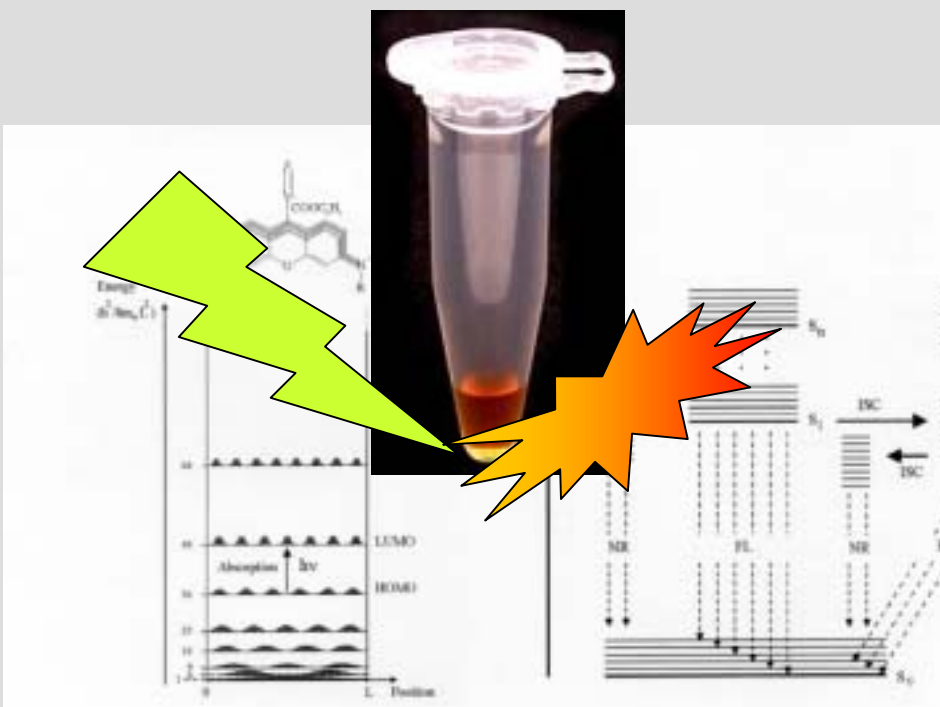


Finding the needle in the haystack
LNA bases enhance SNP detection dramatically

Olfert Landt Weihenstephan, September 2005

Real-Time PCR is based on Fluorescence



<u>Fluorescence</u> is less sensitive when compared to radioactivity or enzyme-linked reactions

- λ wavelenght (nm)
- ε extinction coefficien
- q quantum yield

- Absorbtion Maximum
- Emission Maximum
- Stoke's Shift
- Quench
- FRET (Fluorescence Resonance Energy Transfe
- Photobleaching
- Background fluorescence
- Lifetime



