

Real-time PCR in Microbiology

Gaëtan Muyldermans



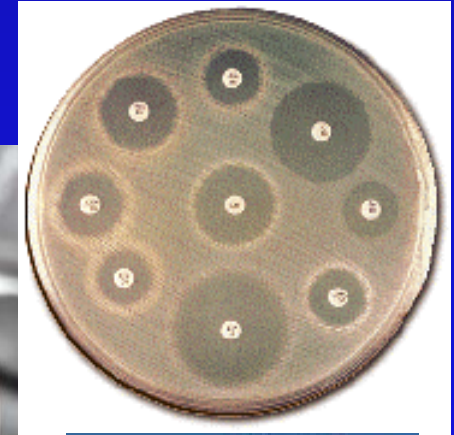
Traditional diagnostic microbiological assays

- **Microscopy**
- **Microbial culture**
- **Serology**



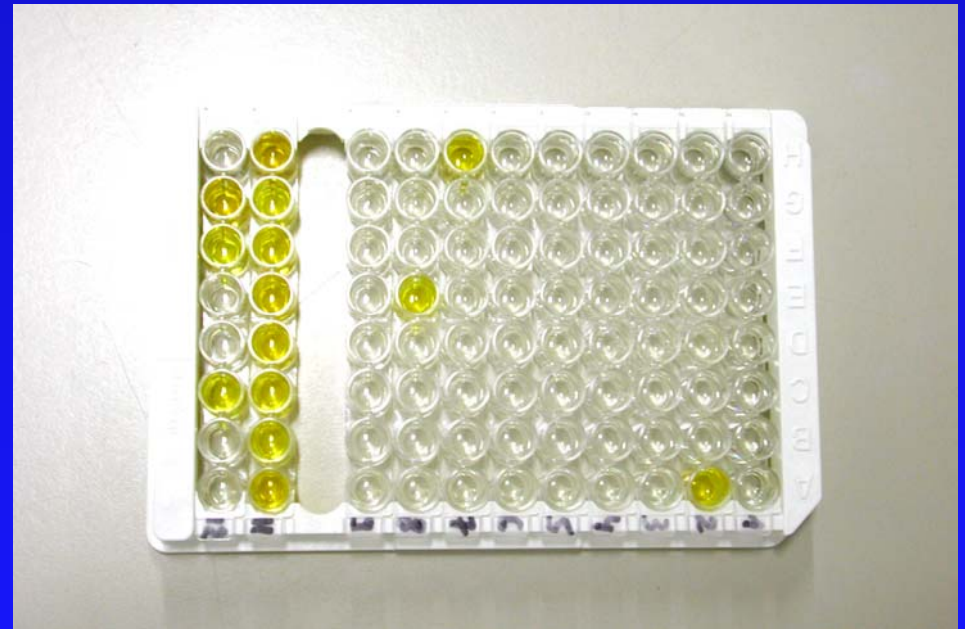
Traditional diagnostic microbiological assays

- Microscopy
- **Microbial culture**
- Serology



Traditional diagnostic microbiological assays

- Microscopy
- Microbial culture
- Serology



Traditional diagnostic microbiological assays

Still important because:

- **Epidemiological data**
- **Reveal new, uncharacterized or atypical microbes**
- **Recover intact and infectious organisms for further study**

Molecular diagnostic assays in microbiology laboratory

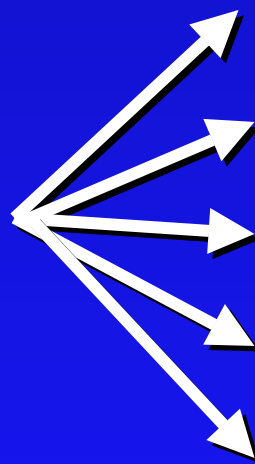


-
- **detection**
 - **identification**
 - **typing/epidemiology**
 - **resistance testing**
 - **quantitation**

Real-time PCR in microbiology laboratory



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- **detection**
- **identification**
- **typing/epidemiology**
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Applications of real-time PCR for detection/identification

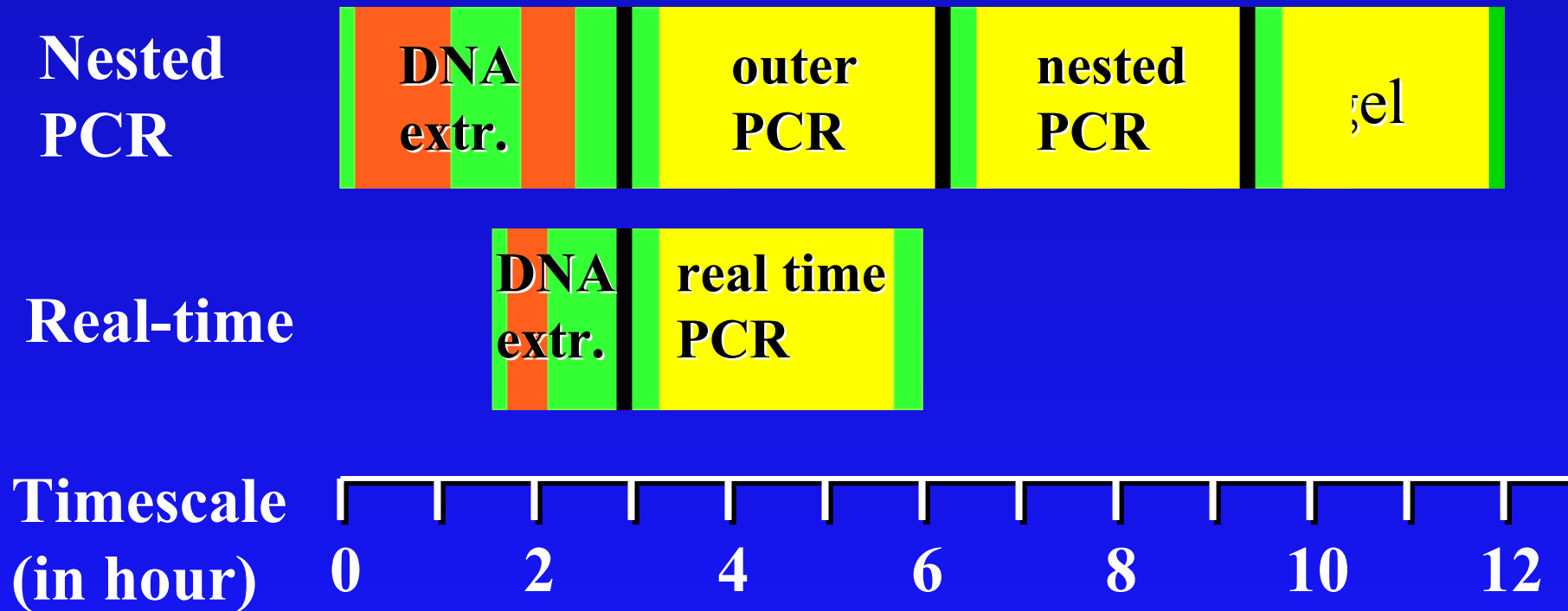
- **Viruses**
- **Bacteria**
- **Fungi, parasites and protozoa**

In house assay > < commercial based assay

Improvement of real-time PCR for detection/identification

- **Visualisation of progression of amplicon production**
- **Close system → less contamination**
- **Speed**

Comparison conventional PCR versus real-time PCR



Improvement of real-time PCR for detection/identification: speed

Rapid real-time PCR assay

- Limit potential for toxicity**
- Reduce duration of hospital stay**
- Prevent improper use of antibiotics**
- Minimising the potential for resistant strains to emerge**

