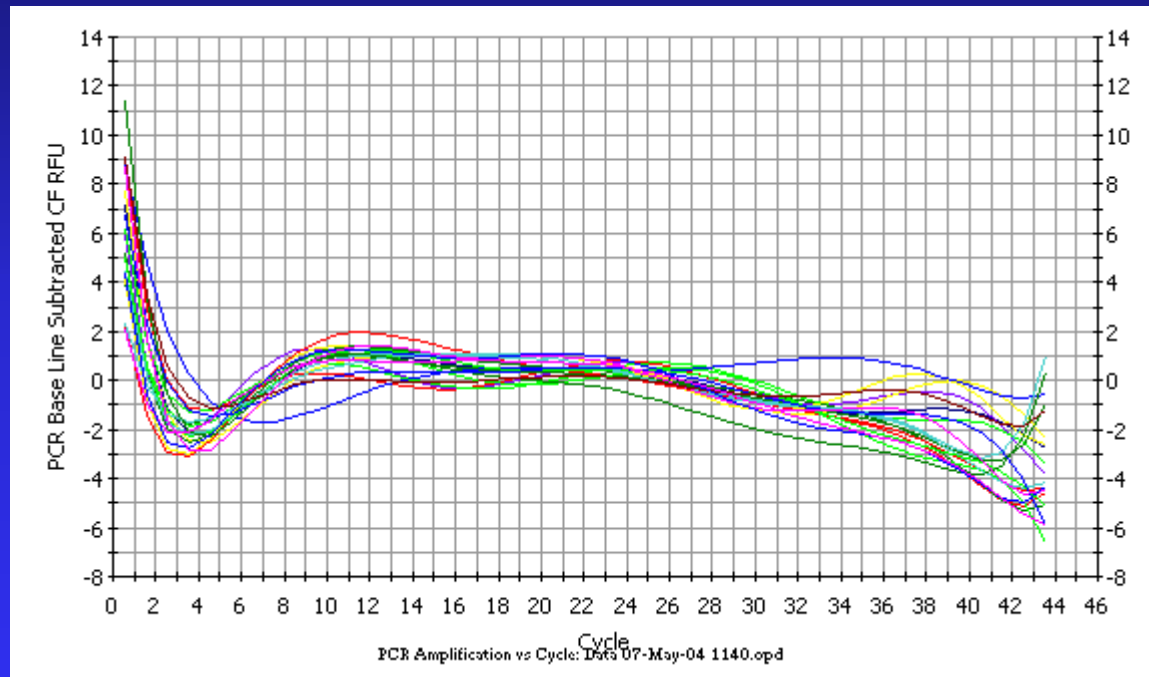
Unknowns  
Standards



Standard: Control DNA from healthy volunteers

# Failure

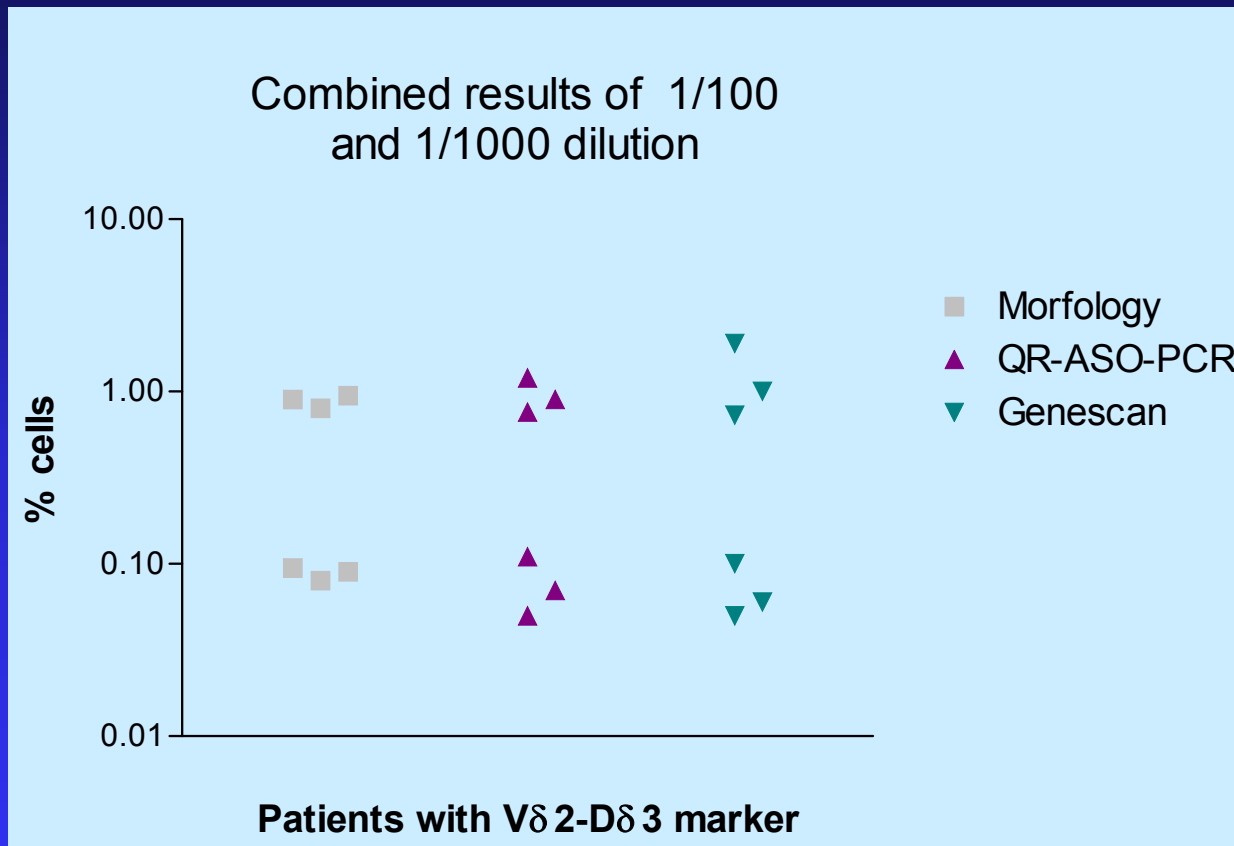
FR2 temperature gradient 58-66



# Ig/TCR RQ-PCR results

<b>PCR target</b>	<b>Suitable ASO (%)</b>	<b>sensitivity</b>
IgH (n=6)	50	$\geq 5 \cdot 10^{-4}$
