



Finding the needle in the haystack

LNA bases enhance SNP detection dramatically

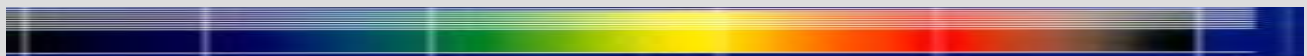
Olfert Landt

Weihenstephan, September 2005

Real-Time PCR is based on Fluorescence

Fluorescence is less sensitive when compared to radioactivity or enzyme-linked reactions

- λ wavelength (nm)
- ϵ extinction coefficient
- q quantum yield
- Absorption Maximum
- Emission Maximum
- Stoke's Shift
- Quench
- FRET (Fluorescence Resonance Energy Transfer)
- Photobleaching
- Background fluorescence
- Lifetime



Real-Time-PCR Instruments



LightCycler480
Roche Diagnostics

LightTyper
Roche Diagnostics



SmartCycler
Cepheid

SDS7700
Applied Biosystems



Opticon
MJ Research

Cycler
BioRad



MiniOpticon
MJ Research-BioRad



SDS7000
Applied Biosystems



LightCycler
Roche Diagnostics



7900HT
Applied Biosystems



RotorGene
Corbett Research



Mx3000/4000
Stratagene



Superconvector
AlphaHelix

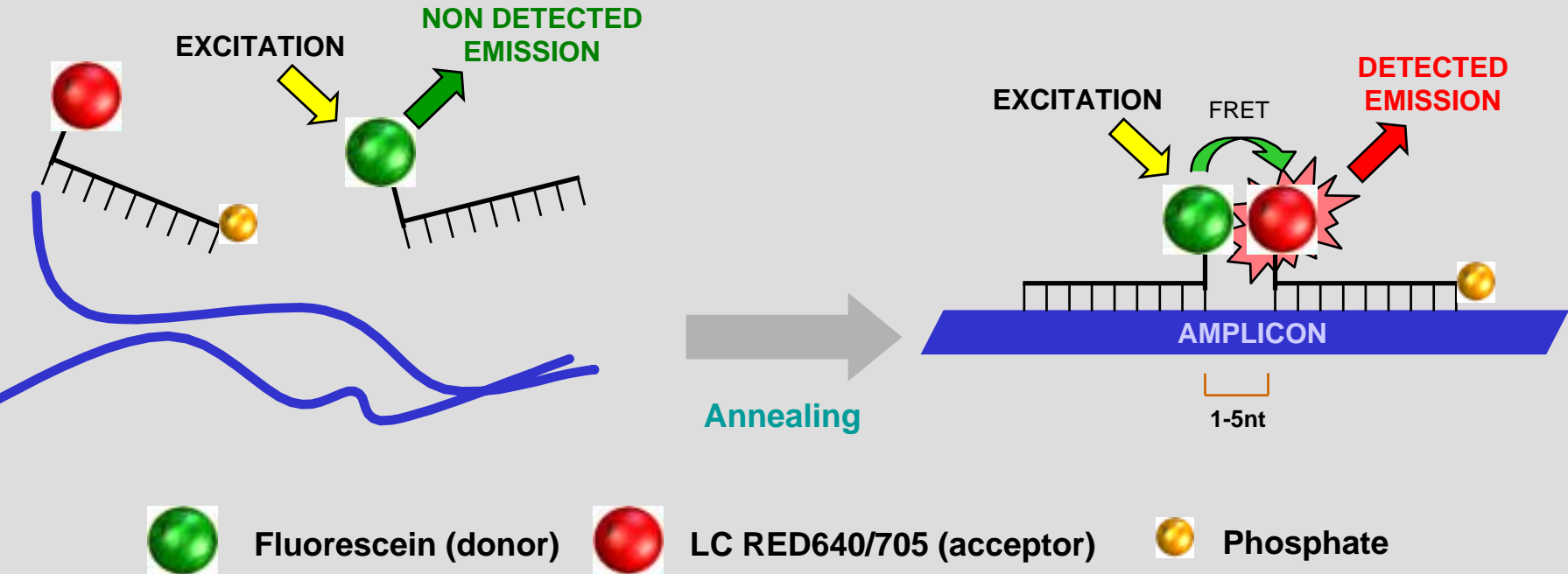


As a producer of fluorescent probes we are interested in new PCR detection technologies



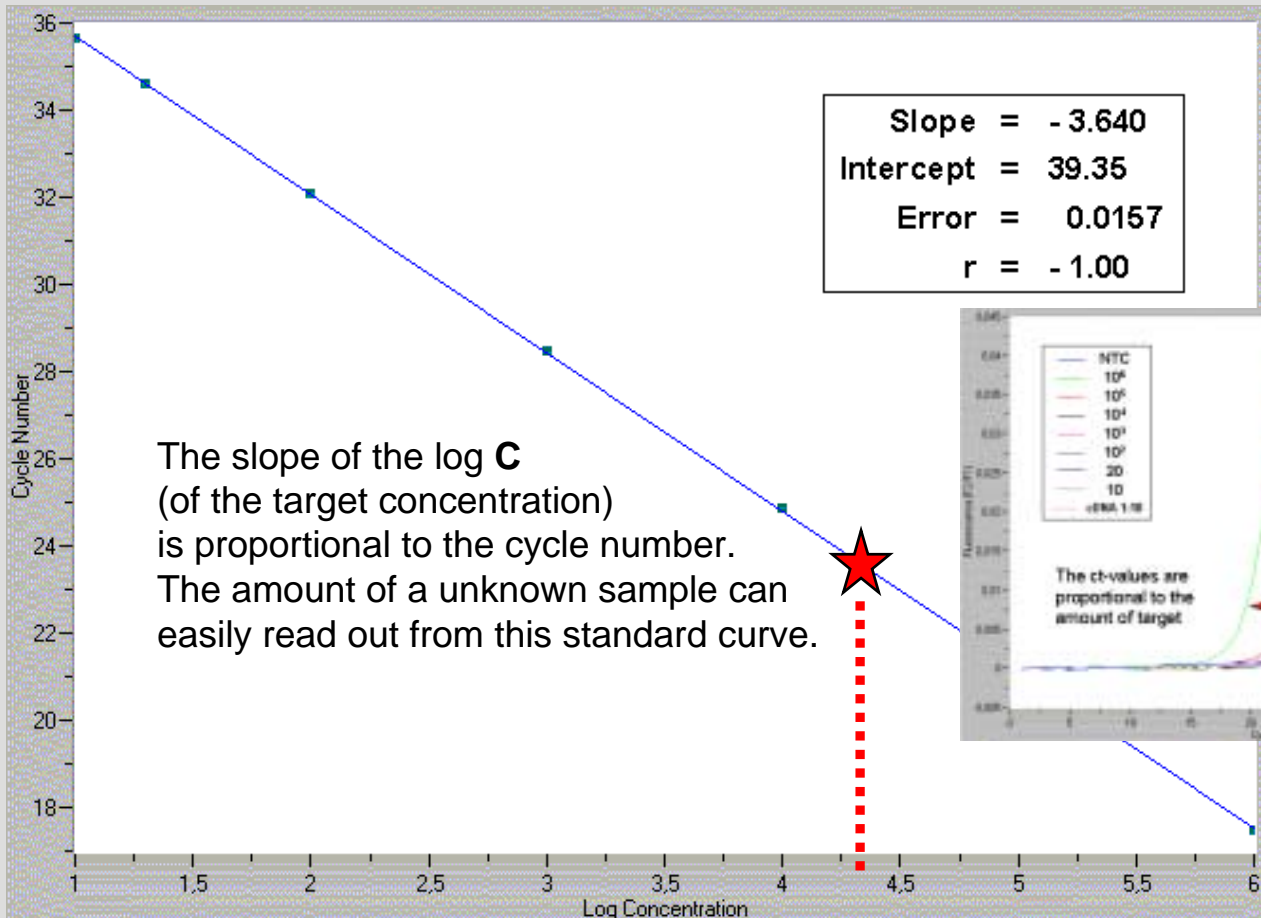
Molecular Concepts: Hybridization Probes