**Combination of
automated
nucleic acid
extraction
+
real-time PCR**



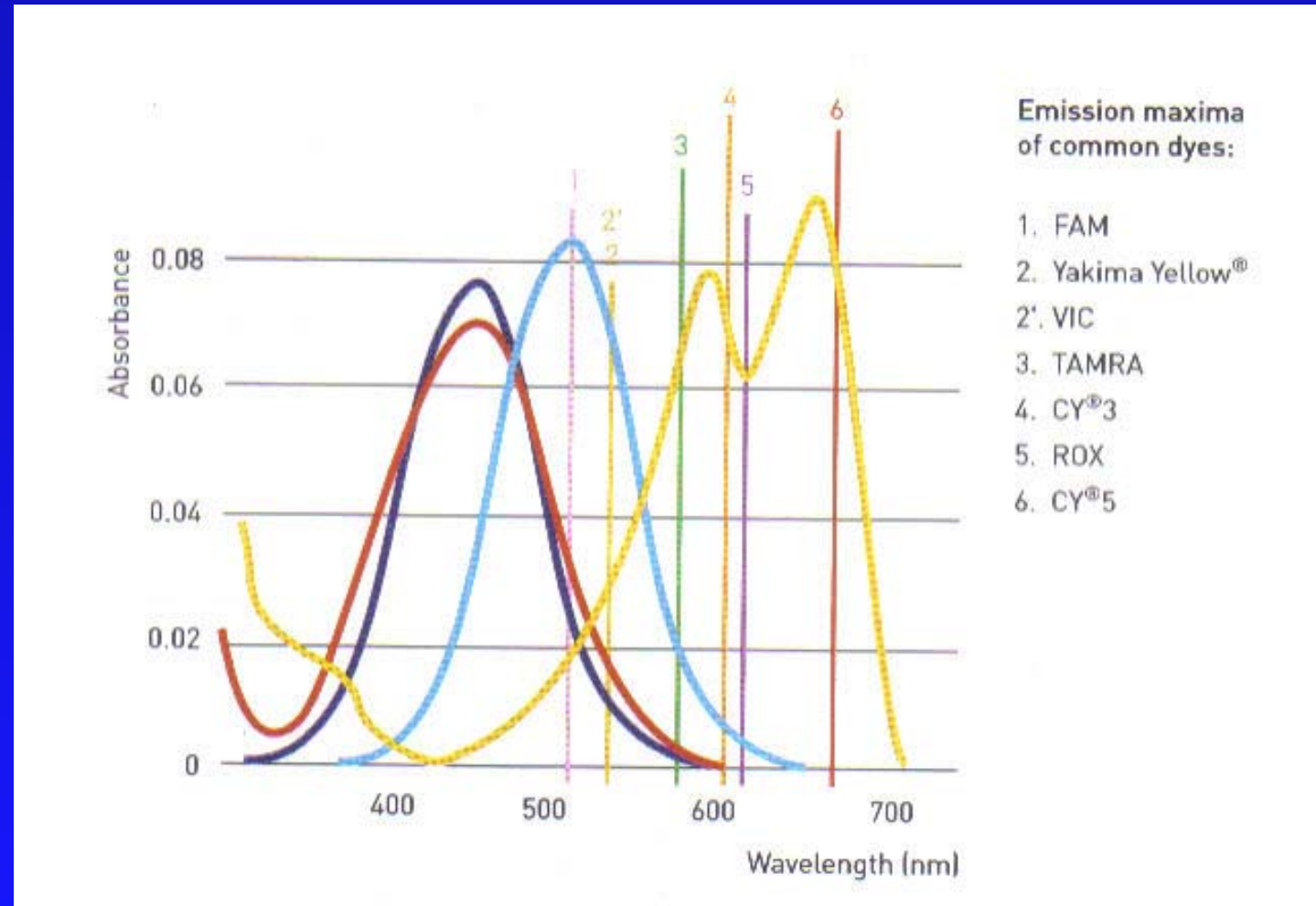
Disadvantage real-time PCR for detection/identification

- **Incompatibility of certain platforms with some fluorescent chemistries**
- **Start-up expense**
- **Relatively restricted multiplex capabilities**

Multiplex PCR

Development is problematic because of limited number of fluorophores

Cross-talk



Optimization of multiplex real-time PCR



Multiplex PCR

- **Only a few multiplex PCR described**
- **Need for further development of novel chemistries**
- **Need for improved real time instrumentation and software**

Real-time PCR in microbiology laboratory



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- detection
- identification
- **typing/epidemiology**
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- quantitation

Microbial genotyping

**Nucleotide sequencing is still “Golden Standard”
for characterizing unknown nucleic acids**

It is still a lengthy process

Microbial genotyping : real-time PCR methodology

- **SybrGreen**
- **Taqman probes**
- **Molecular beacons**

Typing of Belgian *Bordetella pertussis* isolates

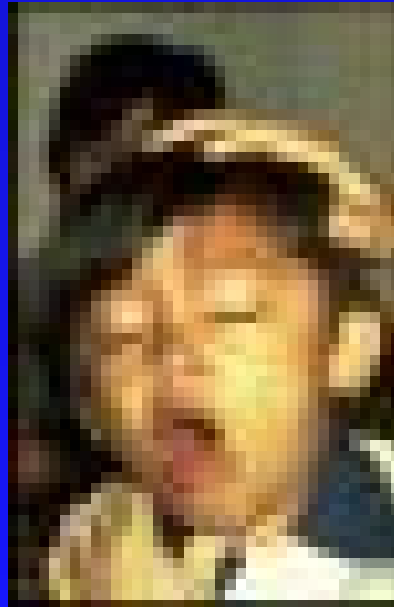
Aim:

- Antigenic drift after the introduction of the acellular vaccin?
- Evolution of the MLST in Belgium

Vaccination

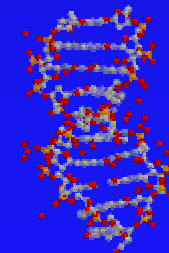
Whole cell vaccin

- Inactivated *B.pertussis* organism
- Side-effects
- From 1950



Acellular vaccin

- few *B.pertussis* antigens
- Less side-effects
- Possibility to booster
- From 2001 in Belgium

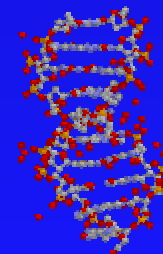


ptxS1

- ptxS1D CTCGAACAT-//-TATTCCAAC-//-ATGGCGCCGGTGGTG
- ptxS1B CTCGACCAT-//-TATTCCAAC-//-ATGGCGCCGGTGATA
- ptxS1A CTCGACCAT-//-TATTCCAAC-//-ATAGCGCCGGTGATA
- ptxS1E CTCGAACAT-//-TATCCCAAC-//-ATGGCGCCGGTGATG

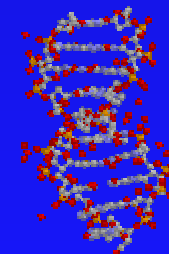
ptxS3

- ptxS3AATATGCTGA
- ptxS3BATATGTTGA



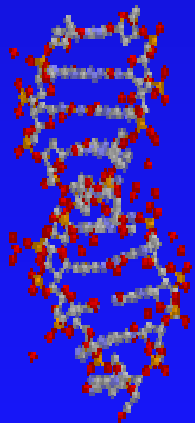
tcfA

- tcfA1 GAGCTG-//-GGGGGGGGGCACGCATGAA-//-CCG-[NNN]25-GAT-//-GCATCGGGT
- tcfA2 AAGCTG-//-GGGGGGGGGCACGCATGAA-//-CCG-[XXX]25-GAT-//-GCATCGGGT
- tcfA3 AAGCTG-//-GGGGGGGGGCACGCATGAA-//-CCG-[XXX]25-GAT-//-GCATCGGAT
- tcfA4 AAGCTG-//-GGGGGGGGGCACGCATGAA-//-CCG-[XXX]25-GAT-//-GTATCGGGT
- tcfA5 AAGCTG-//-GGGGGGGGGCACGCATGA



Multi Locus Sequence Typing (MLST)

- MLST :
combination of
ptxS1, ptxS3 and
tcfA type



MLST	Allele
MLST-1	ptxS1D,ptxS3A,tcfA2
MLST-2	ptxS1B,ptxS3A,tcfA2
MLST-3	ptxS1A,ptxS3A,tcfA2
MLST-4	ptxS1A,ptxS3A,tcfA3
MLST-5	ptxS1A,ptxS3B,tcfA2
MLST-7	ptxS1A,ptxS3A,tcfA5
MLST-8	ptxS1B,ptxS3B,tcfA2
MLST-9	ptxS1E,ptxS3A,tcfA1
MLST-10	ptxS1A,ptxS3A,tcfA4

684

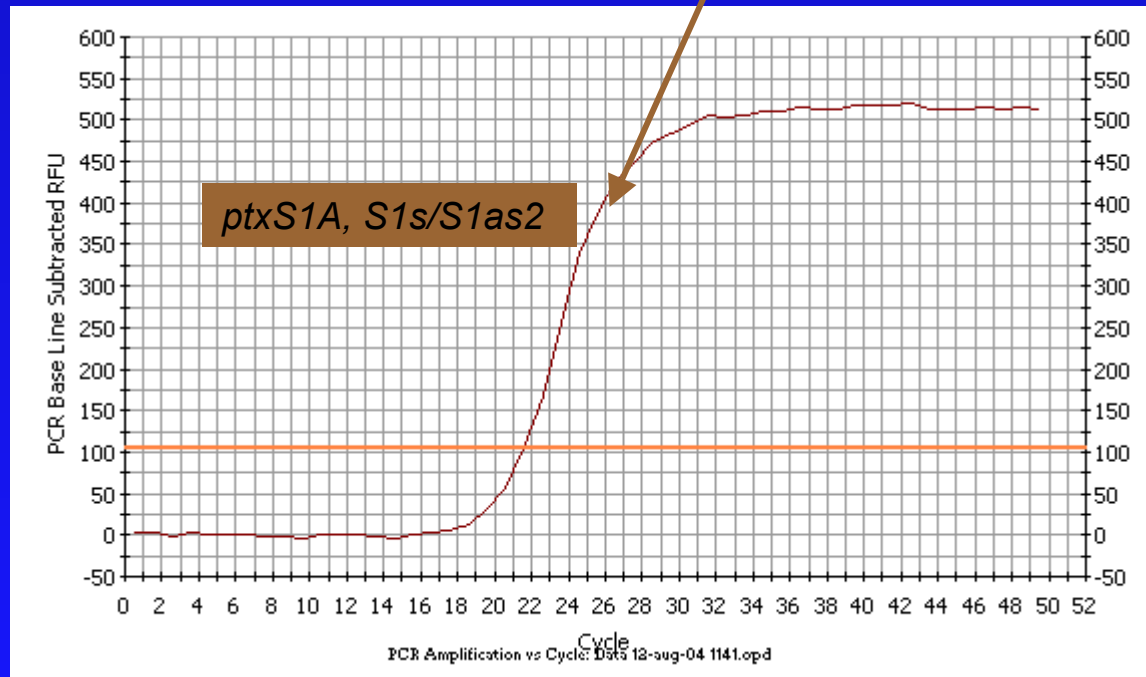
ptxS1B/D/EATGGCGCCGGT.....

ptxS1AATAGCGCCGGT.....