Real-Time-PCR Instruments



LightCycler480
Roche Diagnostics

LightTyper
Roche Diagnostics





SDS7700
Applied Biosystems

SmartCycler
Cepheid



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

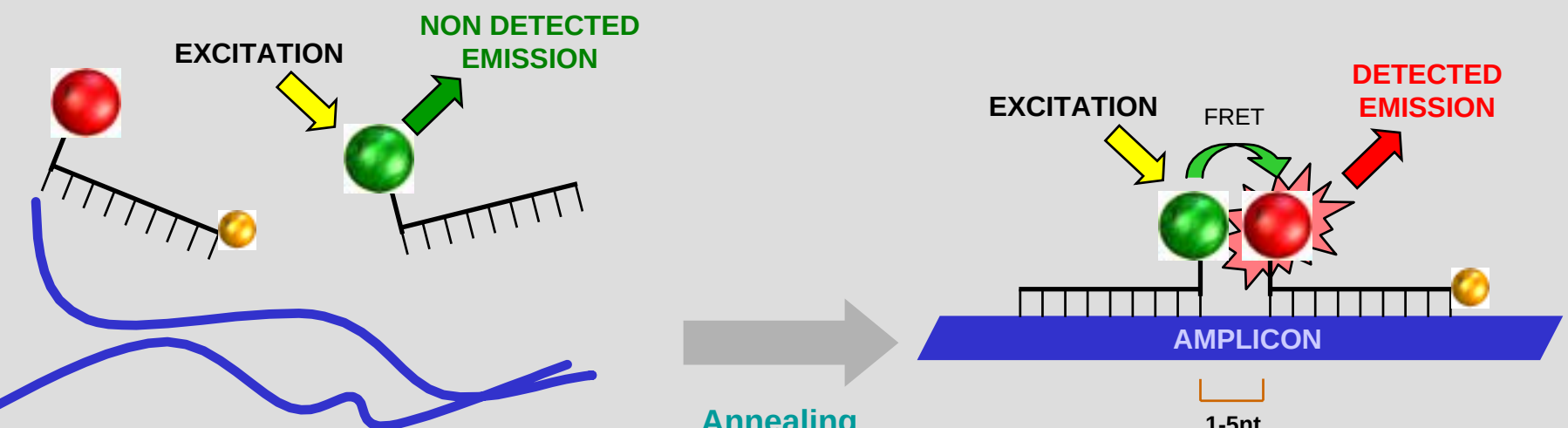