IgK-Kde (n=11)	91	$\geq 1 \cdot 10^{-4}$
TCR $\delta$ (n=4)	100	$\geq 1 \cdot 10^{-4}$
TCR $\gamma$ (n=4)	75	$\geq 1 \cdot 10^{-4}$

# Quantification with RQ-ASO-PCR and Genescan



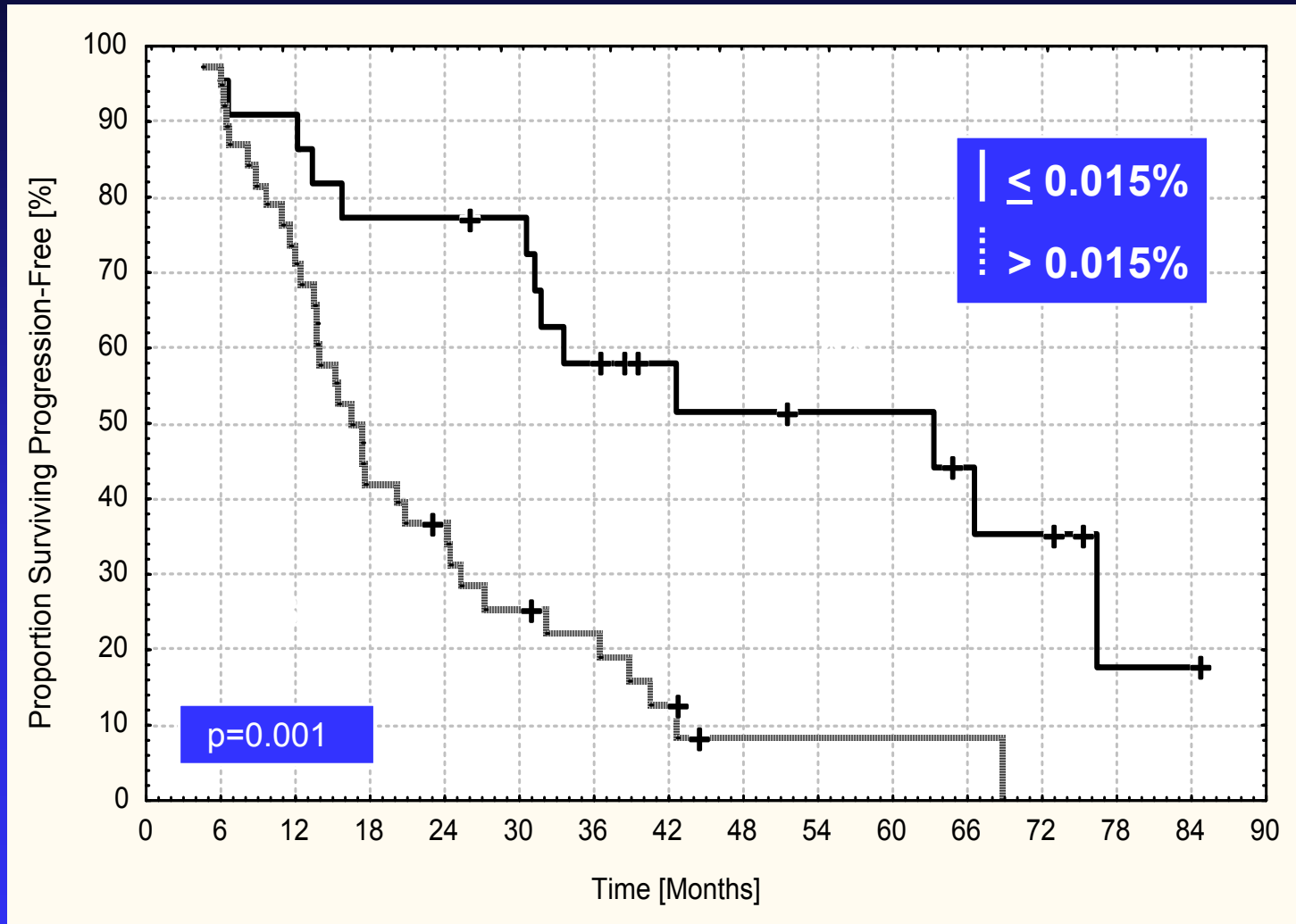
⇒ No differences

# RQ-PCR Ig/TCR interpretation guidelines

(recommended by the European study group on MRD detection in ALL)