→
ptxS1s

T

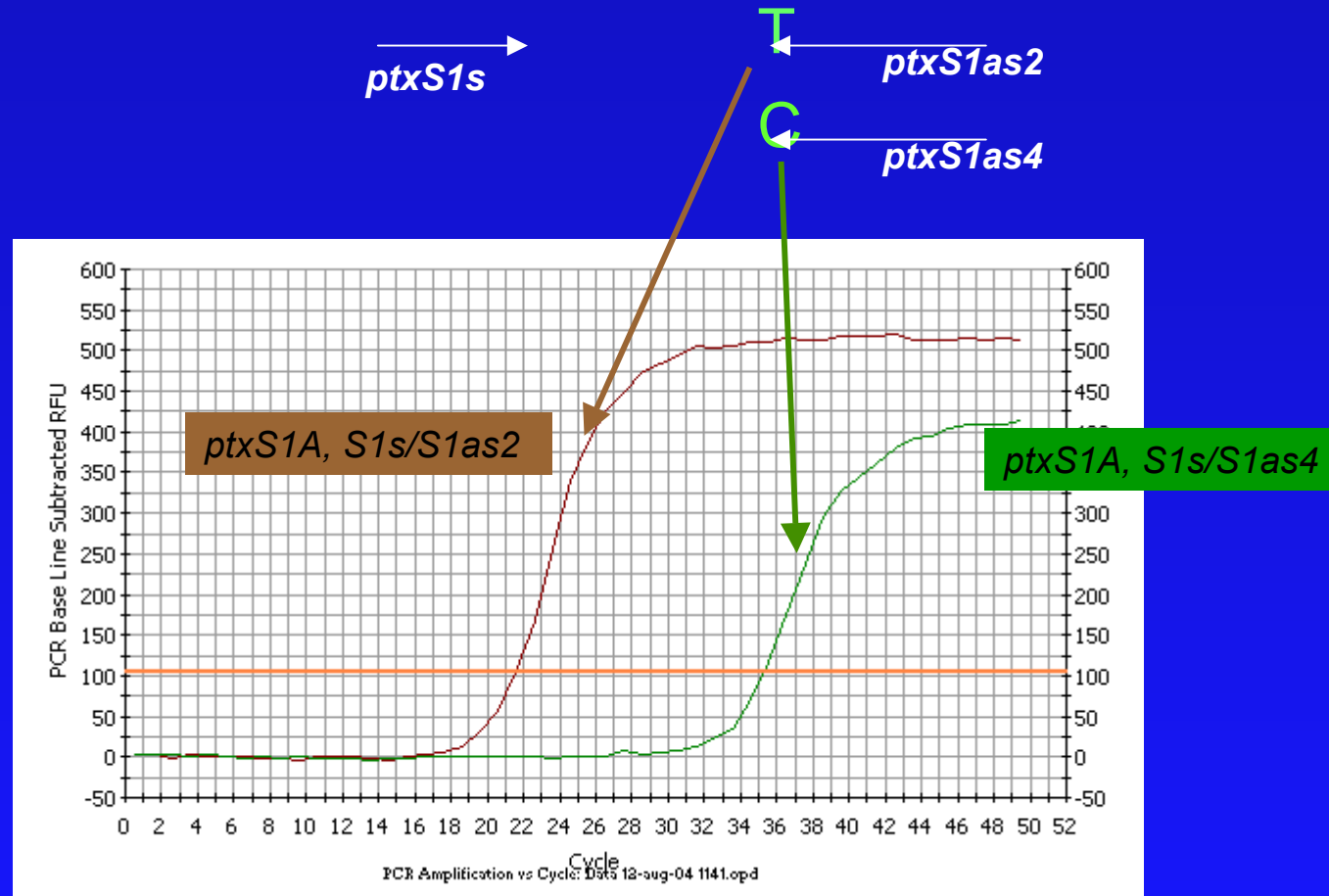
←
ptxS1as2



684

ptxS1B/D/EATGGCGCCGGT.....

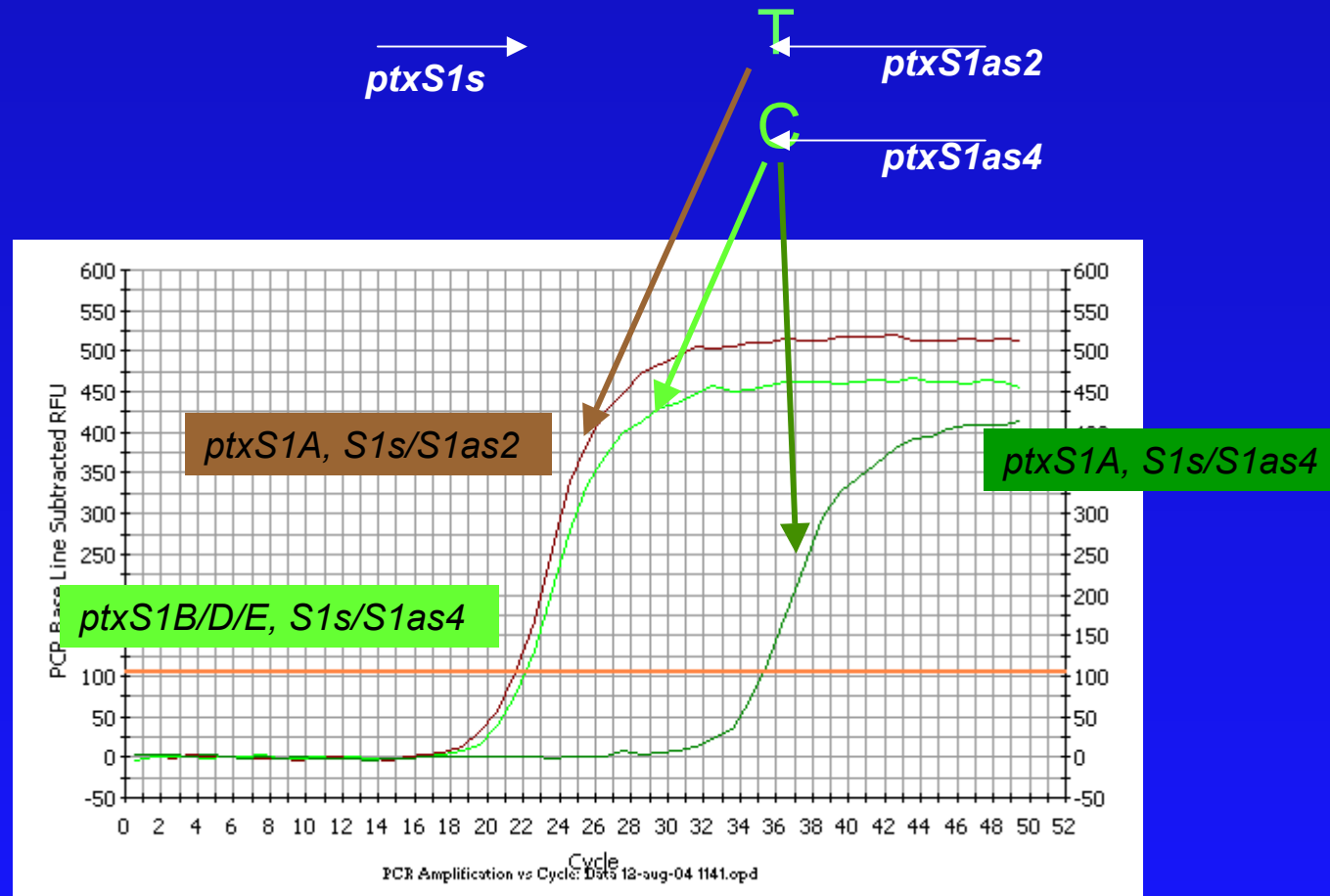
ptxS1AATAGCGCCGGT.....



684

ptxS1B/D/EATGGCGCCGGT.....