As a producer of fluorescent probes we are interested in new PCR detection technologies



Molecular Concepts: Hybridization Probes

Principle: adjacent hybridisation and FRET



Fluorescein (donor)

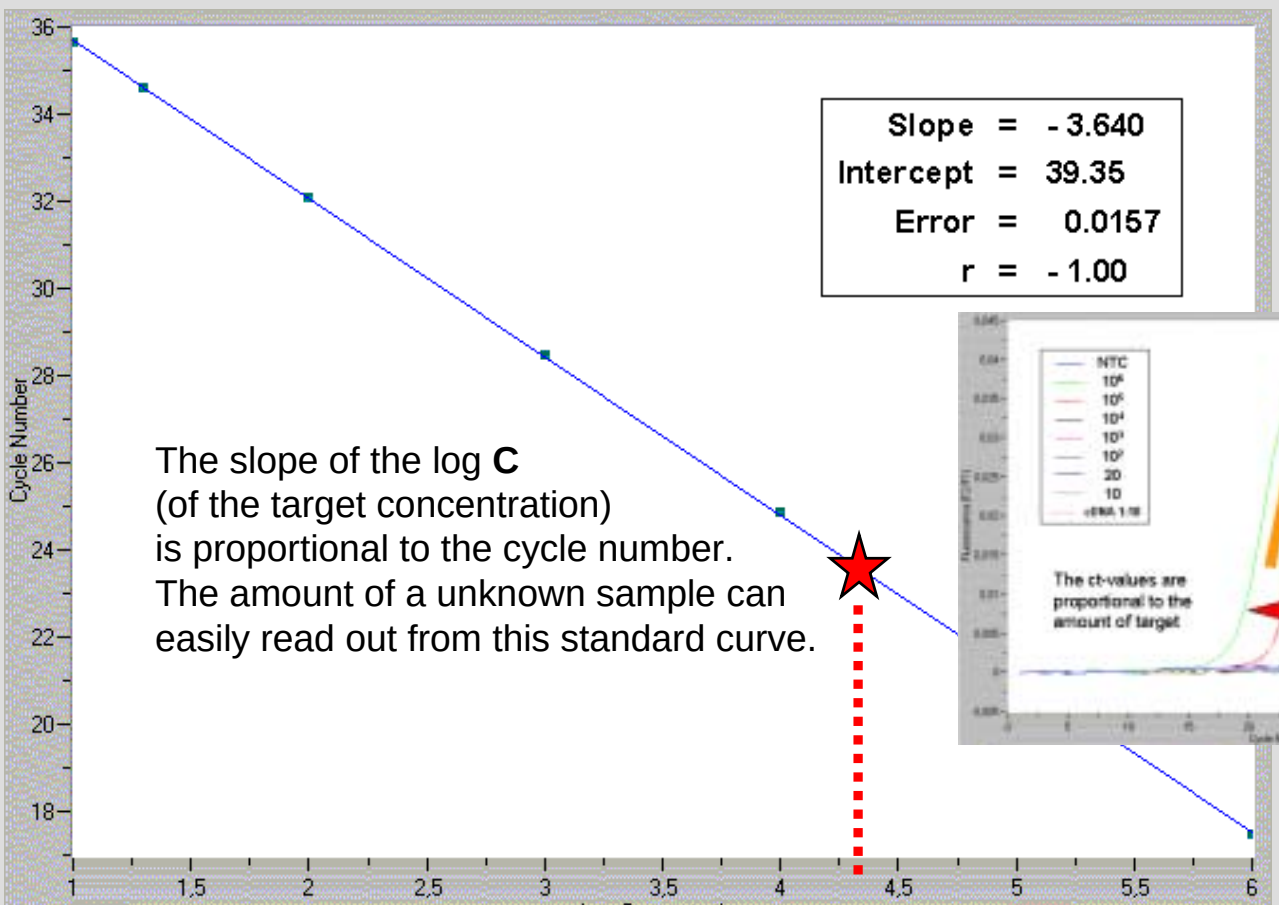
LC RED640/705 (acceptor)