Principle: adjacent hybridisation and FRET

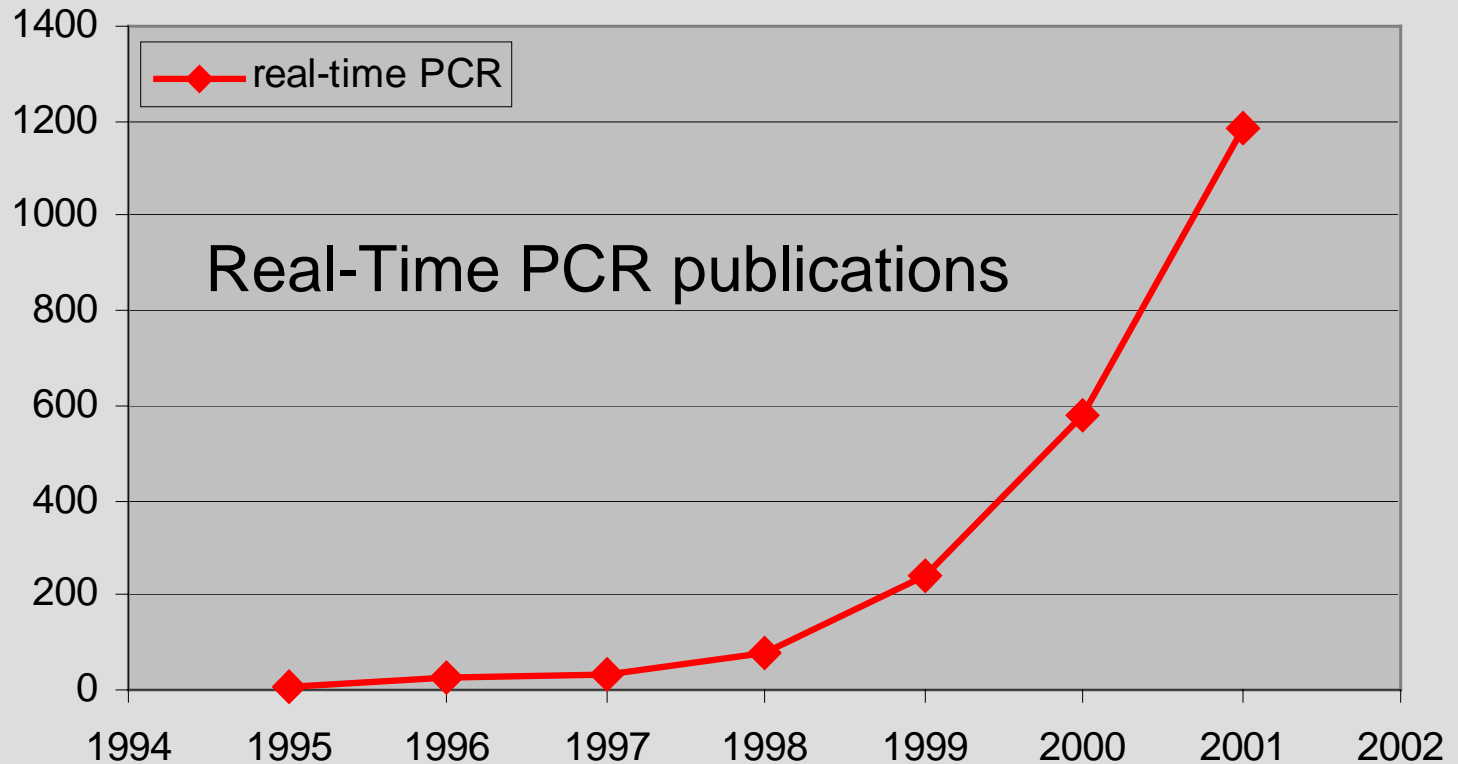


How quantification works

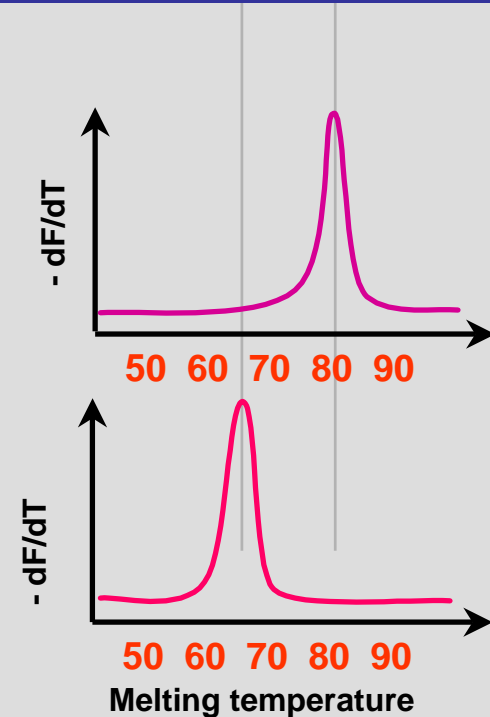
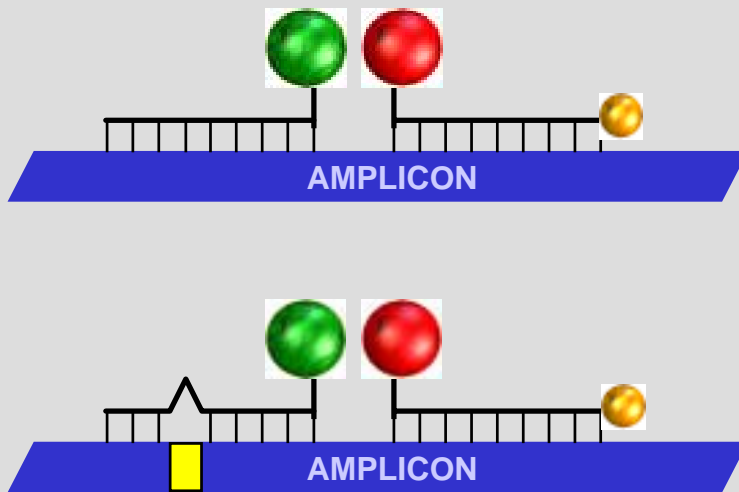
The probes monitor the actual amount of PCR-product



Real-Time qPCR itself is not very exciting



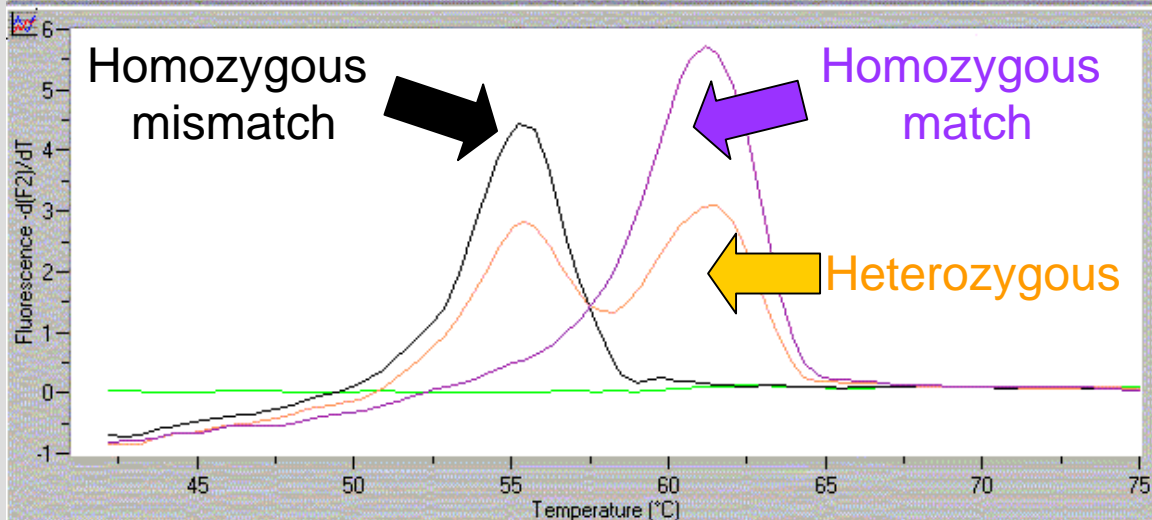
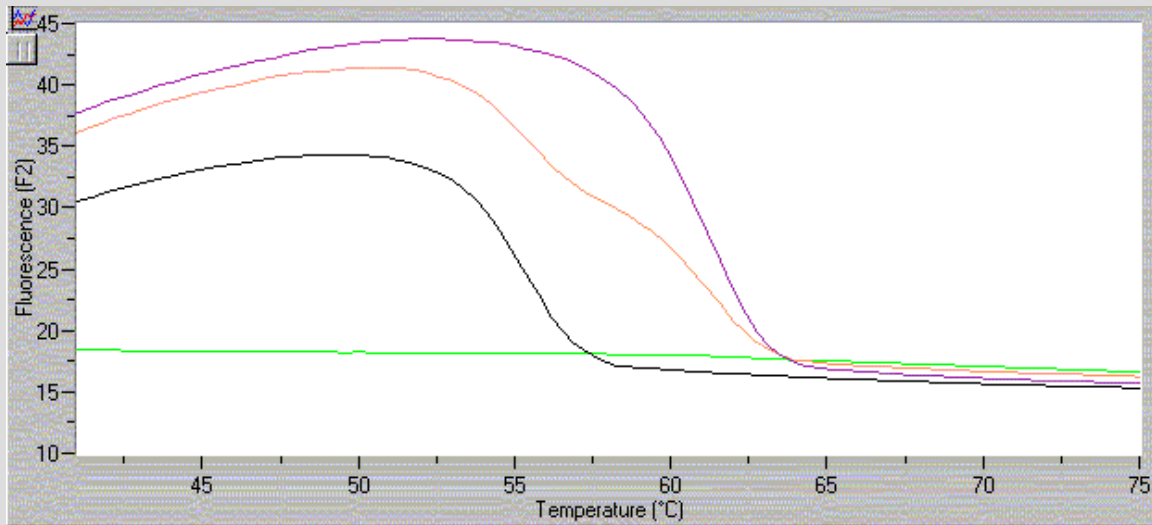
How genotyping works



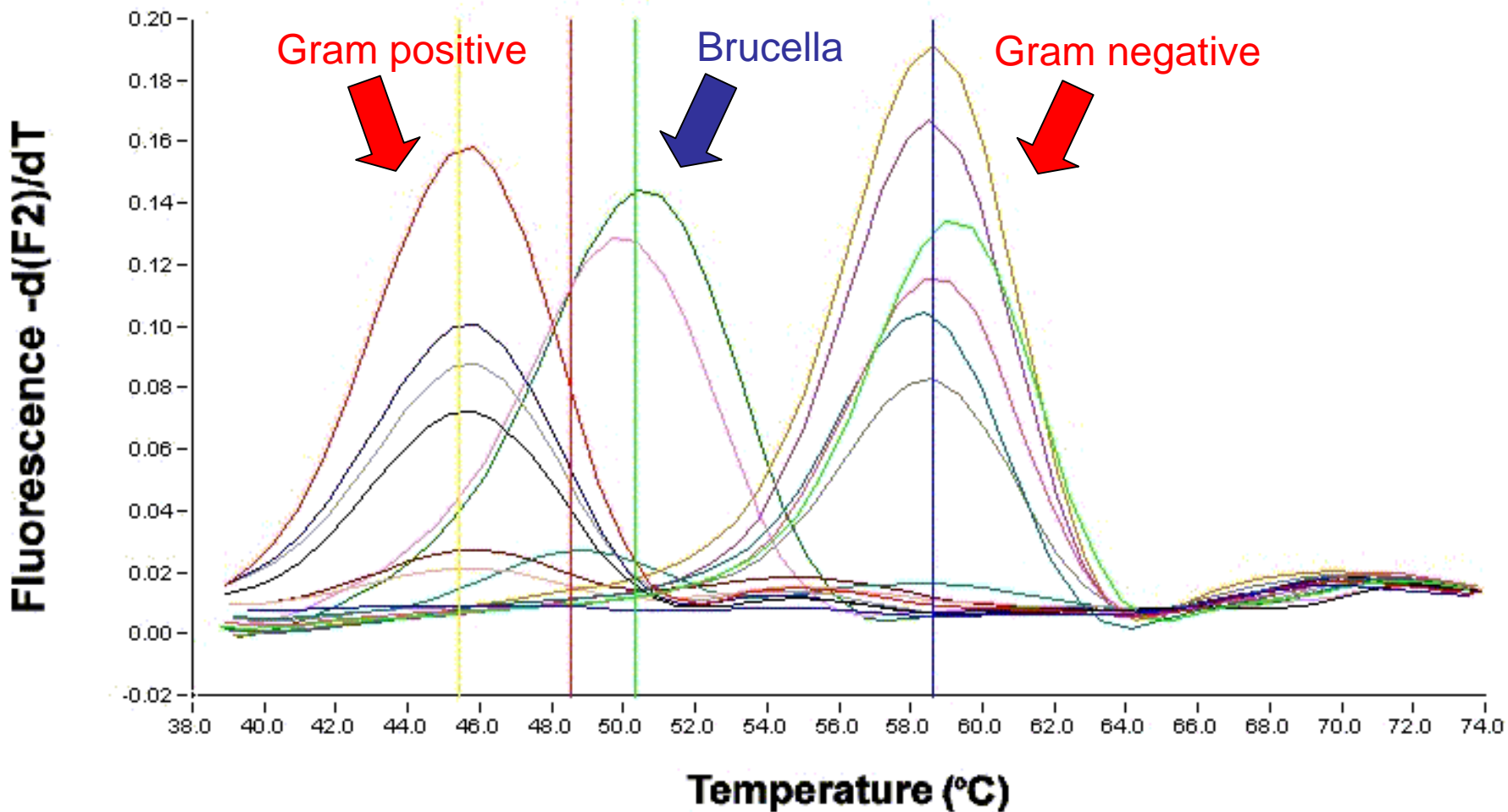
- **Single Nucleotide Polymorphism (SNP)**
- each mismatch destabilizes hybridisation strength
- the melting temperature is lowered for mismatched probes
- Use a pair of long (high T_m) anchor and short sensor probe



Example : hemachromatosis (HFE)