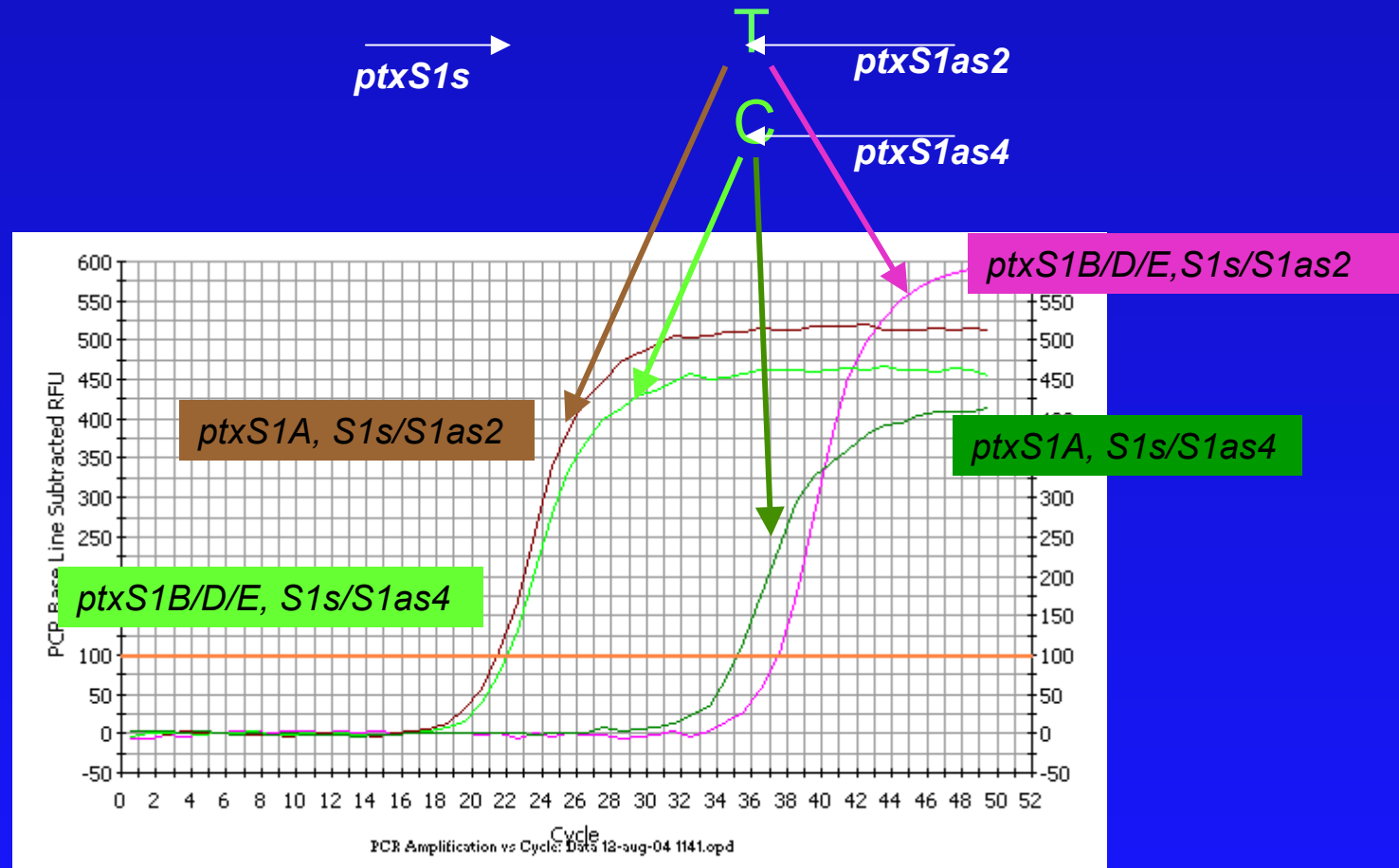
ptxS1AATAGCGCCGGT.....




684

ptxS1B/D/EATGGCGCCGGT.....

ptxS1AATAGCGCCGGT.....

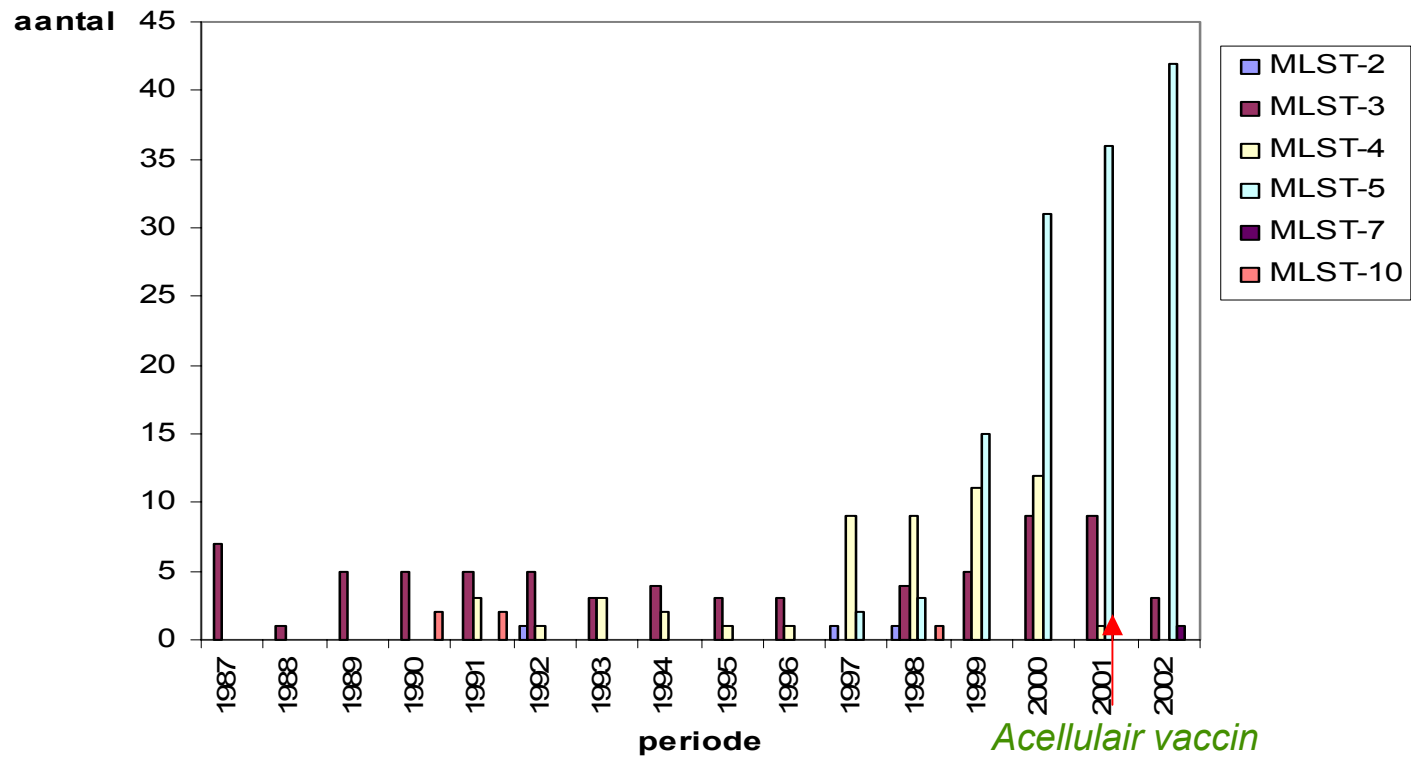


Benefit

- “Golden standard”:
PCR → sequencing → data analysis

16 €/sample
- Real-time PCR

MLST

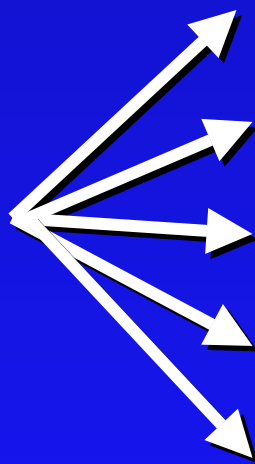
Verdeling van MLST typen in België gedurende 1987-2002