- Standard curve:  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  dilutions in DNA from normal PB MNC (mixture of at least 5 healthy controls) in at least duplicate. Slope between  $-3.1$  and  $-3.9$  (E= 80,5% – 110%) and corr.coefficient  $\geq 0.98$
- At least 250 ng DNA and maximally 1.0  $\mu\text{g}$  of DNA in each PCR tube (we use 300 ng which corresponds to +/- 50.000 cells). Triplicate analyse evaluates  $\sim 1-5 \times 10^5$  cells.
- Quantitative range: lowest dilution, that:
  - ◆ Gives reproducible amplification:  $\Delta\text{Ct}$  replicates  $\leq 1.5$
  - ◆ Has highest Ct  $\geq 3$  lower than lowest Ct of background signal
- Sensitivity: lowest dilution, that:
  - ◆ Has at least one well positive ( $\Delta\text{CT}$  replicates not relevant)
  - ◆ Has the highest Ct  $\geq 1$  lower than the lowest Ct of the background signals

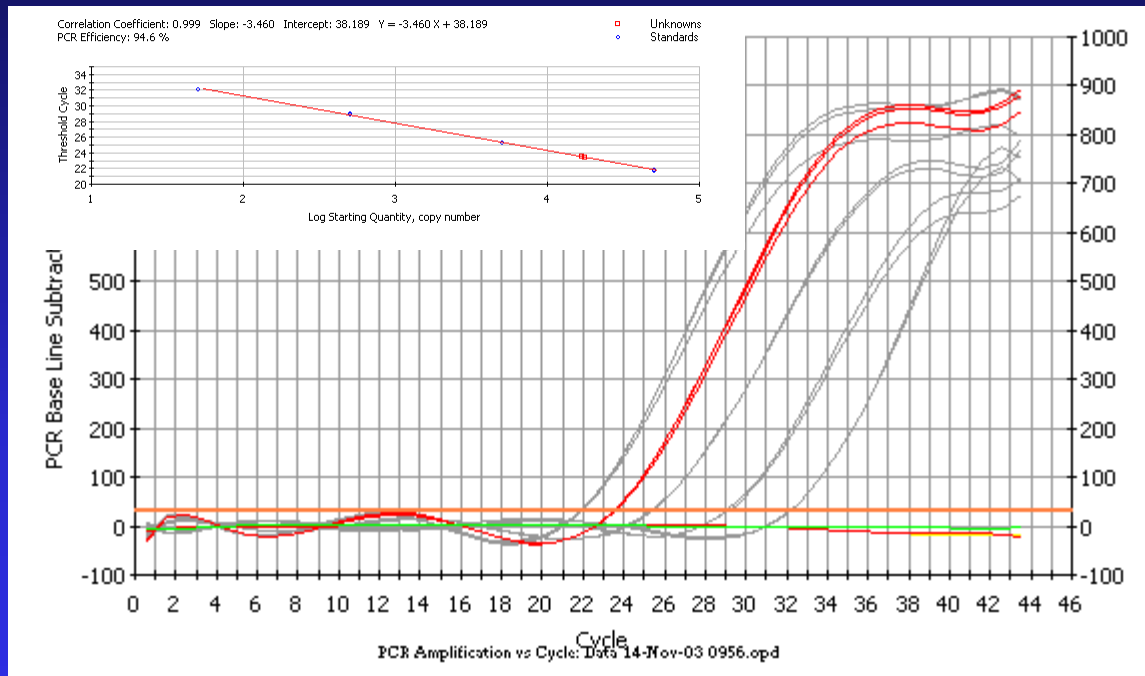
# PFS in MM according to tumor load at 3 months post PBSCT (n=60)



# WT1

- A panleukemic marker for MRD detection?
- Highly expressed in haematopoietic precursor cells and in AML, ALL, CML and MDS.
- Prediction of relapse?