Example : Identification of bacteria

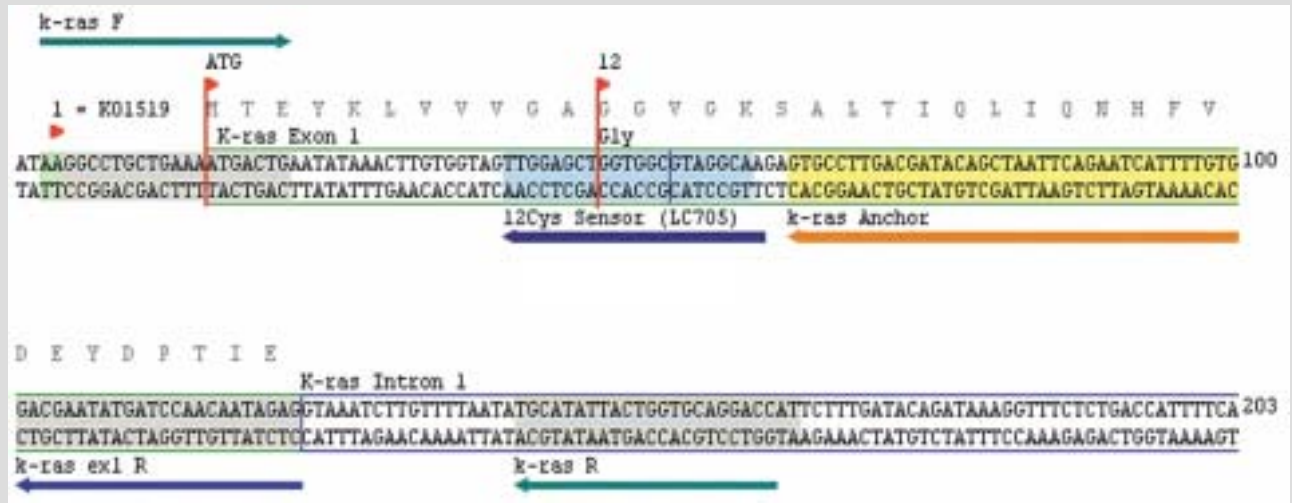


q(Geno-)Typing

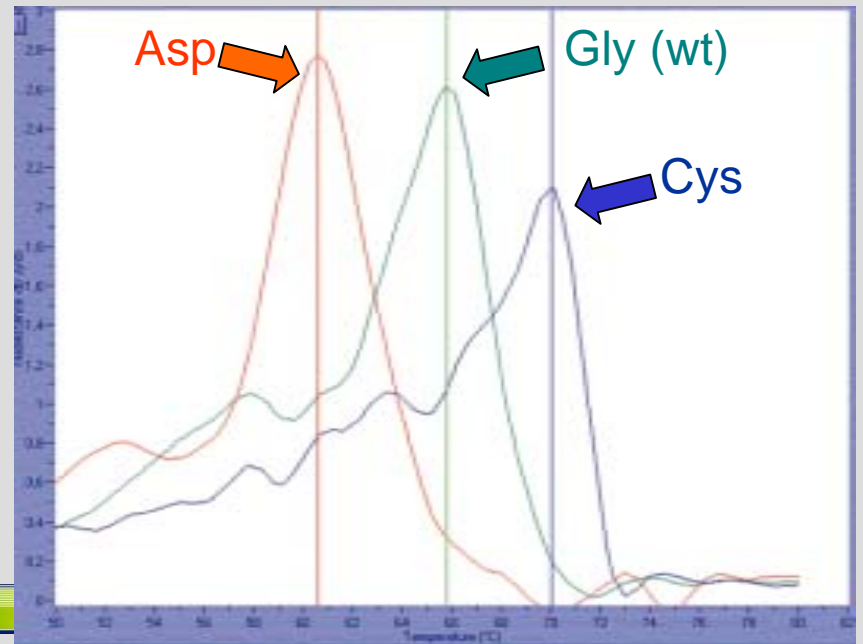
Detection of
sequence variations (mutations)
and how to find **minimal amounts**
of variants, e.g. contaminations,
minimal residual diseases, or
growing resistant populations,
varying in just one base.



Example I : k-ras Codon Gly12



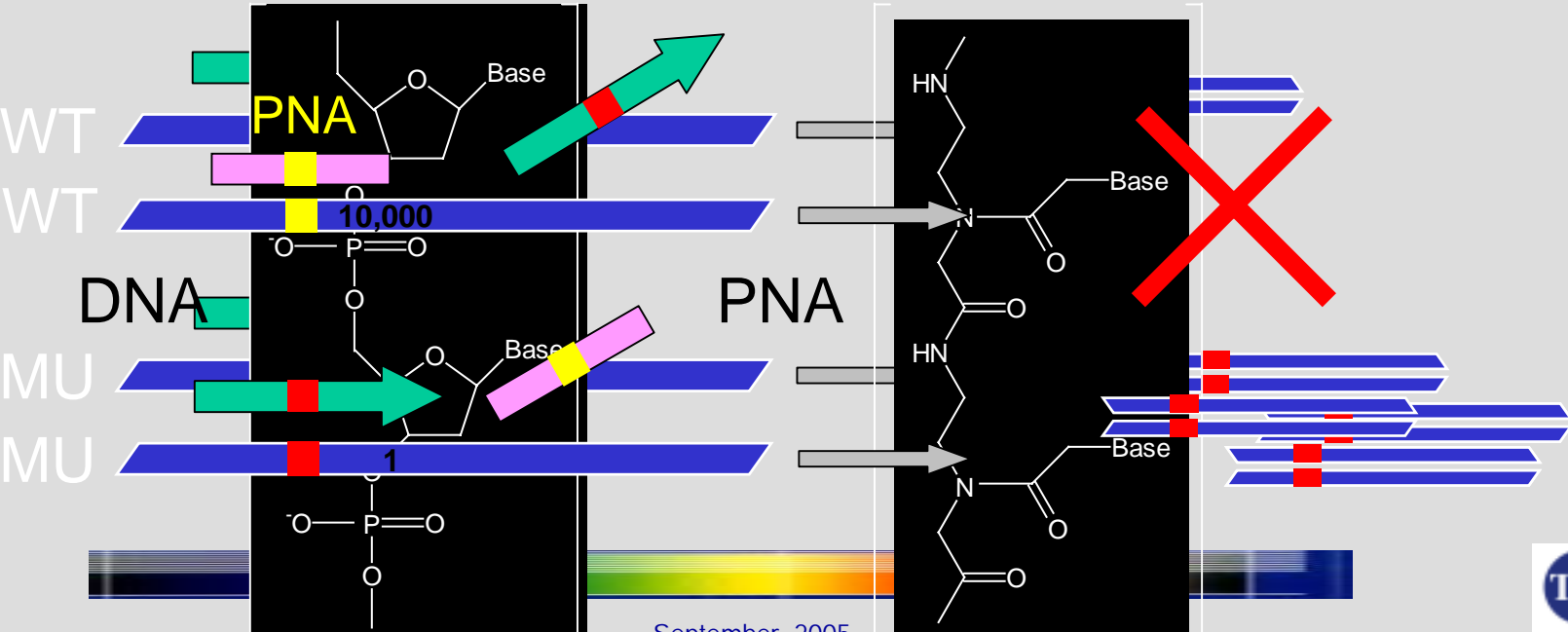
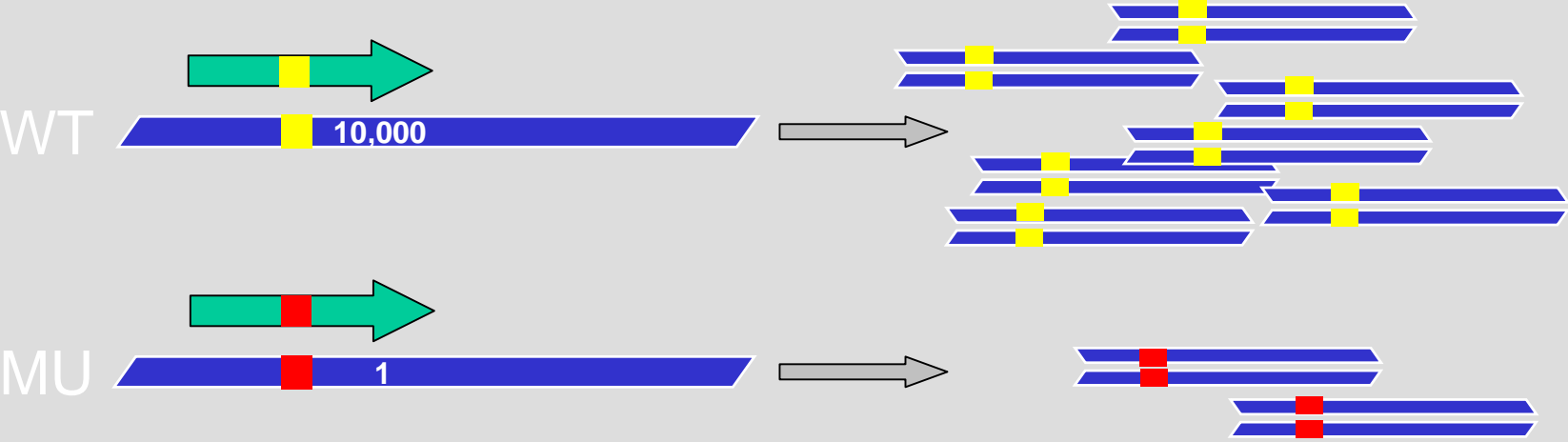
Several mutations
Ratio: 1:10,000



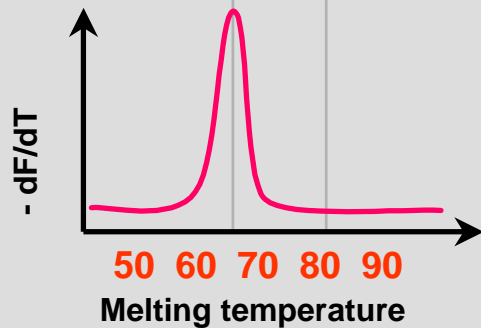
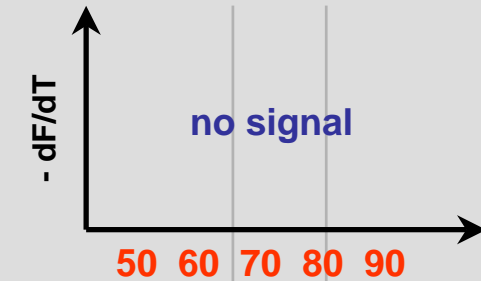
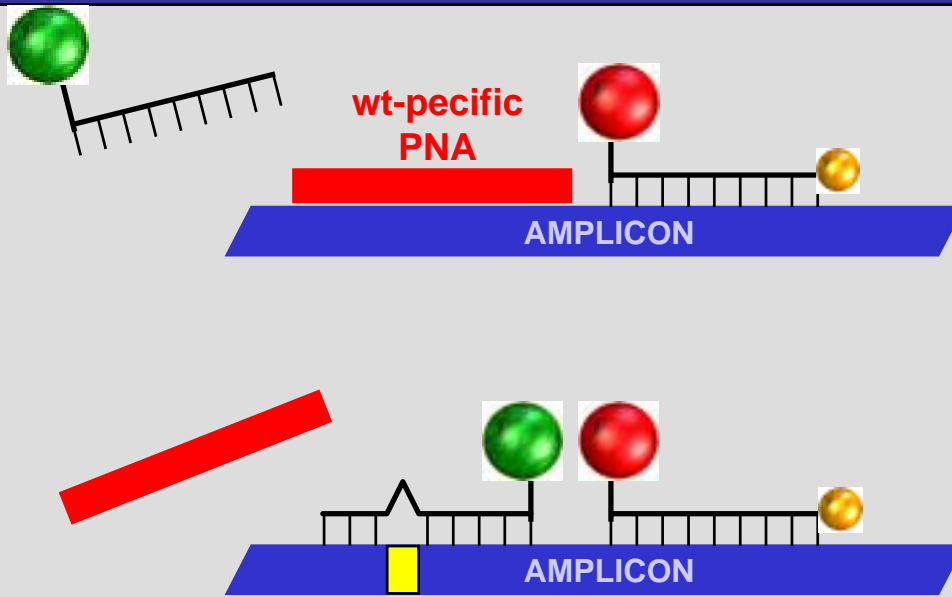
Melting on artificial targets