Real-time PCR in microbiology laboratory



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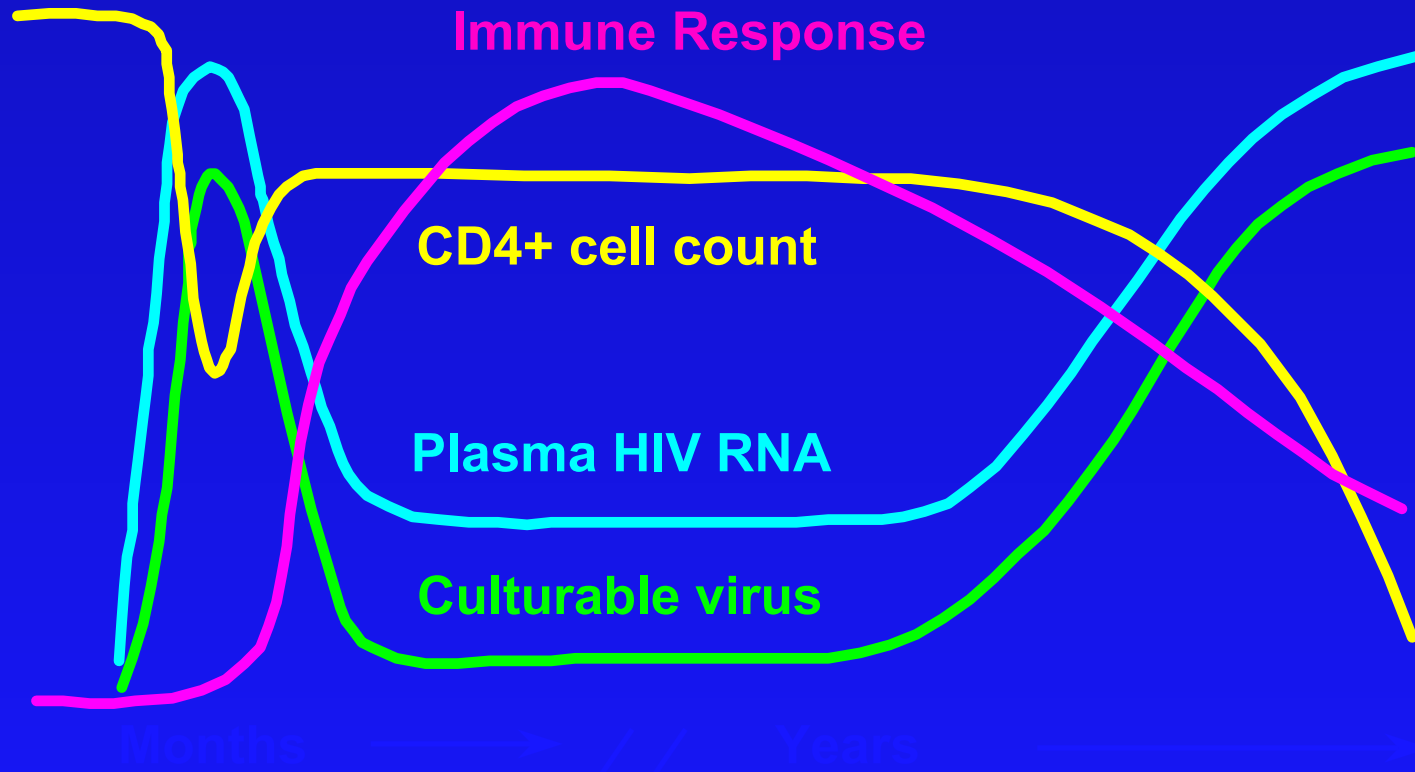


- detection
- identification
- typing/epidemiology
- resistance testing
- **quantitation**

Applications for microbiology: viral load

- **To study the interactions between virus and host**
- **For studying the role of viral reactivation**
- **It provides a marker for disease progression**
- **To monitor the change in VL as a result of antiviral therapy**

Virologic and Immunologic course of HIV



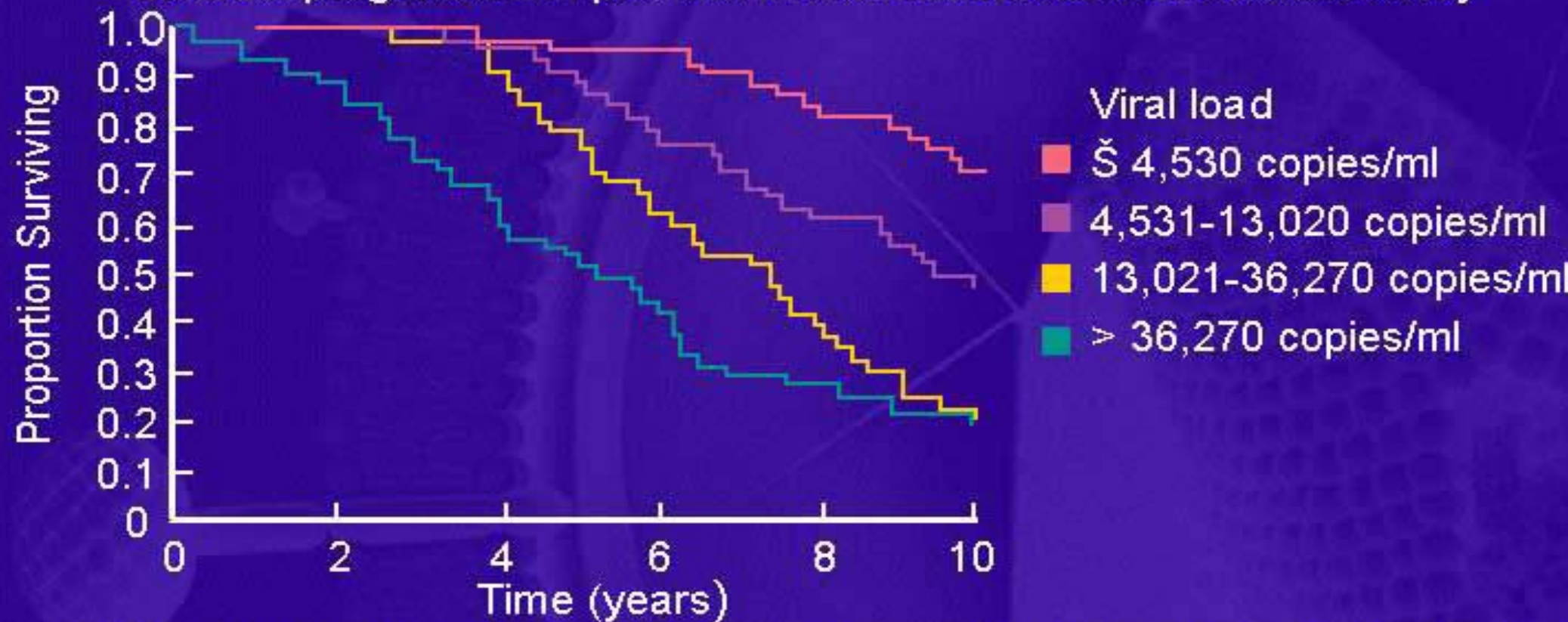
Applications for microbiology: viral load

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Diagnosis and Monitoring of HIV

Correlation of Viral Load and Disease Progression

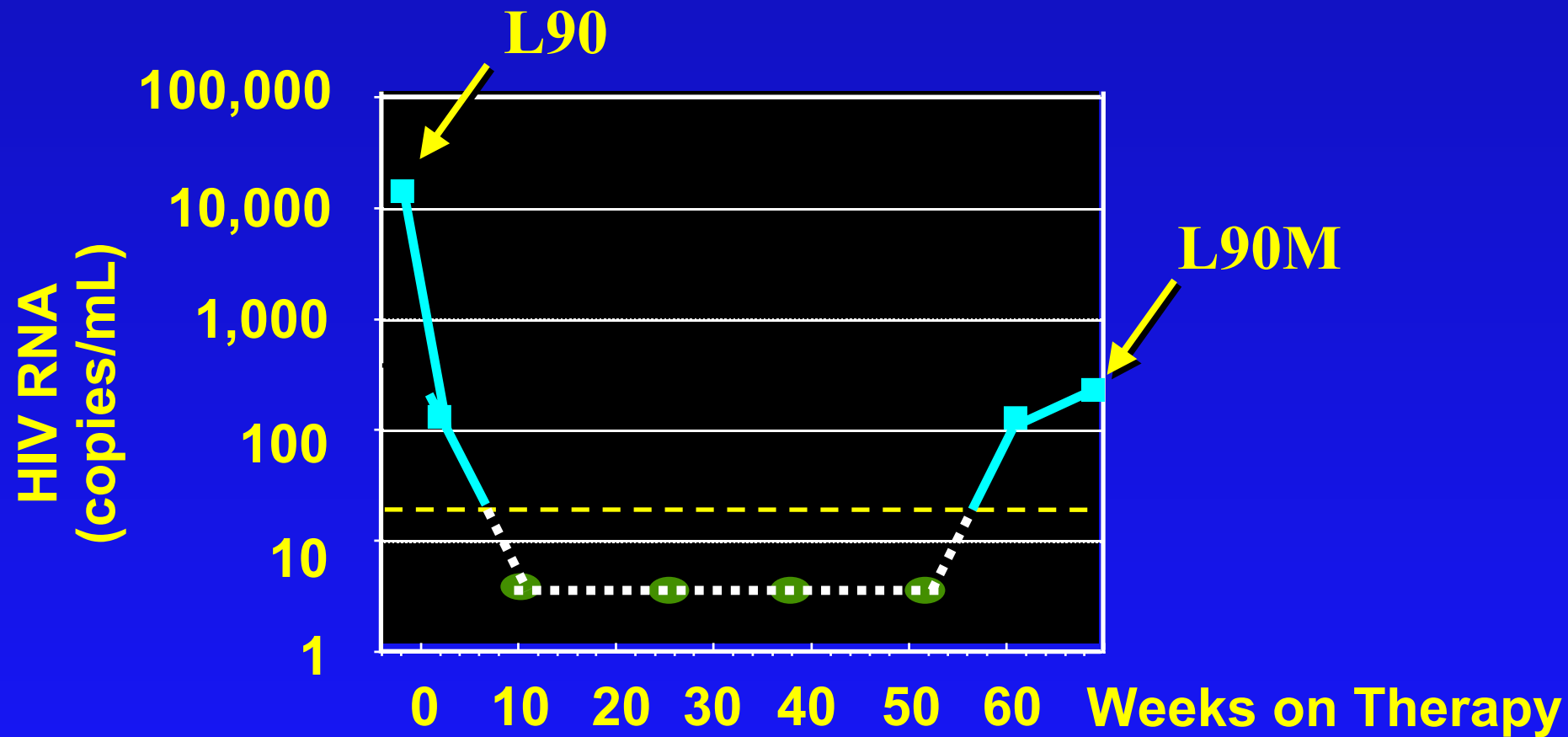
- Disease progression in patients in the Multicenter AIDS Cohort Study¹