# RQ-PCR WT1

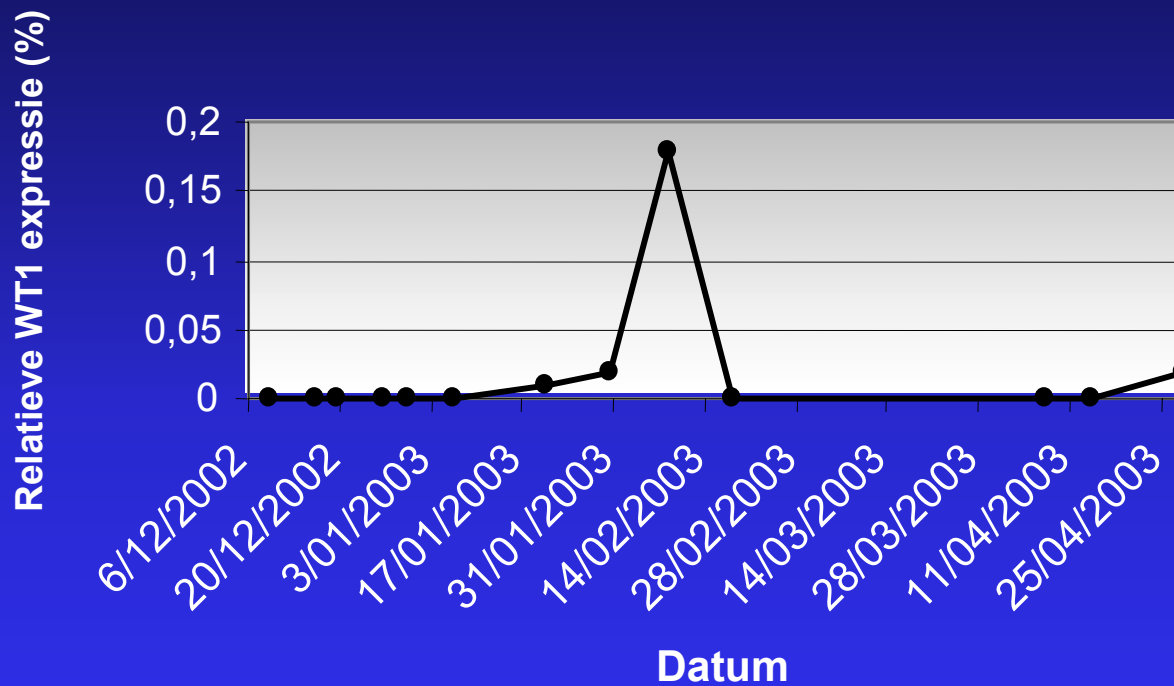


— ALL BM  
34.6%  
K562

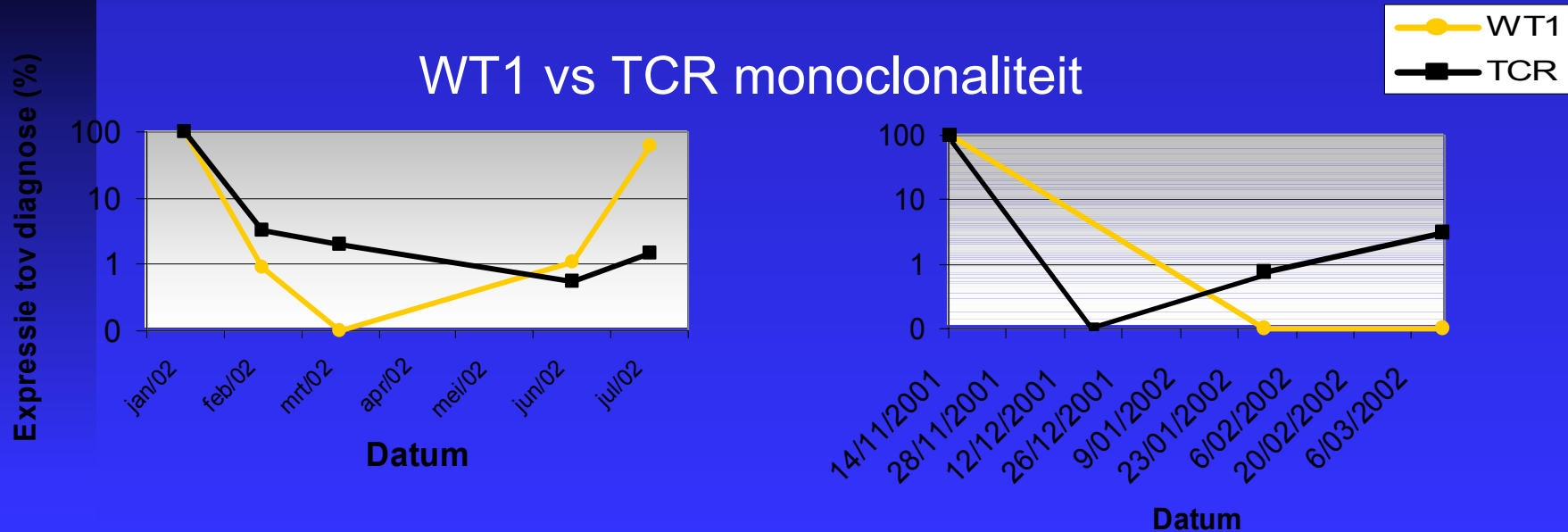
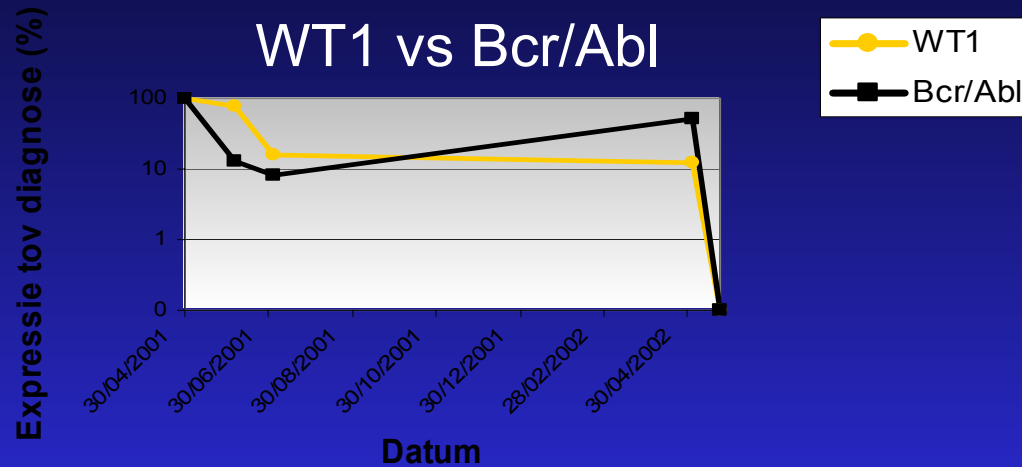
Primer and probe design in exon 6 and 7.  
Standard : K562 cDNA, 50-5.10<sup>4</sup> pg/well  
Control gene: PBGD or Abl