PNA/LNA-mediated PCR-clamping



Molecular concepts: Clamped-Probe-Assay

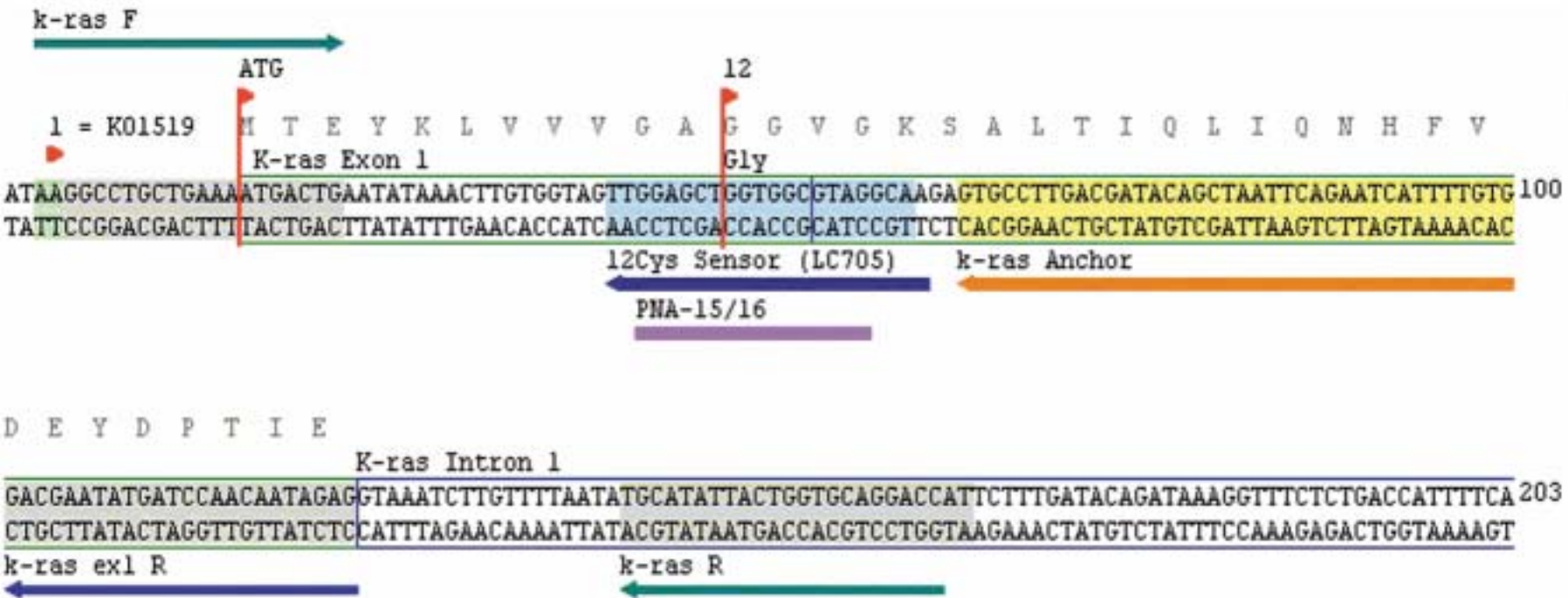


Possible applications :

- detection of minor variants (e.g. k-ras codon 12,13)
- minimal residual diseases (MRD)
- developing resistances (STI-571 in abl exon 6 in CML, developing lamivudine resistance in HBV, ...)

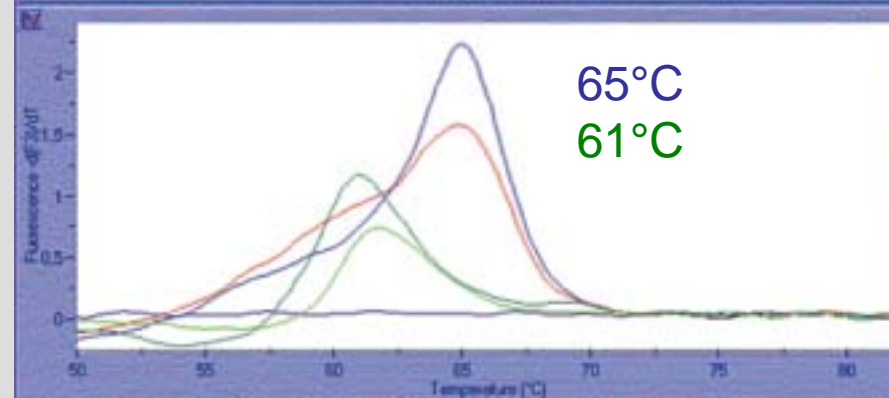
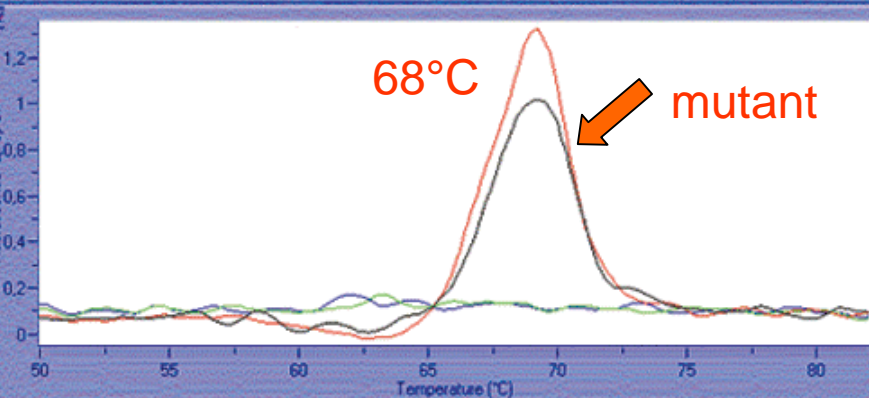
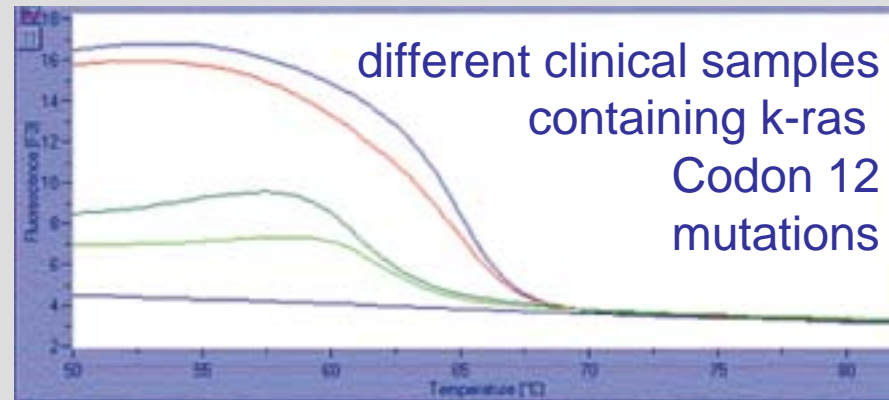
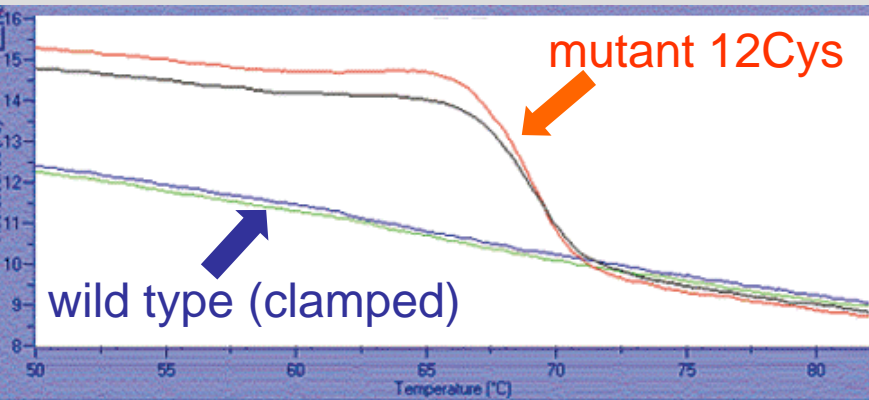


Example I : k-ras Codon 12



Melting curves from different patients

Melting curve (fluorescence vs. temperature)

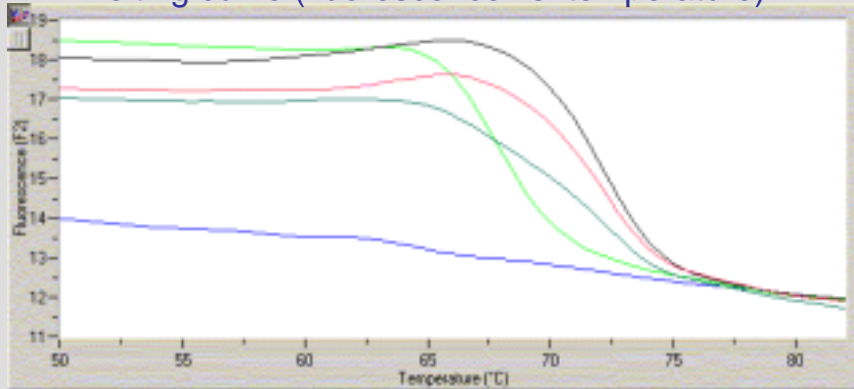


Melting curve (dFl/dT vs. temperature)