Phosphate



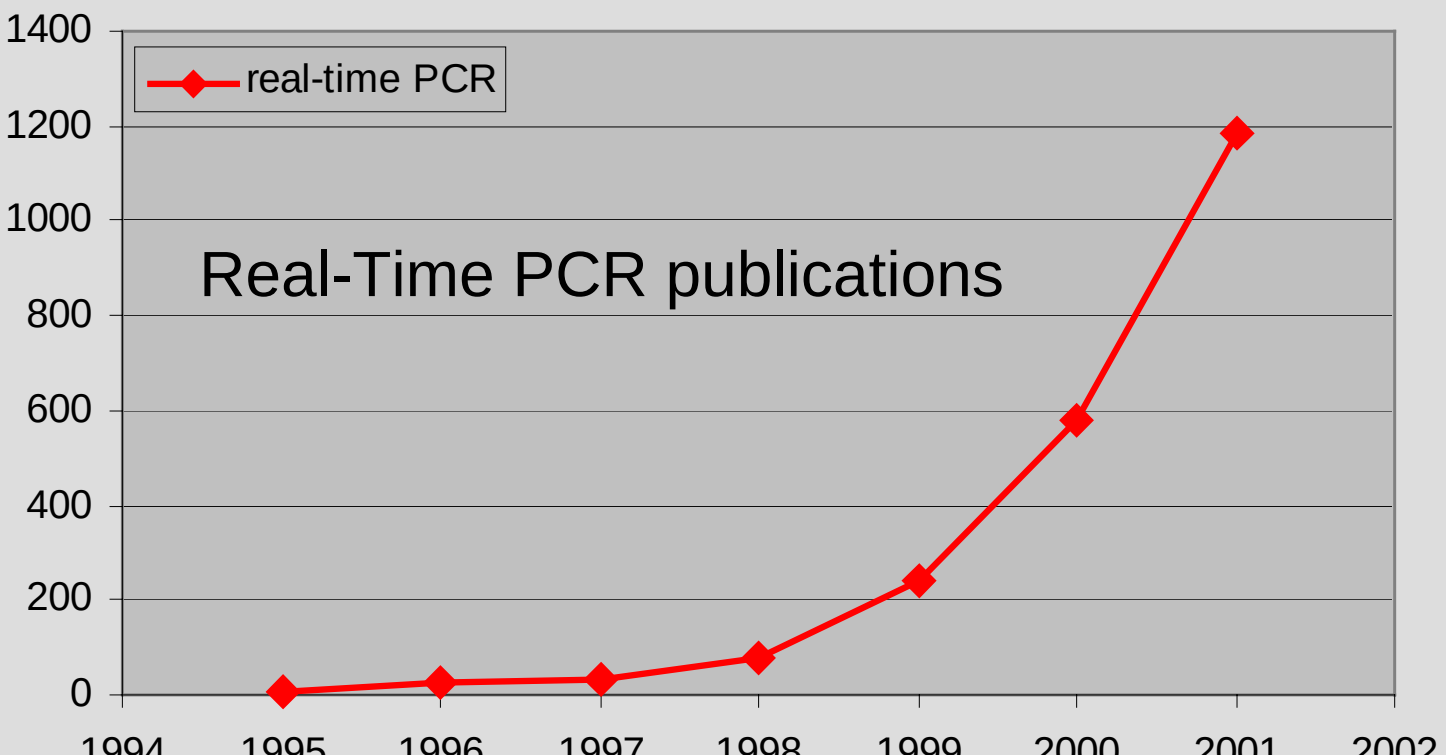
How quantification works

The probes monitor the actual amount of PCR-product

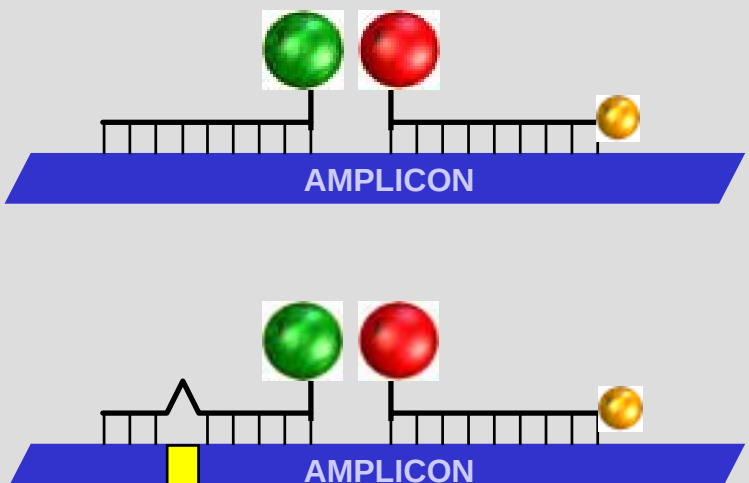


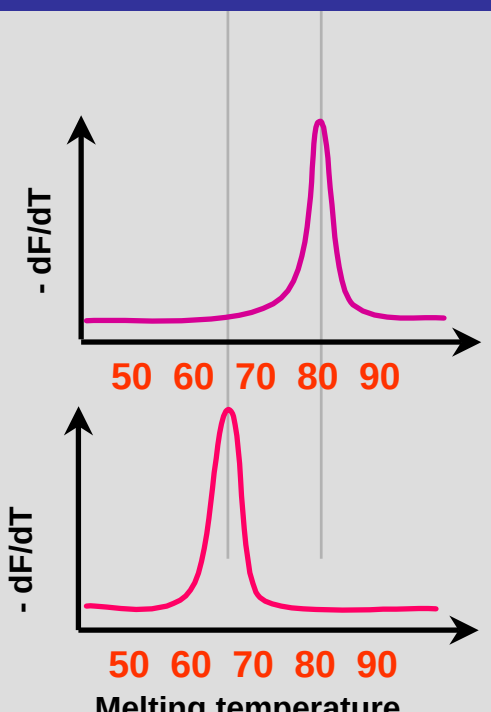
The slope of the log **C**
(of the target concentration)
is proportional to the cycle number.
The amount of a unknown sample can
easily read out from this standard curve.