# Follow-up after SCT



# Comparison with other tumor markers



# WT1 after SCT

- WT1 as panleukemic MRD marker is interesting
- But sensitivity is low due to background expression and increase in WT1 is usually found at time of relapse, no predictive value
- Our policy: use it when no other markers are available

# Communication to the clinic

- Negative?

- ◆ Better: below the detection level.

- Positive?

- ◆ Information about increase or decrease to previous sample may be important and normal background levels as well.

# Standardisation

- Sample transport
- Sample treatment
- Sample storage
- DNA/RNA extraction
- RT step
- Primer and probe design and production
- Housekeeping genes

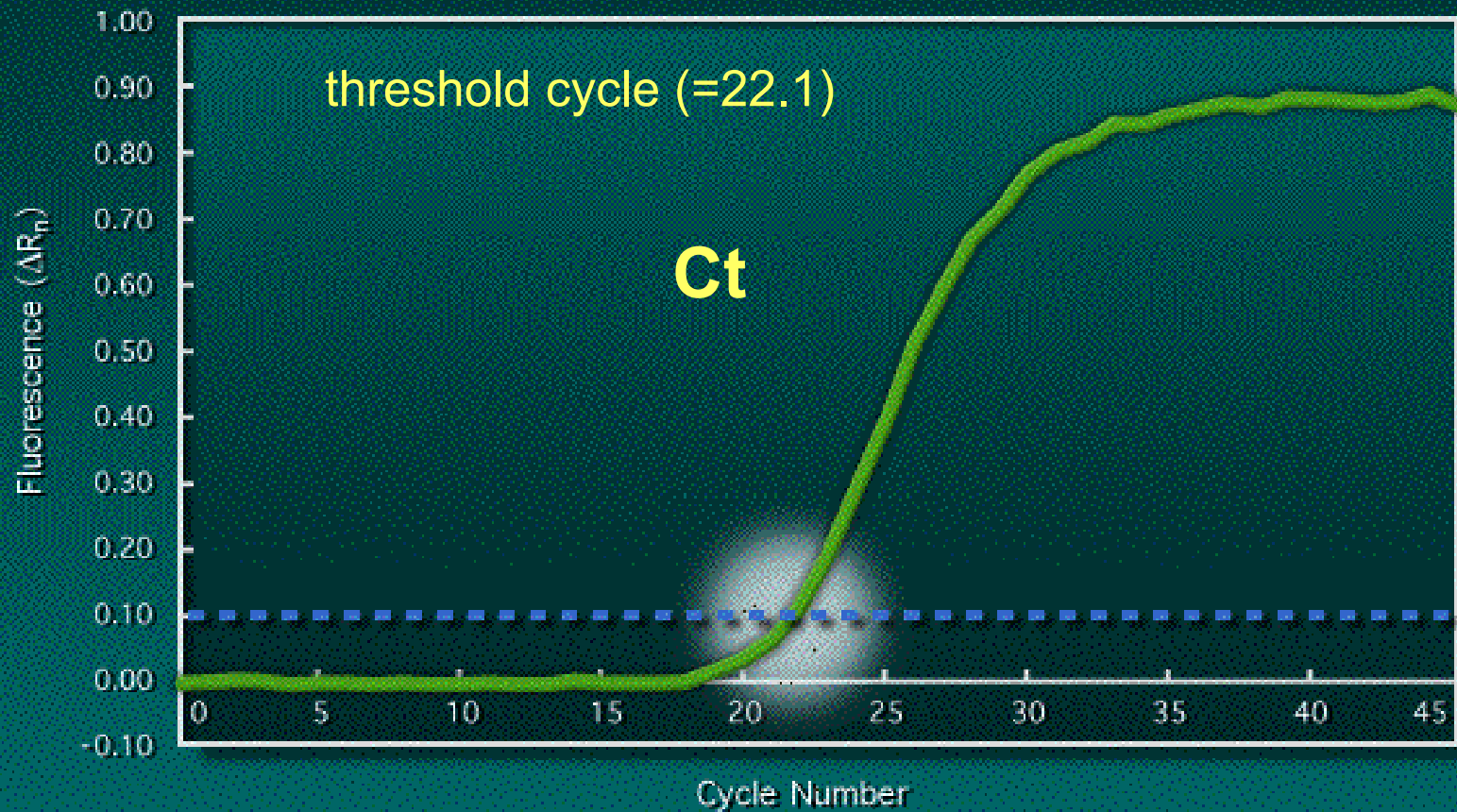
Participation in national and international QC rounds:  
-CMD (B)  
-MODHEM (NL)  
<http://www.modhem.nl/>  
-ESG-MRD-ALL