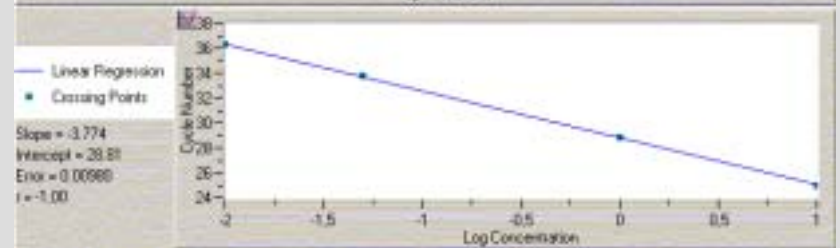
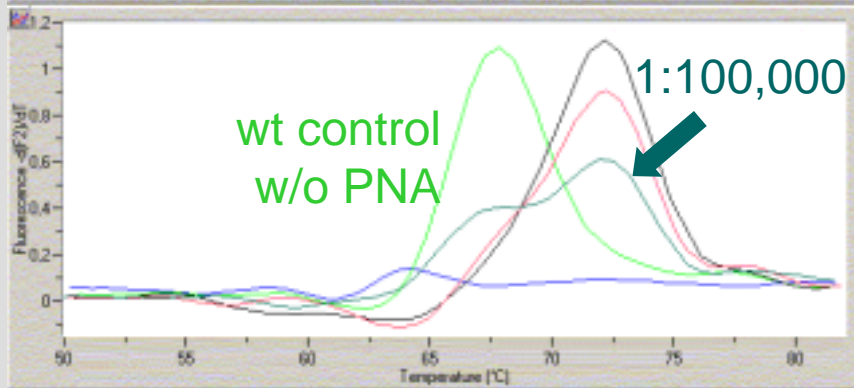
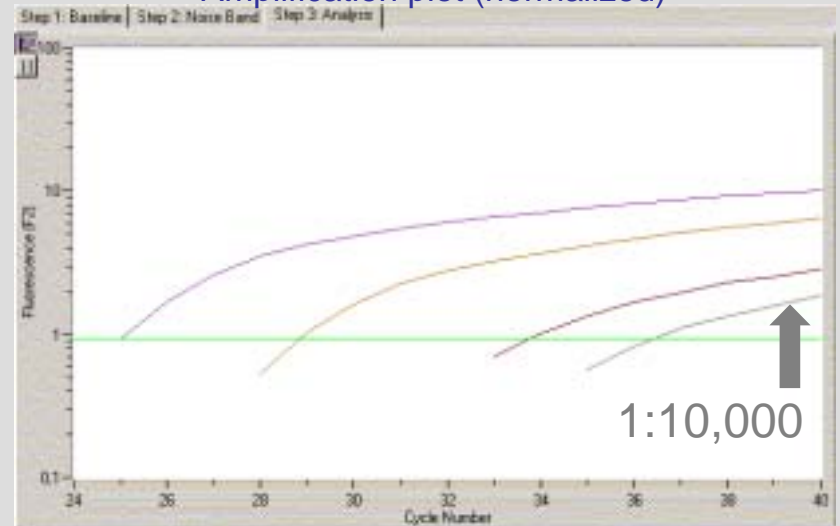


Dilution row of mutant in wt DNA

Melting curve (fluorescence vs. temperature)



Amplification plot (normalized)



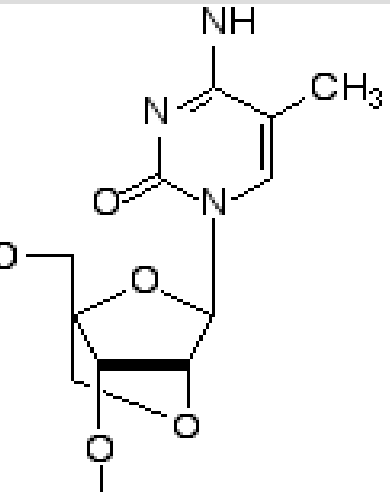
Melting curve (dF/dT vs. temperature)

Rapid detection of K-ras mutations in bile by peptide nucleic acid-mediated PCR clamping and melting curve analysis: comparison with restriction fragment length polymorphism analysis. Chen CY, Shiesh SC, Wu SJ. Clin Chem. 2004 Mar;50(3):481-9. Epub 2004 Jan 12

Transrenal DNA as a diagnostic tool: important technical notes. Su YH, Wang M, Block TM, Landt O, Botezatu I, Serdyuk O, Lichtenstein A, Melkonyan H, Tomei LD, Umansky S. Ann N Y Acad Sci. 2004 Jun;1022:81-9



Clamping probes consisting of Locked-Nucleic Acid (LNA) work even well

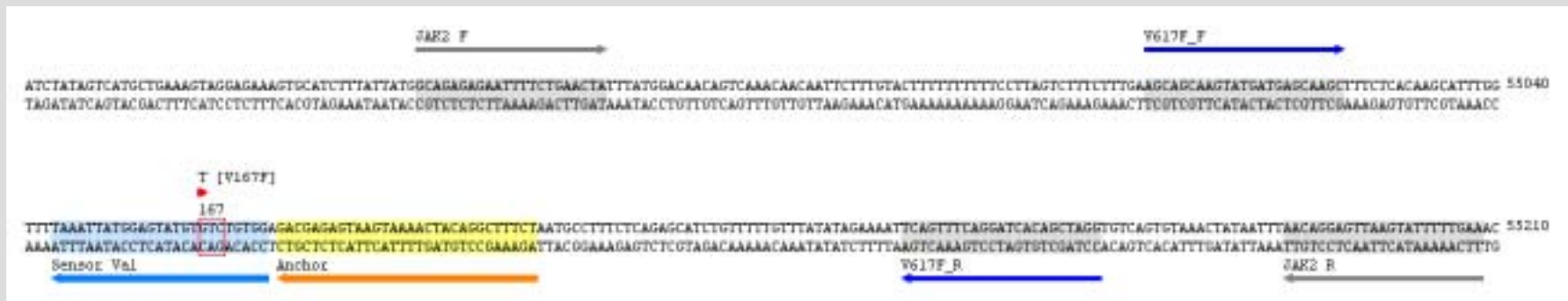
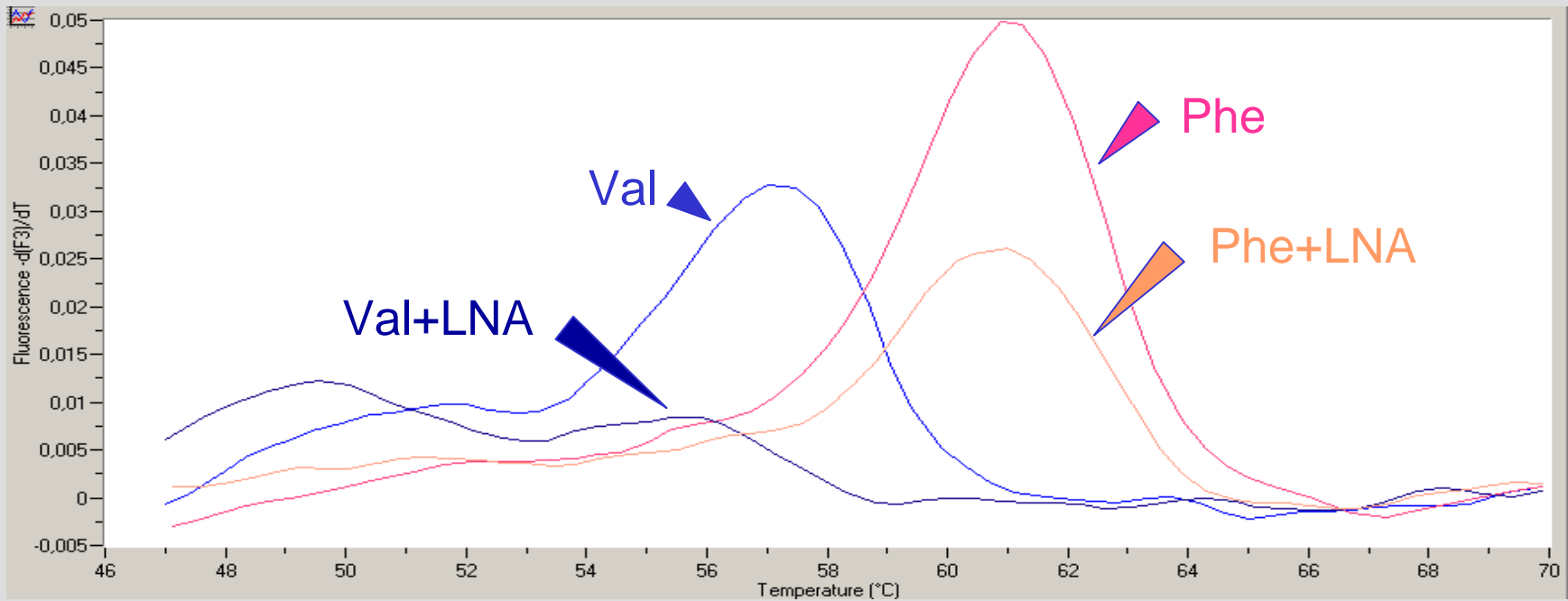


Nr.	Chemistry of the clamping probe	Concentration of probe still suppressing the 12 Gly-variant	Concentration of probe suppressing the 12 Cys-variant (1 mismatch)
1	PNA 15mer 12Gly	2,8 μM^* /3 $\mu\text{M}^{\text{§}}$	
2	PNA 17mer 12Gly	1,0 μM	
3	LNA 17mer 12Gly	0,1 μM	
4	5'-NH LNA 17mer	0,05 μM	0,2 μM
5	5'-MB LNA 17mer	0,02 μM	0,1 μM
6	3'-NH LNA 17mer	0,1 μM	
7	3'-MB LNA 17mer	0,05 μM	

A 3'-terminal attached MB dye boosts the suppression significantly (working concentration is 20-fold lower).



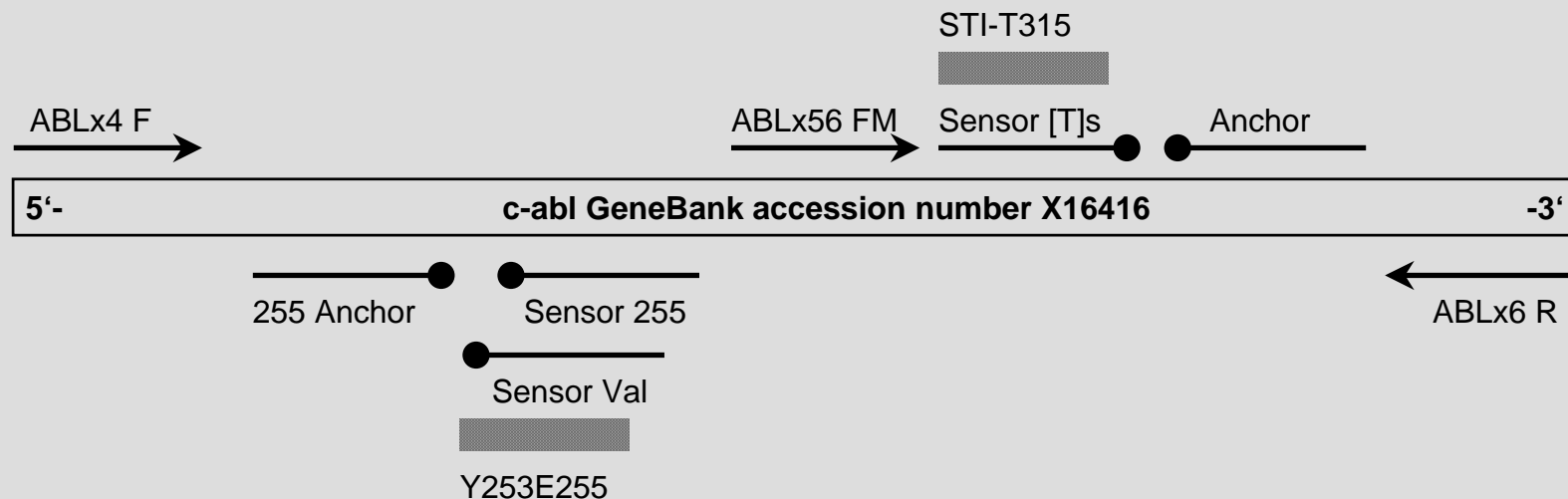
Example II : JAK2 mRNA or gen. V617F



Example III : Leukemia (CML) STI resistance

The M315T mutation in ABL exon 6 and mutations at Y253 and E255 in ABL exon 4 are responsible for the resistance of bcrABL-clones in CML.