1. Mellors J W, 3rd Conference on Retroviruses and Opportunistic Infections, January 1996. Abstract number 52

Applications for microbiology: viral load

- **For studying the role of viral reactivation**
- **To study the interactions between virus and host**
- **It provides a marker for disease progression**
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Saquinavir

Quantitation

examples:

- **HIV**
- **HCV**
- **HBV**
- **CMV**
- **etc**

Quantitation methods

- **Amplicor (Roche Diagnostics)**
- **NASBA (Biomerieux)**
- **branched-DNA (Bayer)**
- **Real-Time PCR (Abbott)**
- **Real-Time PCR (In house)**

Quantitation methods

- **Amplicor (Roche Diagnostics)**
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COBAS AMPLICOR™ System

Routine Diagnostic PCR

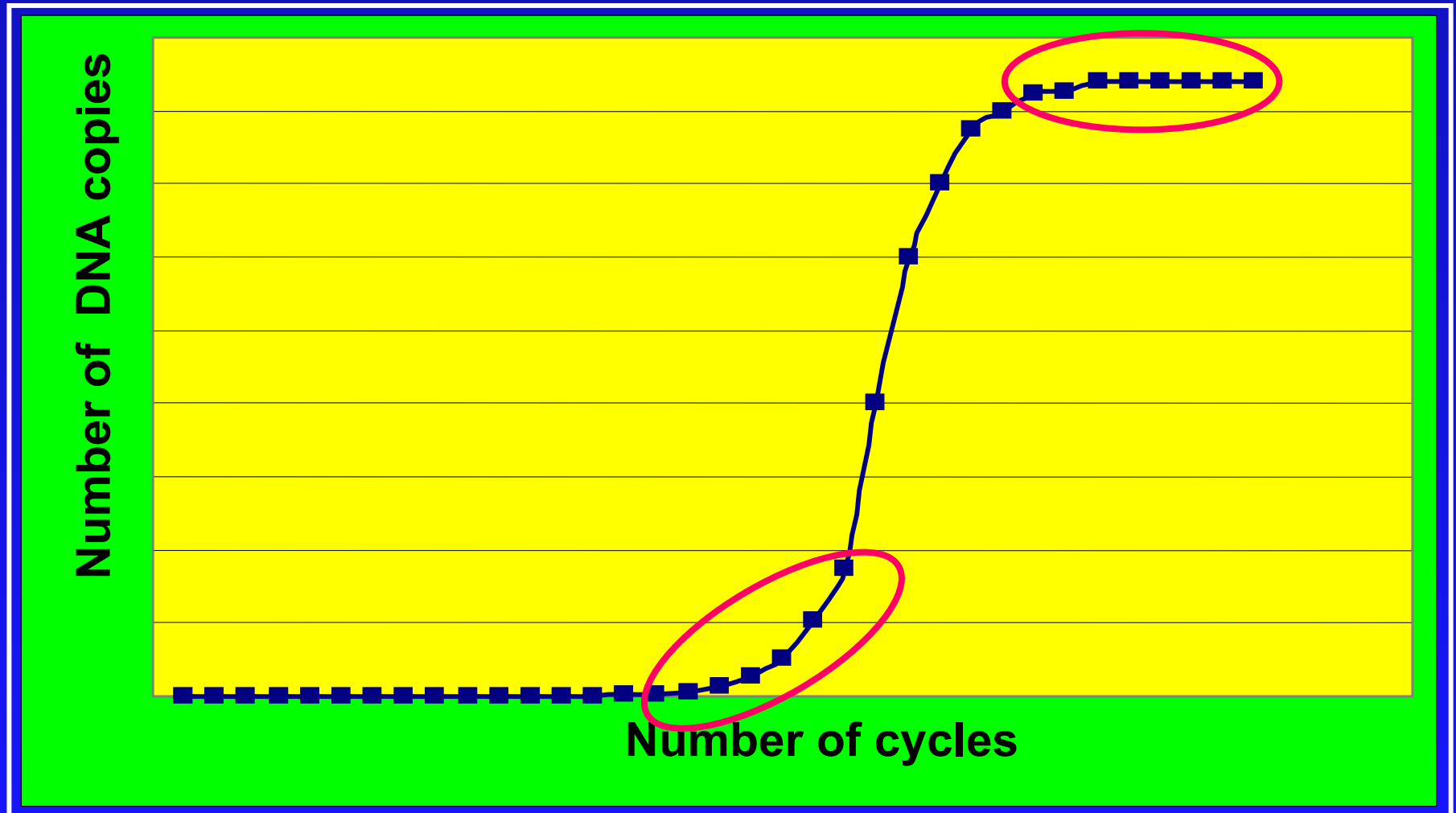


COBAS AMPLICOR™ System

Combining 5 different instruments into one automated system



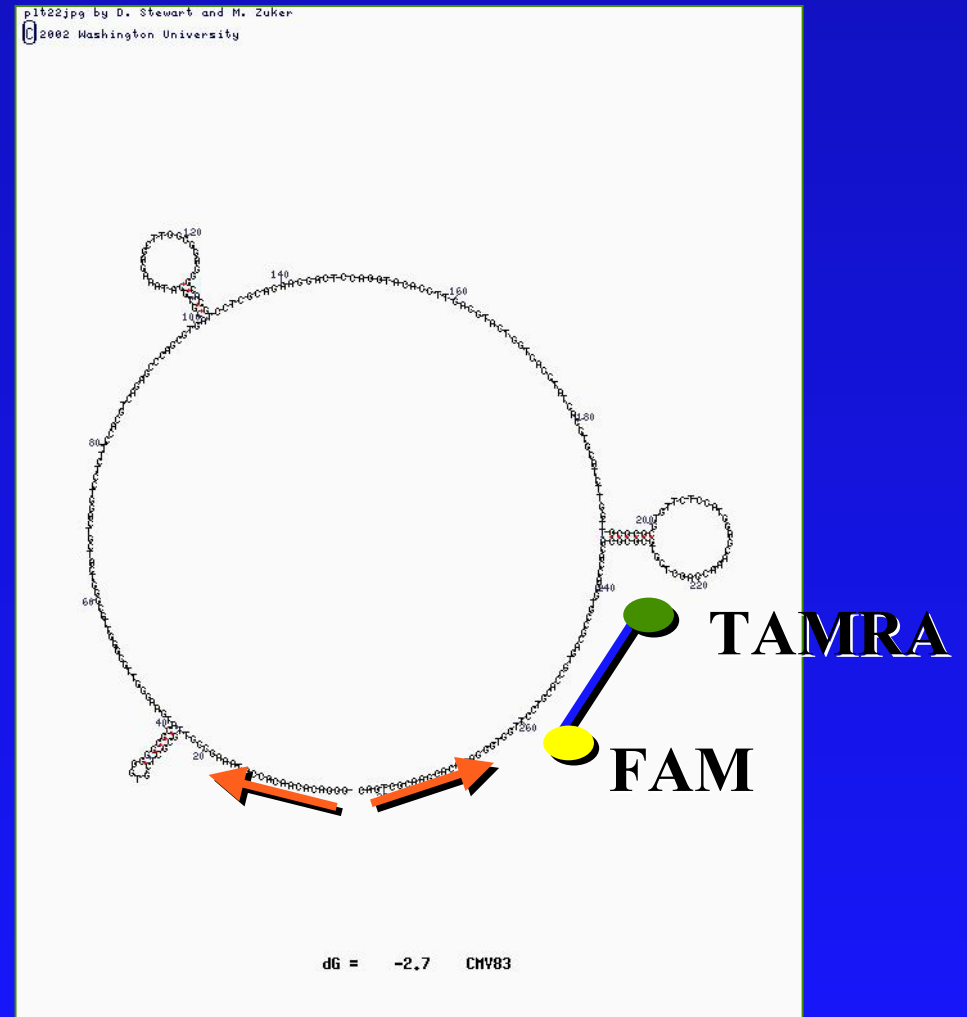
“Real-time” PCR versus “conventional” PCR