Further reading: **Gabert F.**, *Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. **Leukemia 2003, 1-40.***

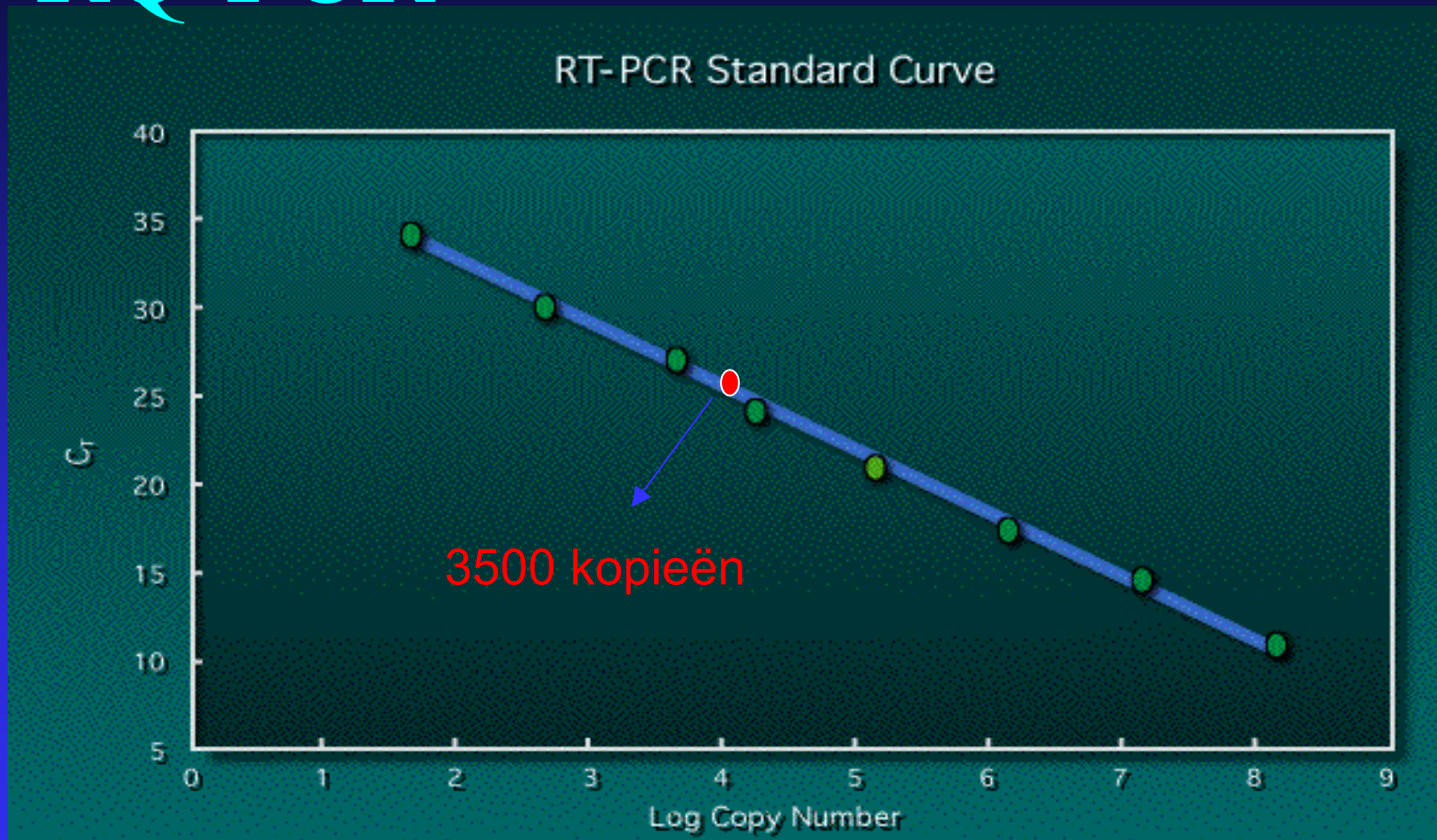


# RQ-PCR

RT-PCR Amplification Plot

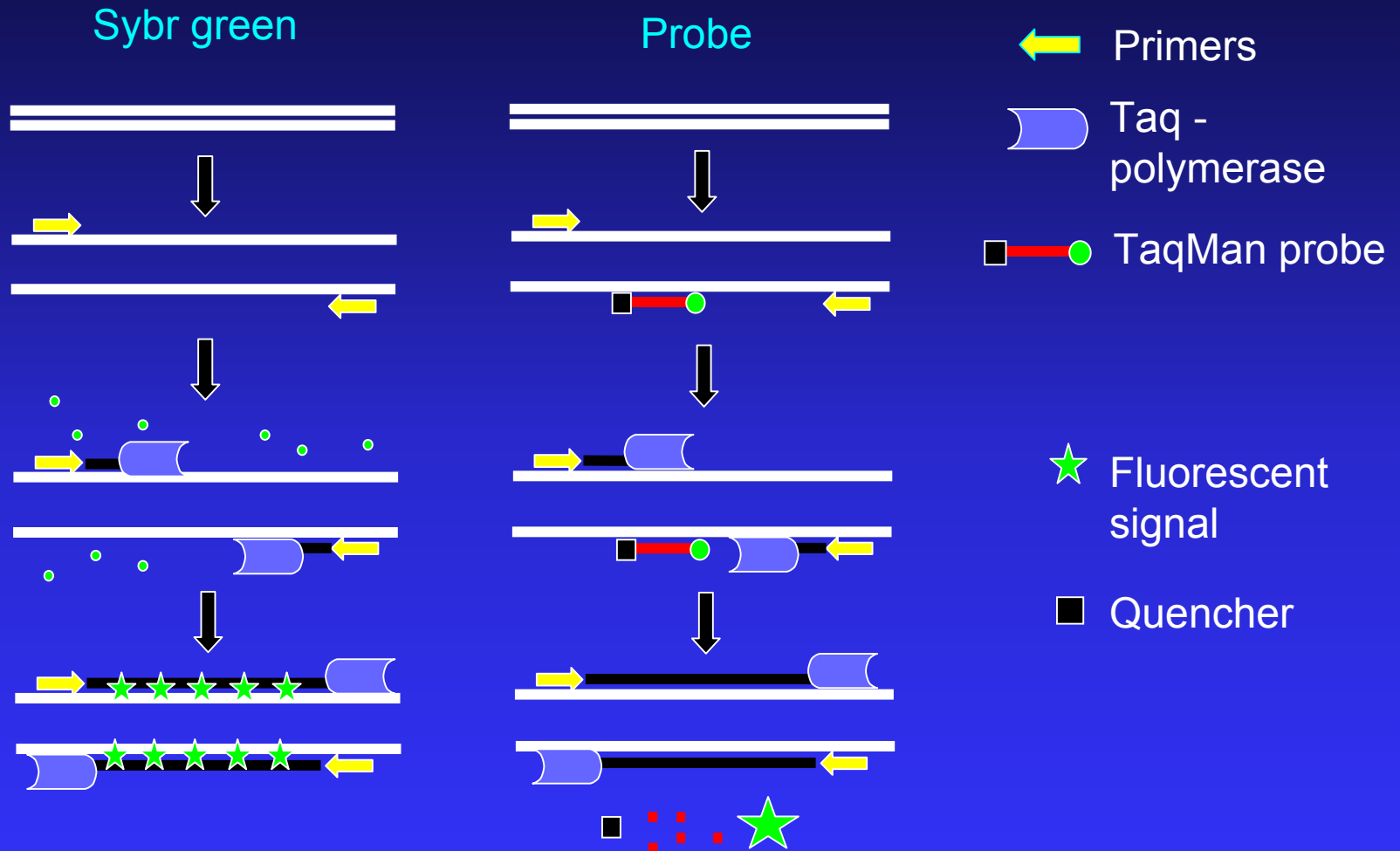


# RQ-PCR



Ideal: slope (s) = -3.32  
PCR efficiency = 100%

# Fluorescence



# QC for RQ-PCR Bcr-Abl

Send	K562 $10^{-2}$	K562 $10^{-5}$	K562 $10^{-6}$	TOM1 $10^{-3}$	TOM1 $10^{-5}$	TOM1 $10^{-3}$
Test <u>Bcr-Abl</u> % Abl	56.43	0.038	0.005	0.76	0.012	1.22

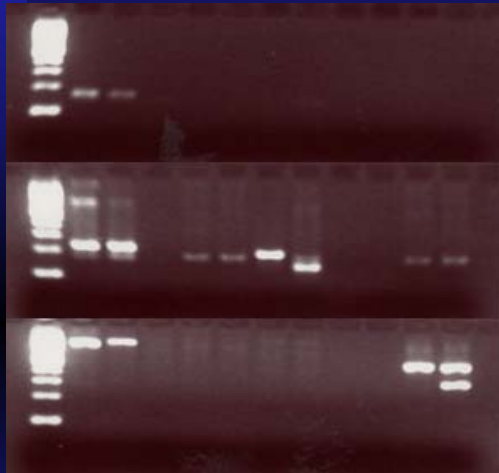
# t(14;18) and t(11;14)

- Used in classification of lymphoma
  - ◆ t(14;18) in 80-90% of FL, 20-30% of DLBCL
  - ◆ t(11;14) in 100% of MCL
- No fusion transcript but deregulation of adjacent genes (Bcl-2 and cyclinD1 resp.)
- Breakpoints are widely scattered.
  - ◆ t(14;18) PCR positive in 60-80%
  - ◆ t(11;14) PCR positive in 50-60%

# t(14;18) en t(11;14) PCR

- Nested PCR at diagnosis and MRD
  - ◆ Primers t(14;18): consensus JH + MBR or MCR
  - ◆ Primers t(11;14): consensus JH + MTC, or cyclinD1 overexpression (also in CLL and MM)
- Sensitivity:  $10^{-5}$ - $10^{-6}$