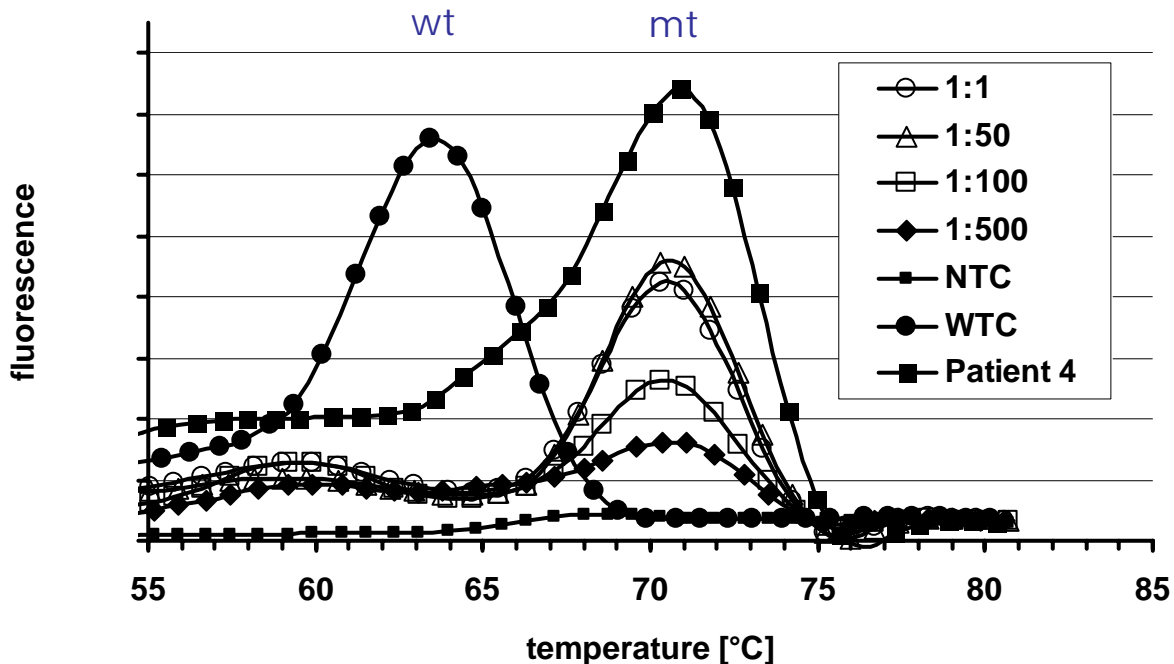
Clinical resistance to STI-571 cancer therapy caused by bcr-abl gene mutation or amplification. Gorre, M.E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P.N., Sawyers, C.L. *Science*, 293: 876-80, 2001



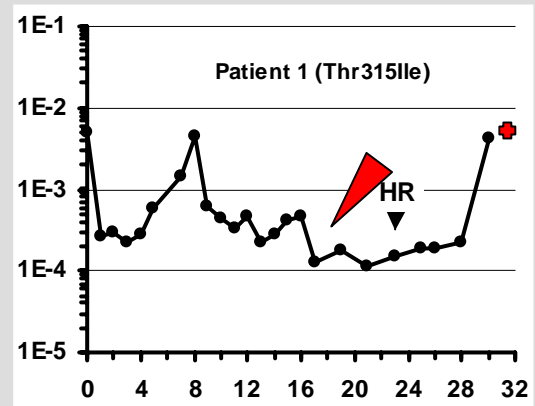
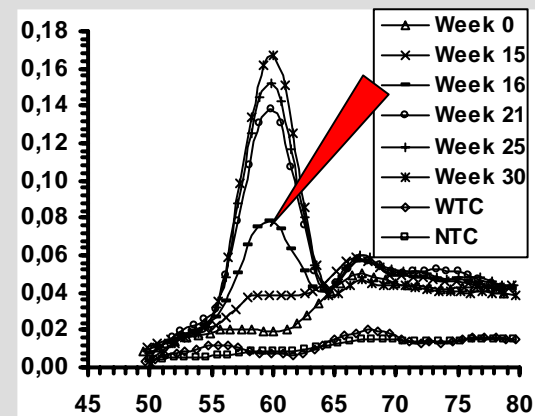
Preexistence and evolution of imatinib mesylate-resistant clones in chronic myelogenous leukemia detected by a PNA-based PCR clamping technique. Kreuzer KA, Le Coutre P, Landt O, Na IK, Schwarz M, Schultheis K, Hochhaus A, Dörken B. *Ann Hematol.* 2003 Apr 12

Example III : Leukemia (CML) STI resistance

Dilution series (bcr/abl Tyr253His)



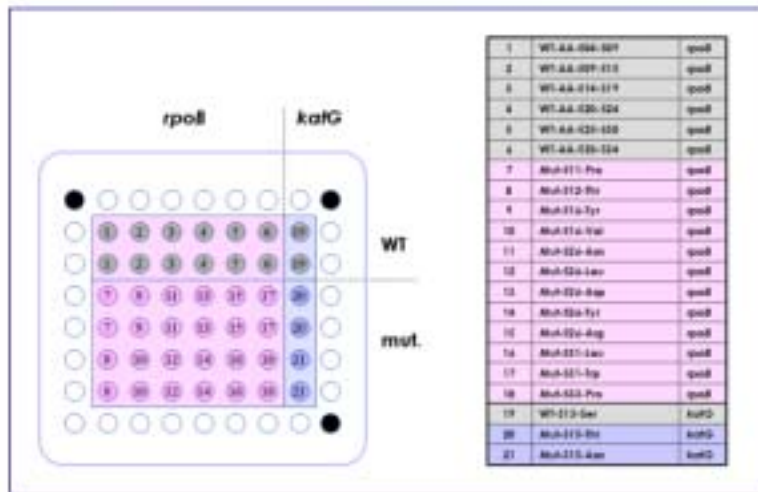
Serial dilutions of mutant in wildtype, wildtype control and patient sample.



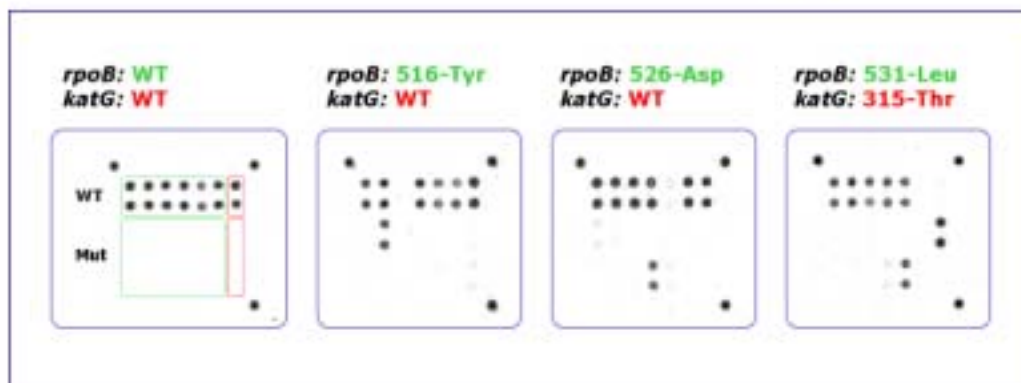
Patient history monitored with the CPA assay.



Example IV : Growing resistances in infectious diseases - Mycobacteria katG (Isoniazid)



- identification of multiresistant strains (MRS) in 2.5 hrs
- *rpoB* gen: 6 wt probes (compl. 81bp region)
12 SNPs associated with RIF^{res}
- *katG* gen: 315-Ser wt probe
2 SNPs associated with INH^{res}
- additional capture probes upon request (e.g. *inhA* gen etc.)



- 1 „duplex“ amplification
- 45 min protocol (excl. PCR)
- optional combination with quantitative Real-Time PCR

Example IV: Mycobacteria katG –Design

TB86



V E T A A L I V G G H T F G K T H G A G P A D L V G P E P E A A P L E Q

CGTCGAAACAGCGGCGCTGATCGTCGGCGGTCACACTTTTCGGTAAGACCCATGGCGCCGGCCCGGCGGATCTGGTCGGCCCCGAACCCGAGGCTGCTCCGCTGGAGCAG 2863
GCAGCTTTTGTGCGCCGCGACTAGCAGCCGCCAGTGTGAAAGCCATTCTGGGTACCGCGCCGGGCGGCTAGACCAGCCGGGGCTTGGGCTCCGACGAGGCGACCTCGTC

TB anchor



TB sensor



katG LNA



C [Ser315Thr]