Design primers and probes

CMV

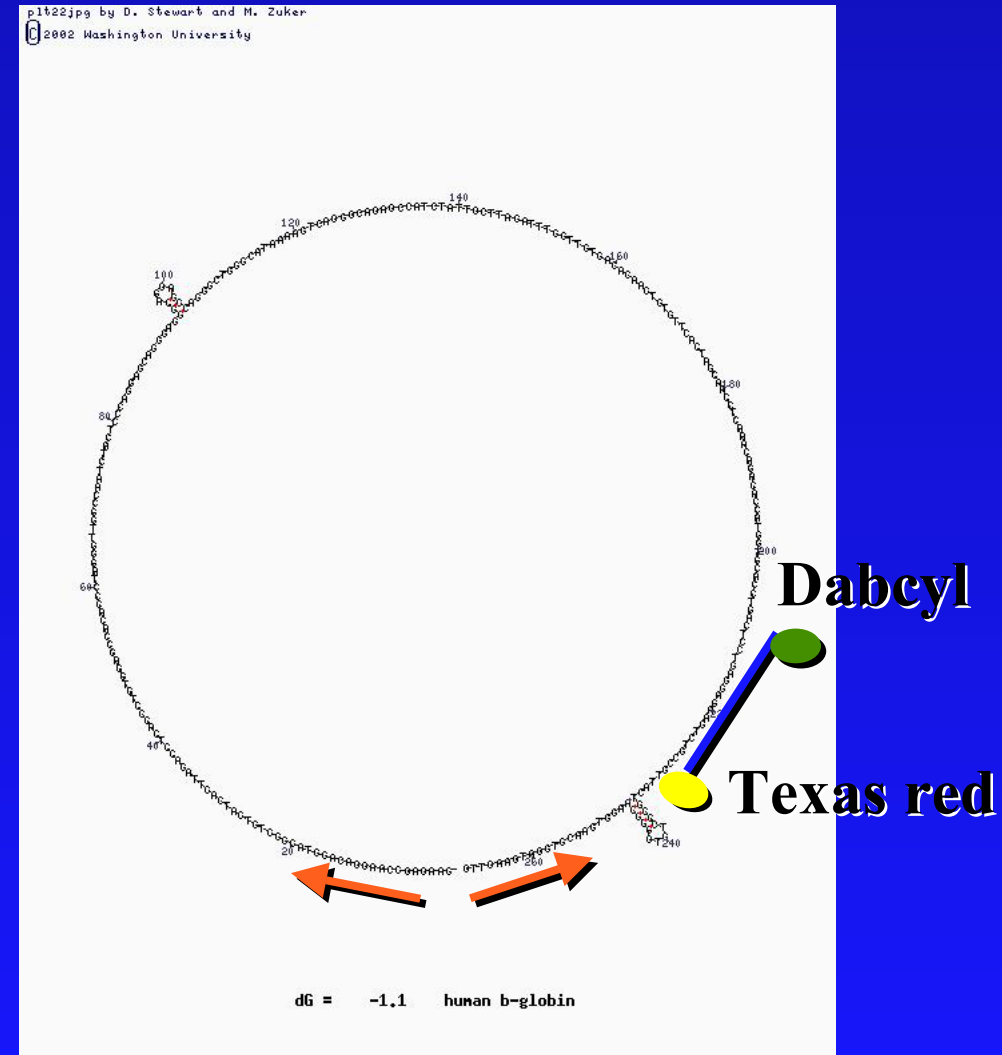
<http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi>



Design primers and probes

Human β -globin

<http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi>



Quantitation of CMV

Isolation of PBMC

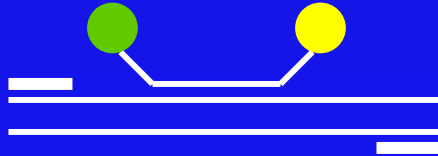


DNA extraction



amplification and detection

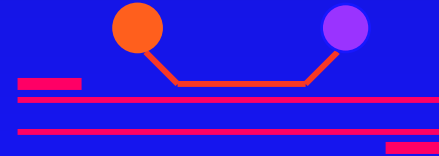
FAM TAMRA



pp56

CMV DNA

TEXASRED DABSYL



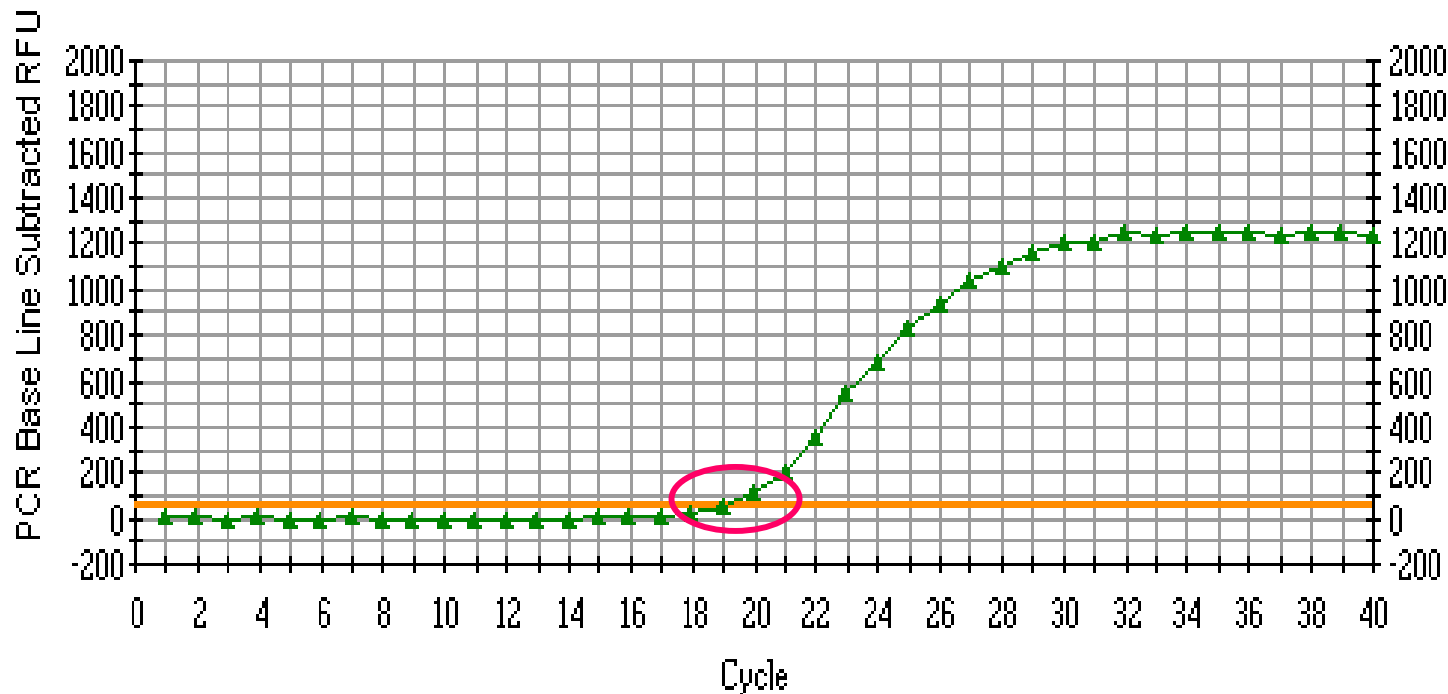
β globine

Human DNA

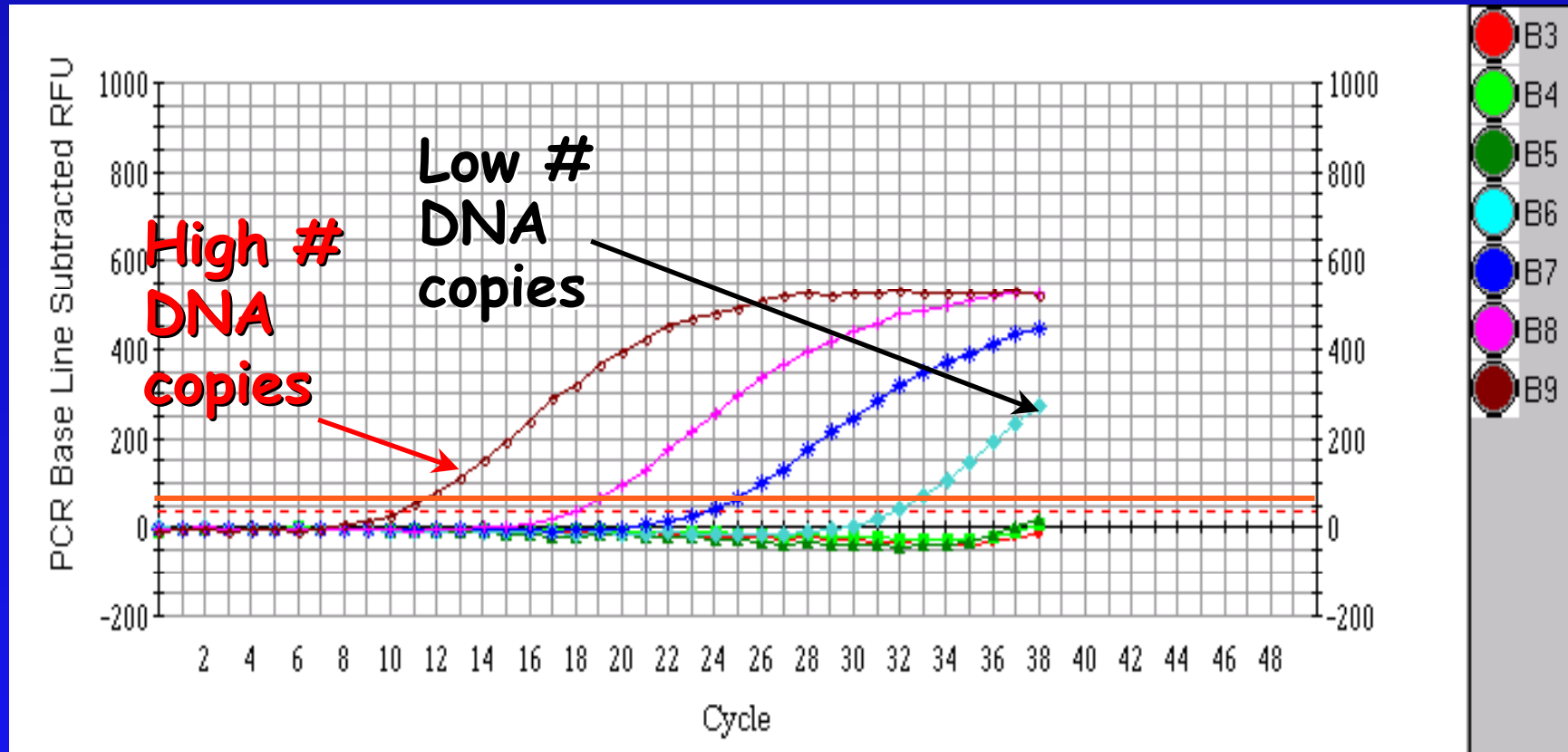
Real-time PCR

“Threshold cycle” or Ct :

The cycle in which the gain in fluorescence generated by the accumulating amplicon exceeds a baseline level.