# t(11;14) en t(14;18) PCR



- t(11;14) ~ bcl-1

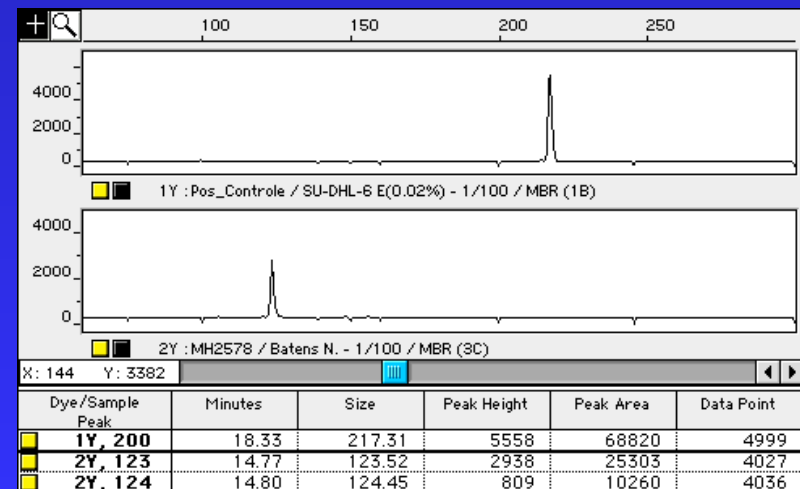
~50%

- t(14;18) MBR ~ bcl-2

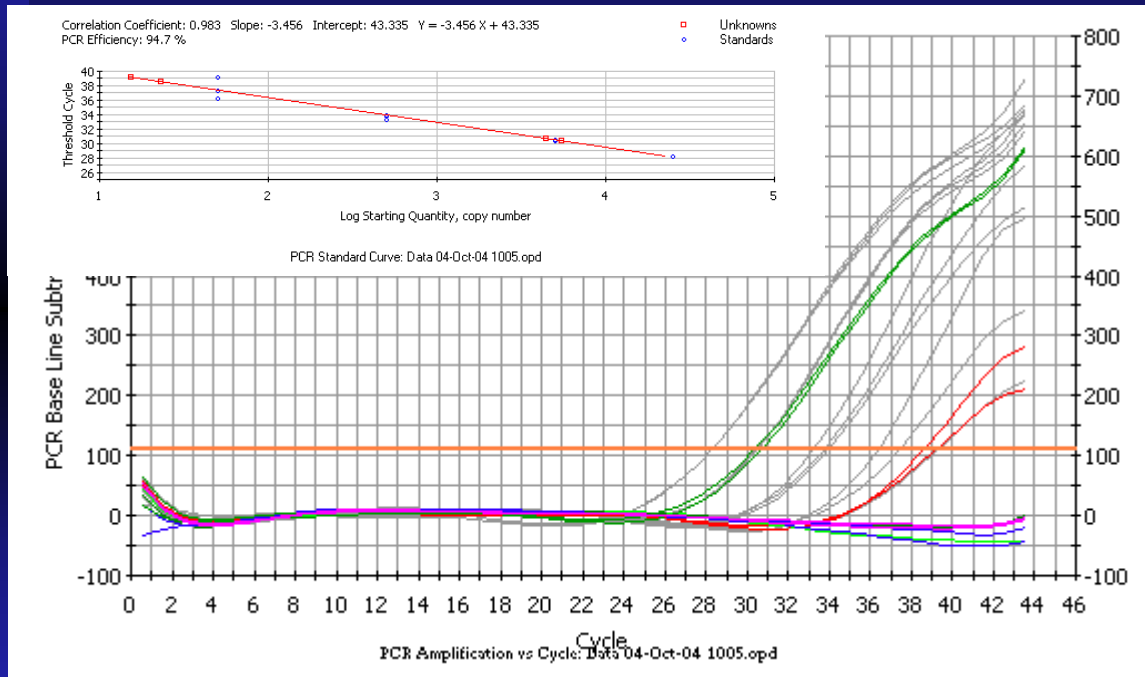
~75%

- t(14;18) MCR ~ bcl-2

t(14;18) in healthy persons  
complicates MRD test!  
Sizing is useful.



# RQ-PCR t(11;14) and t(14;18)



— 0.087%

MTC: 19.2 cop  
Alb: 22100 cop

— 28.68%

MTC: 4960cop  
Alb: 17300 cop

Standard: JVM-2 cell line DNA diluted in healthy control DNA  
150-300 ng DNA/well ~25,000-50,000 cells/well  
Sensitivity:  $10^{-3}$ - $10^{-4}$

# QC for $t(14;18)$

send	1.0%	0.04%	0.02%
	MBR cells	MBR cells	MBR cells
test	1.2%	0.08%	0.02%

# RQ-PCR for t(14;18) and t(11;14)

## Useful?

- ◆ Lack of correlation of t(14;18)+ cells and response to first line treatment (n=43) (Mandigers et al. Blood 2001)
- ◆ Correlation between t(14;18)+ cells in BM or blood and achieving CR (Lee et al. Int. J. Hematol. 2004 and Chang et al. BMT 2003, threshold 0.01%)

# ASO design

- Primer express and Oligo software
- 3' end in or a few bases past the CDR3 region
- $T_m$  +/- the same as reverse primer
- Avoid stretches of G's and C's
- No more than 2 G's or C's in last 5 nt
- No T ending
- Avoid primer-dimer formation and self-complementarity as much as possible