M G L G W K S S Y G T G T G K D A I T S G I E V V W T N T P T K W D N S F

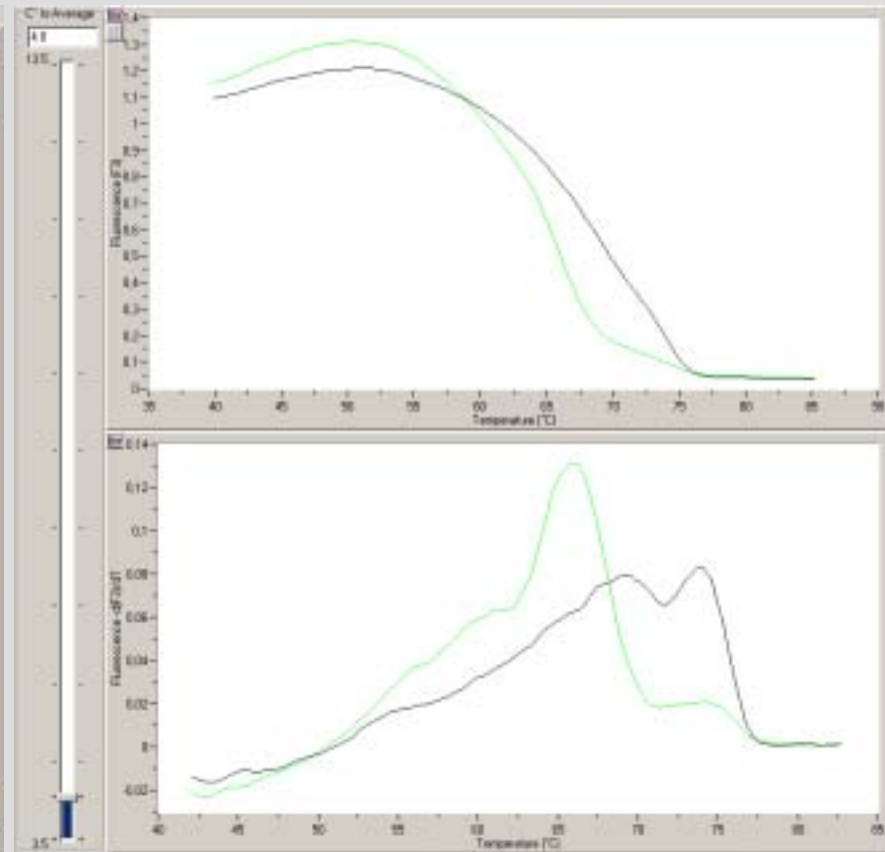
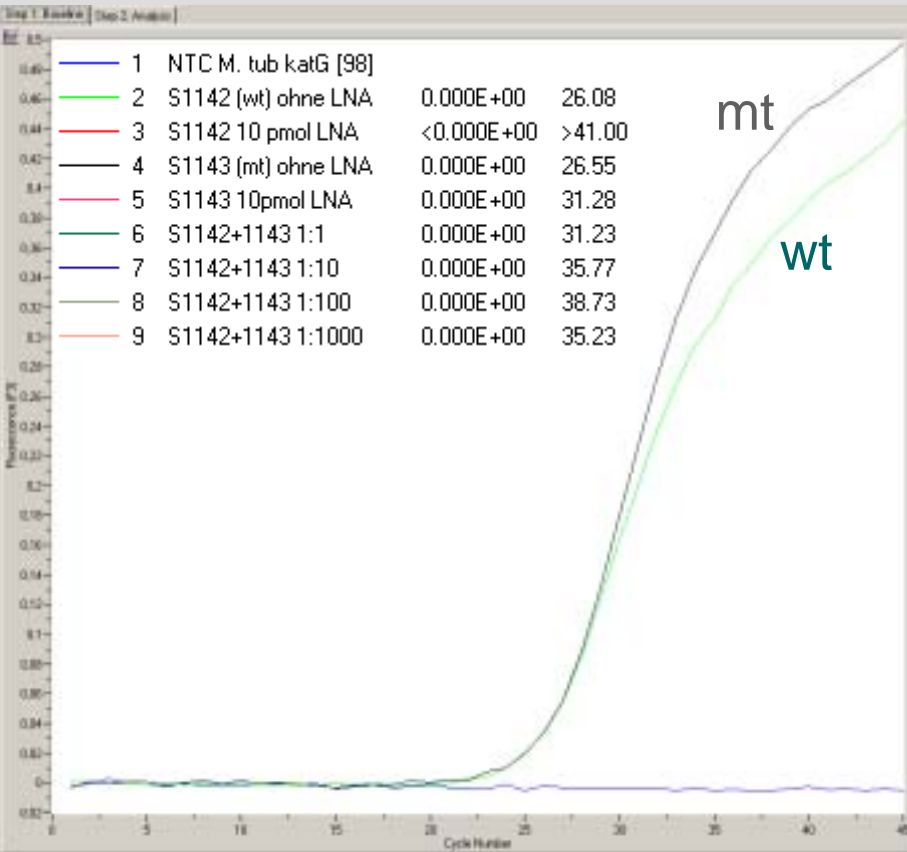
315

ATGGGCTTGGGCTGGAAGAGCTCGTATGGCACCCGGAACCGGTAAGGACGCGGATCACCAGCGGCATCGAGGTCGTATGGACGAACACCCCGACGAAATGGGACAACAGTT 2972
TACCCGAACCCGACCTTCTCGAGCATAACCGTGGCCCTTGGCCATTCTGCGCTAGTGGTCGECGTAGCTCCAGCATAACCTGCTTGTGGGGCTGCTTTACCCTGTTGTCAA

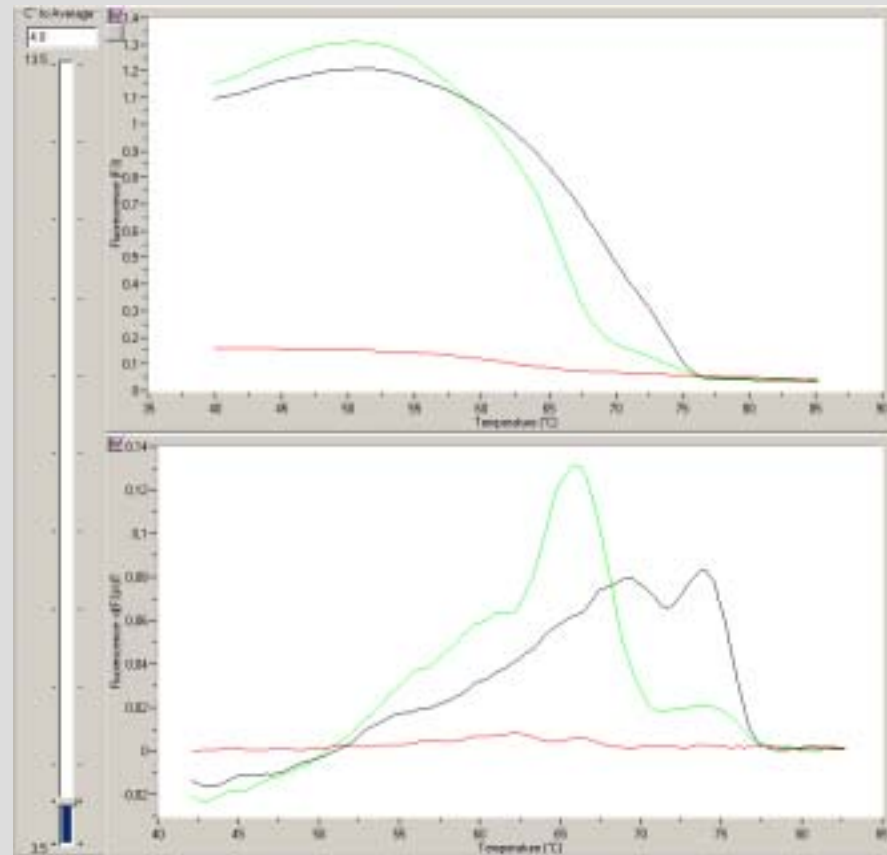
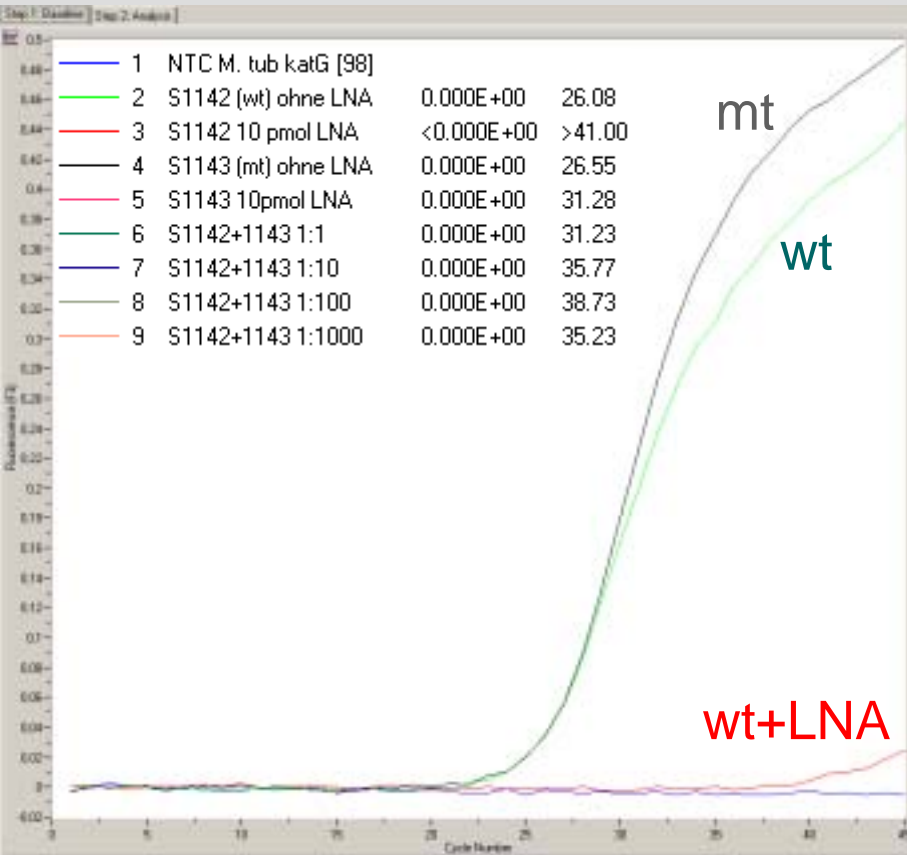
TB87



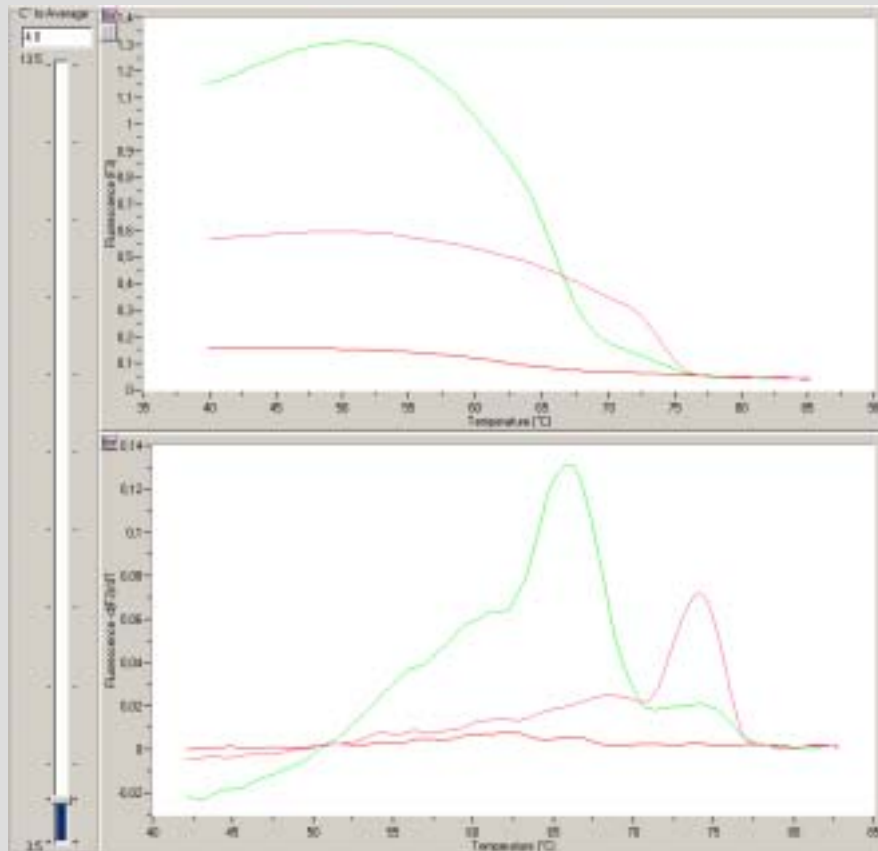
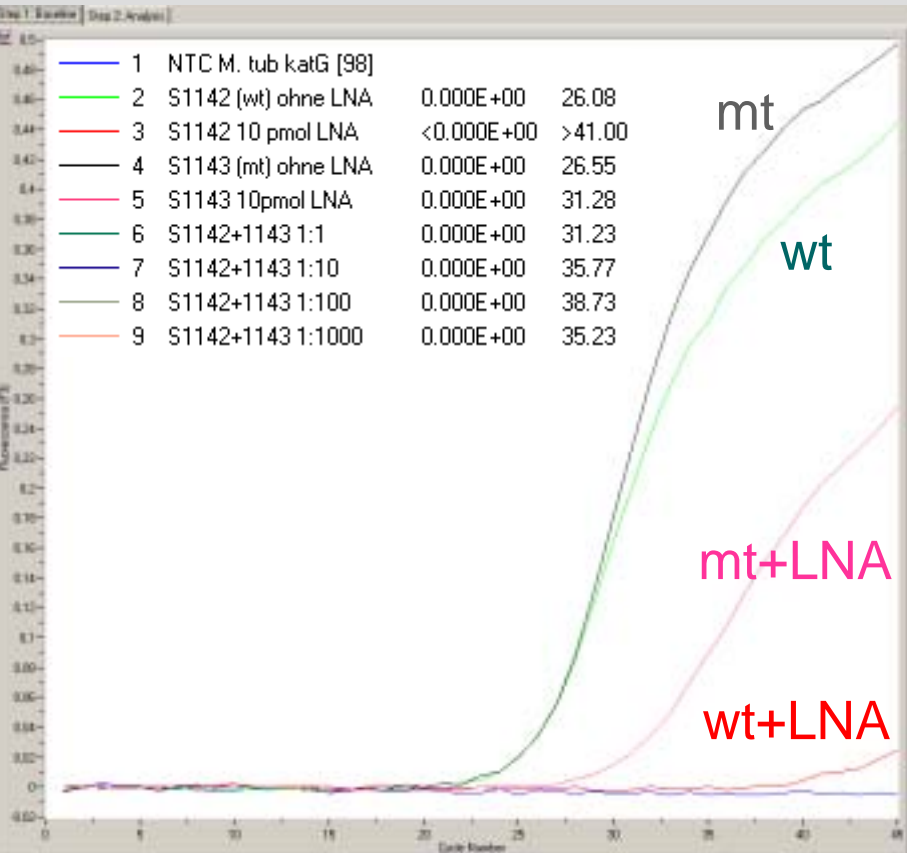
Amplification – Melting Analysis wt/mt