Real-time PCR

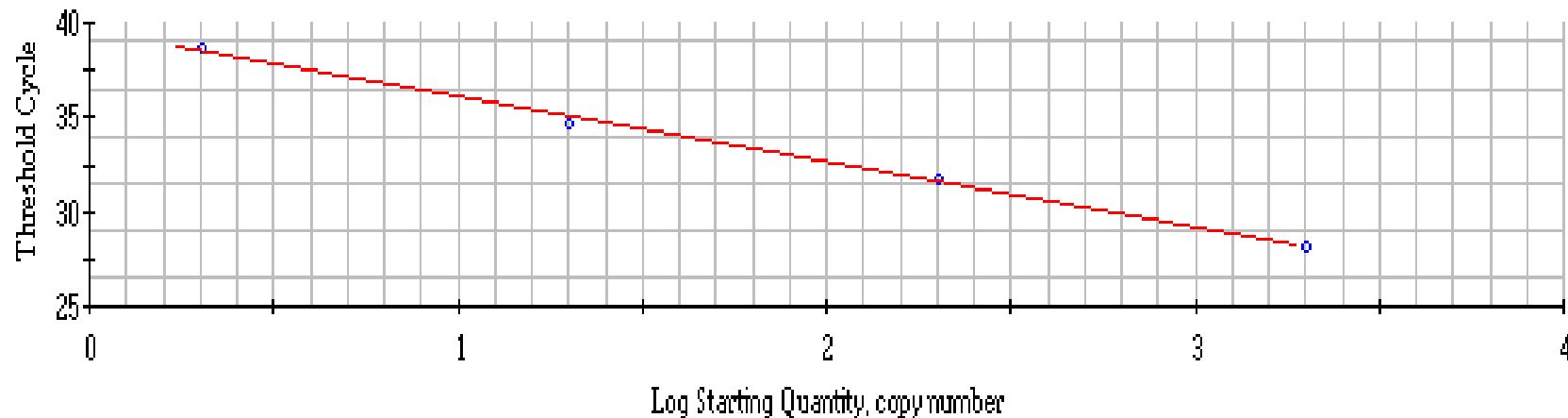


A linear correlation between log initial concentration and C_t

CMV detection

Correlation Coefficient: 0.998 Slope: -3.471 Intercept: 39.648 $Y = -3.471 X + 39.648$

□ Unknowns
○ Standards

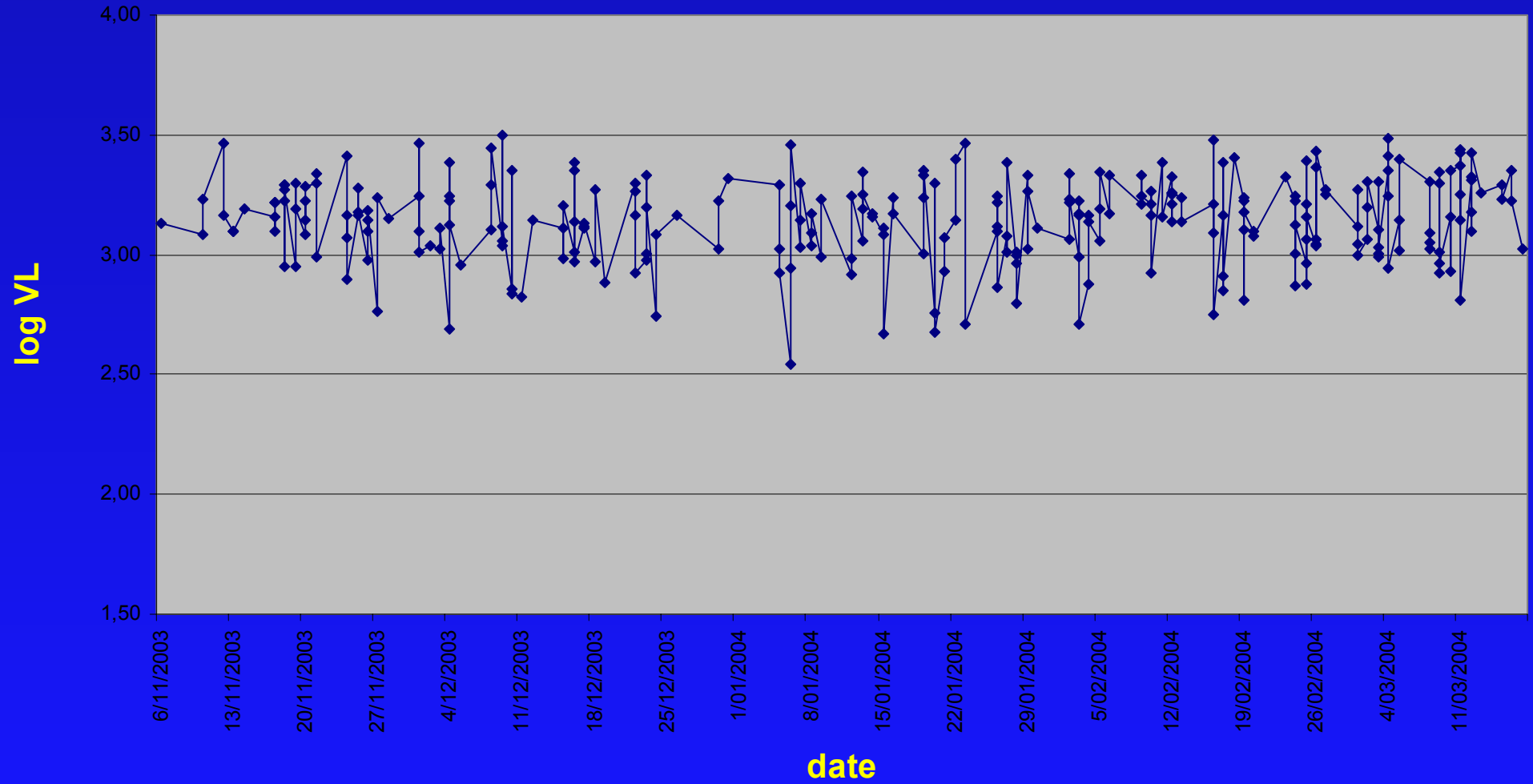


PCR Standard Curve: Data 06-Feb-02 1402.opd

Use of standards and controls

- **Inhibition control**
- **Negative control**
- **Positive control**
- **IRC**
- **Standard dilution**

results of the IRC-VL2



Use of standards and controls

- **Inhibition control**
- **Negative control**
- **Positive control**
- **IRC**
- **Standard dilution**

Standards

- **Cloned amplicon**
- **The target organism**
- **Purified amplicon**

Use of a non-participating or passive internal reference fluorophore (ROX)- to correct for intervessel variability

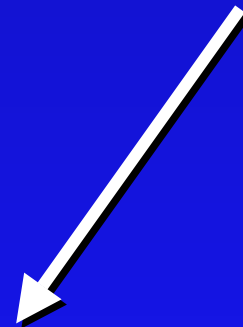
Quantitation: calculation

- **Absolute > < relative amount**
- **Expressed relative to a biological marker (/vol plasma or /#cells)**
- **Allowing comparison between assay results and testing sites**
- **Introduction of international units**

Calculation of CMV viral load

#CMV copies/PCR

#glob copies/PCR



#CMV copies/10⁶ glob copies

Quantitation: calculation

- **Absolute \gg relative amount**
- **Expressed relative to a biological marker (/vol plasma or /#cells)**
- **Allowing comparison between assay results and testing sites**
- **Introduction of international units**

Improved quantitation using real-time PCR

- **Dynamic range ($8 \log_{10}$ copies)**
- **Low inter-assay and intra-assay variability**
- **Equivalent or improved sensitivity compared to microbial culture, or conventional PCR**

Real-time PCR in microbiology: Conclusion

- **Popularity is still expanding**
- **Commercial based application were developed for diagnosis**
- **Need for uniformization**
- **Robotic nucleic acid extraction and real time PCR is attractive for routine diagnostic laboratory**
- **Development in multiplex real time PCR will improve the easy identification, genotyping and quantitation of microbial targets**
- **The technology is only reliable if associated Qa programmes are established.**