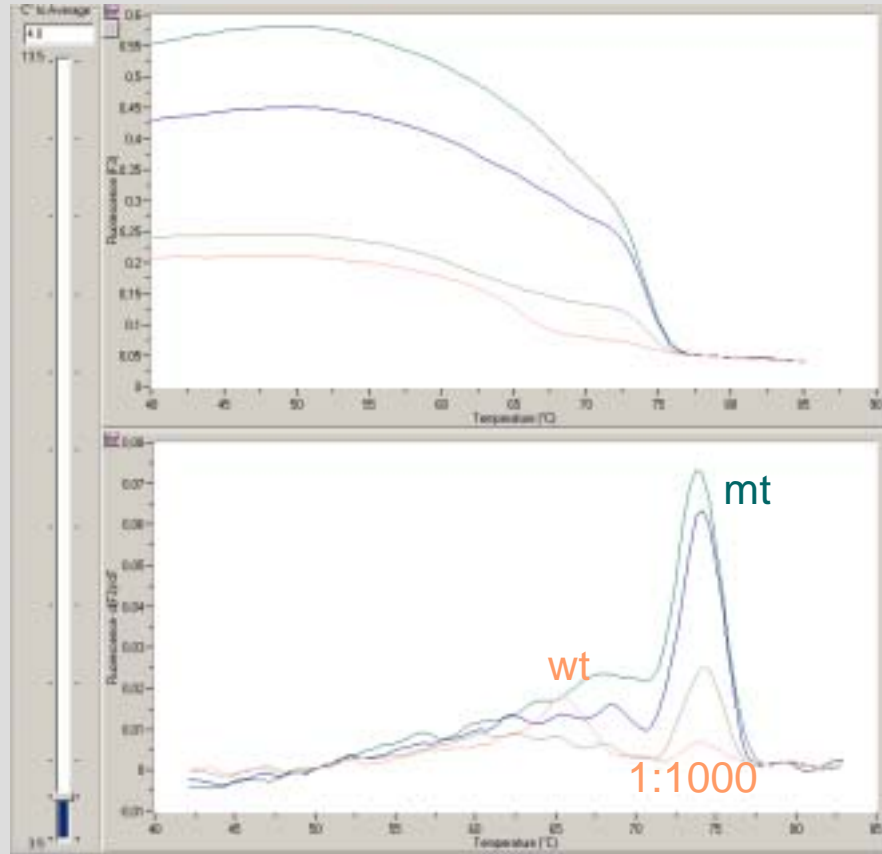
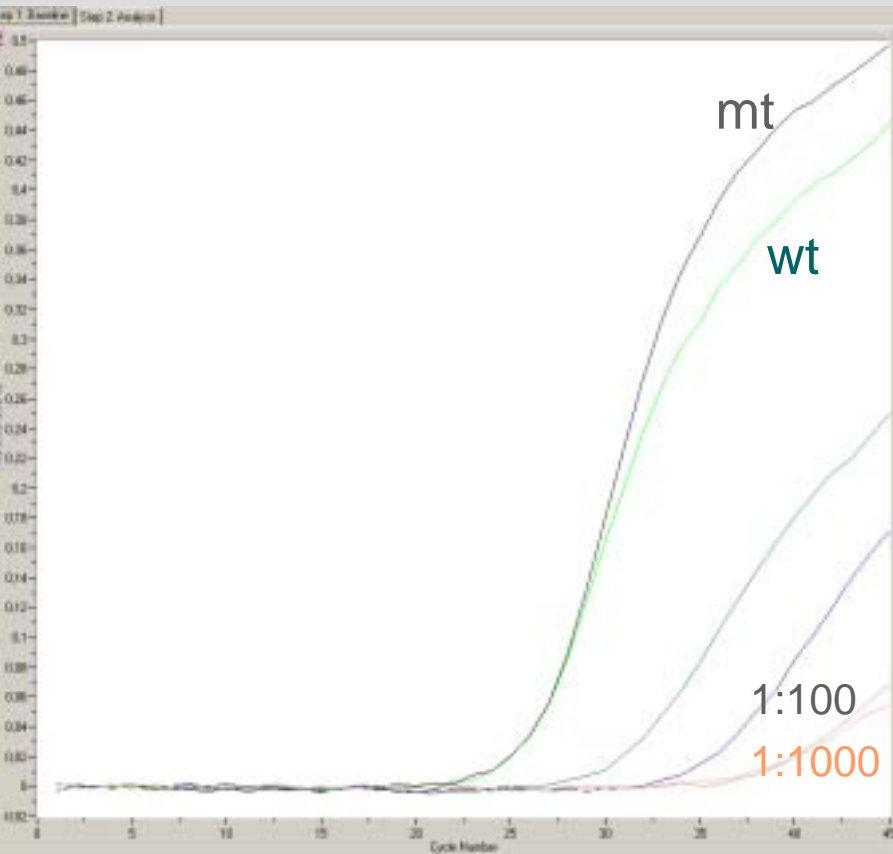
Amplification – Melting Analysis wt/mt



Amplification – Melting Analysis wt/mt

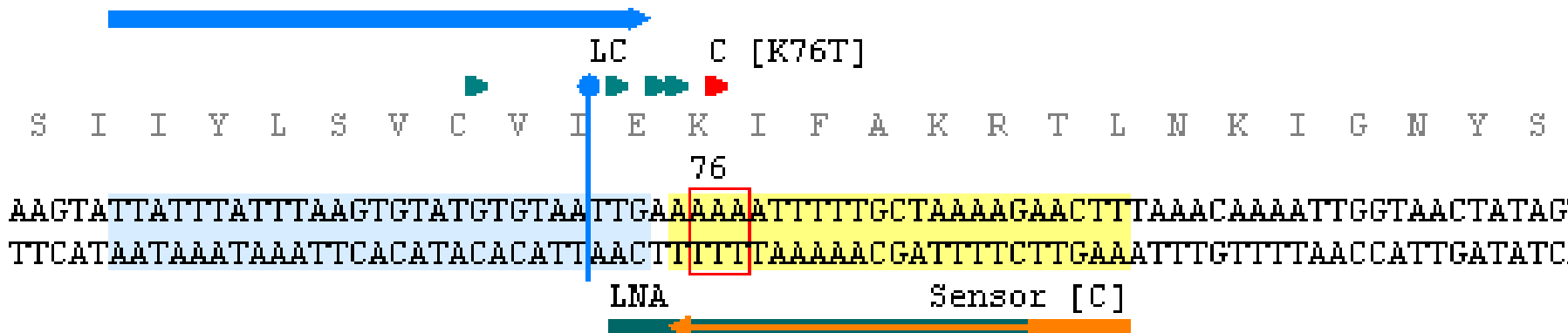


Amplification – Melting Analysis wt/mt



Expl. V: Plasmodium chloroquine resistance

Plas iLC S



Use of a locked-nucleic-acid oligomer in the clamped-probe assay for detection of a minority Pfert K76T mutant population of Plasmodium falciparum. Senescau A, Berry A, Benoit-Vical F, Landt O, Fabre R, Lelievre J, Cassaing S, Magnaval JF. *J Clin Microbiol.* 2005 Jul;43(7):3304-8.



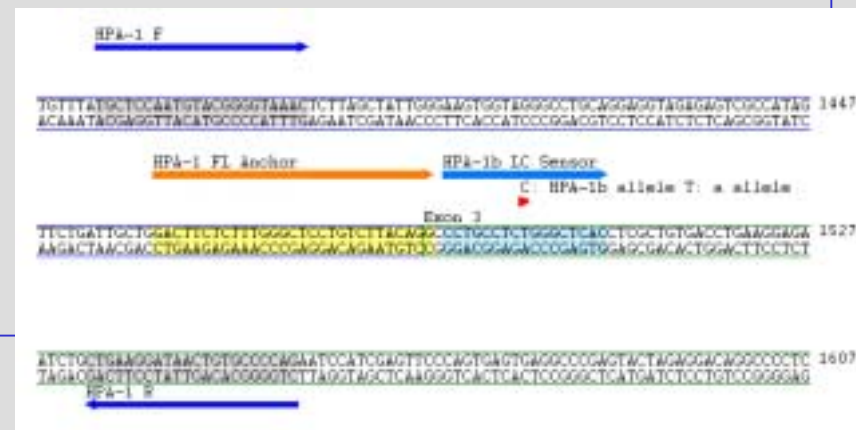
Example VI – current projects – HPA-1

Should all pregnant women be tested for their platelet PLA (Zw, HPA-1) phenotype? Br J Haematol. 1994 Jan;86(1):1-5.

About 97% of the population has the HPA-1a allele

Human platelet antigens (HPA) can be targets for antibody responses that cause life-threatening thrombocytopenia following platelet transfusions or pregnancy.

Search for fetal HPA-1b alleles **in the maternal blood** (HPA-1a background)





MOLBIOL



*Locked
Nucleic Acid*

opens new perspective