Real-Time qPCR itself is not very exciting



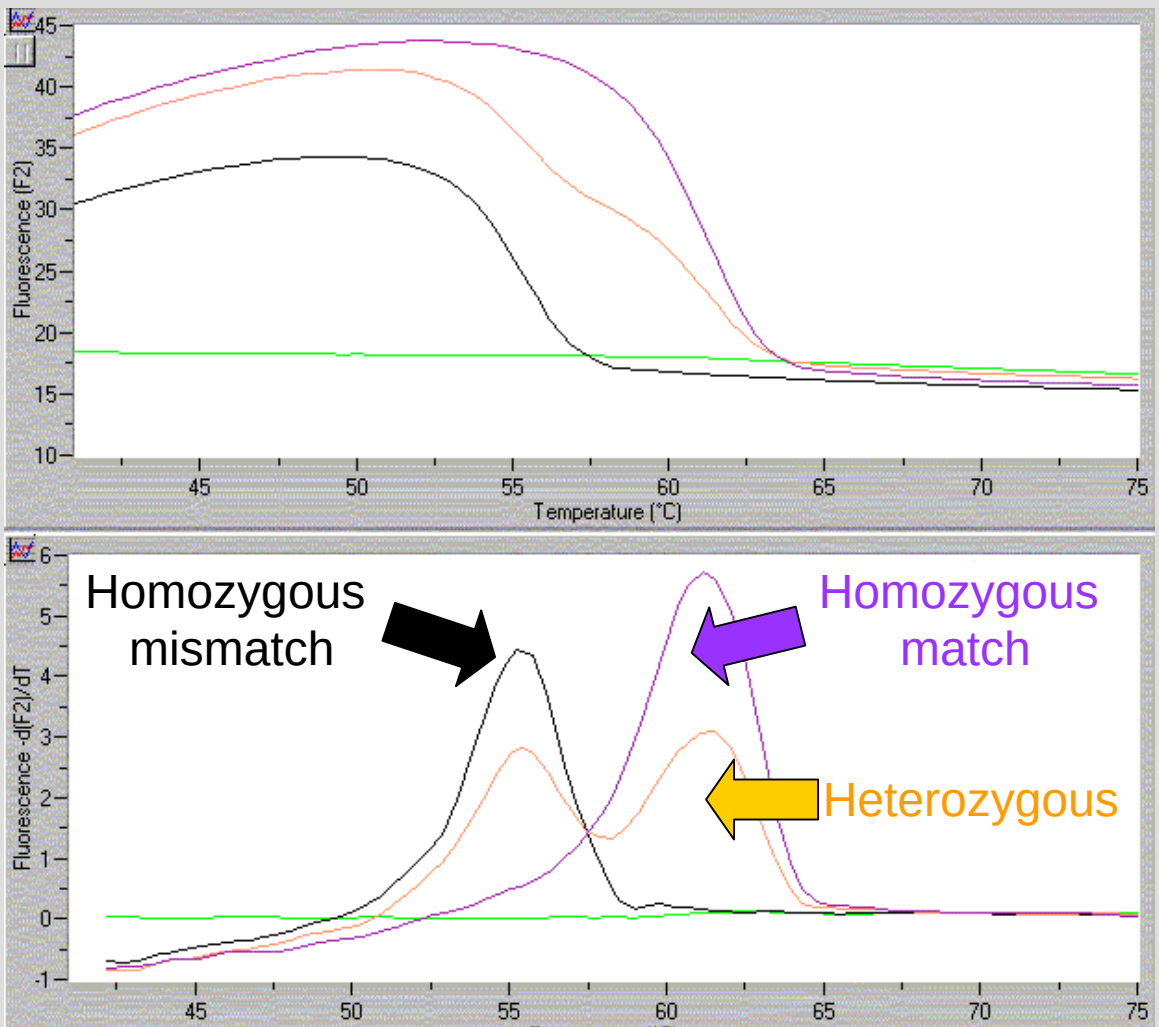
How genotyping works



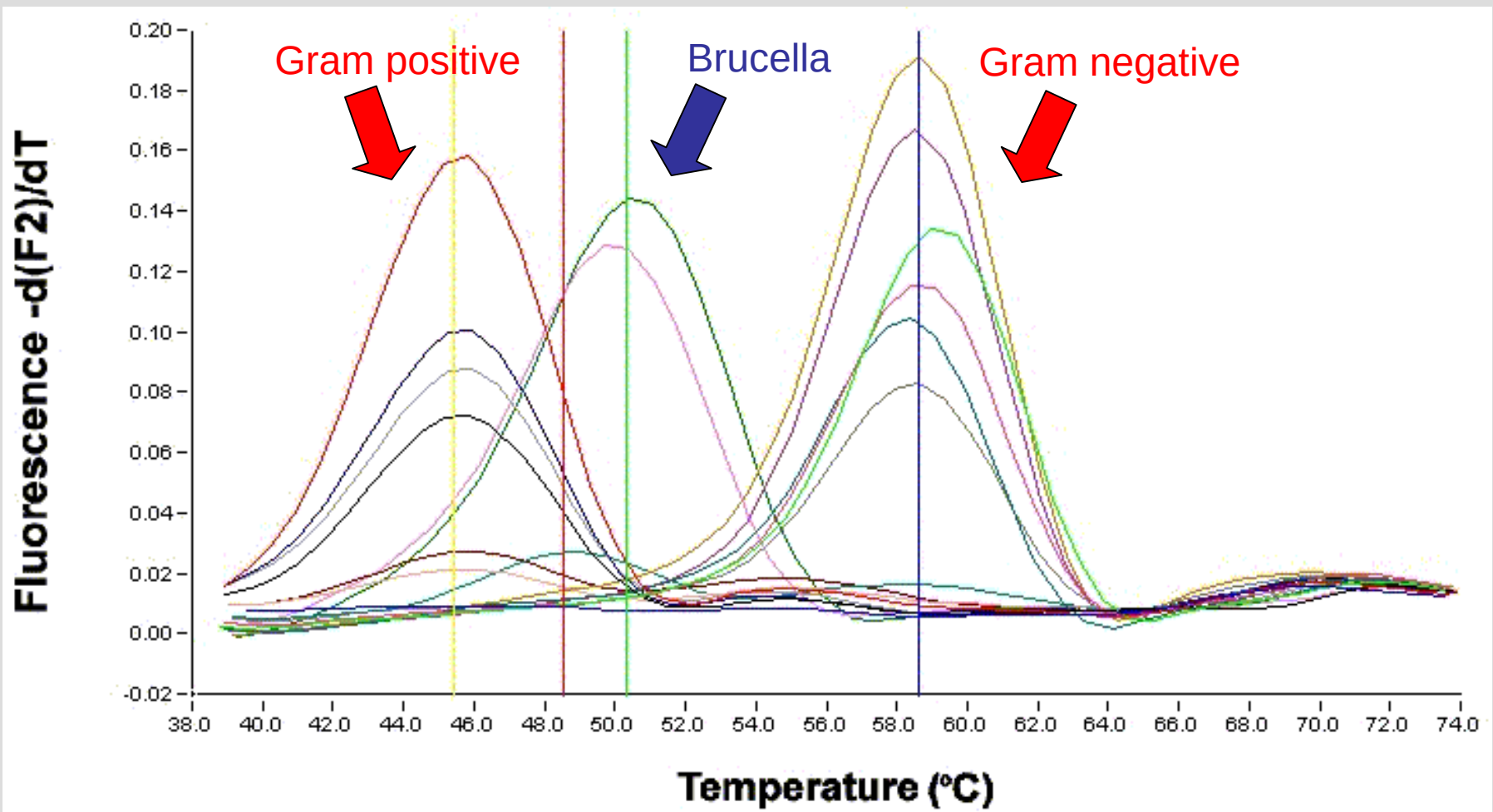


- Single Nucleotide Polymorphism (SNP)
- each mismatch destabilizes hybridisation strenght
- the melting temperature is lowered for mismatched probes
- Use a pair of long (high Tm) anchor and short sensor probe

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.

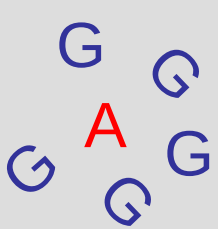


Problem : Low-abundance (geno)types

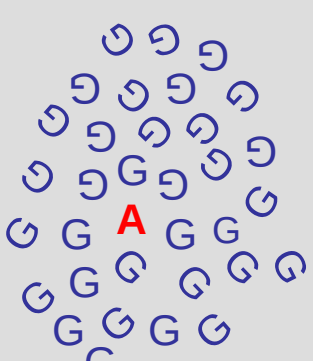
G A

G A

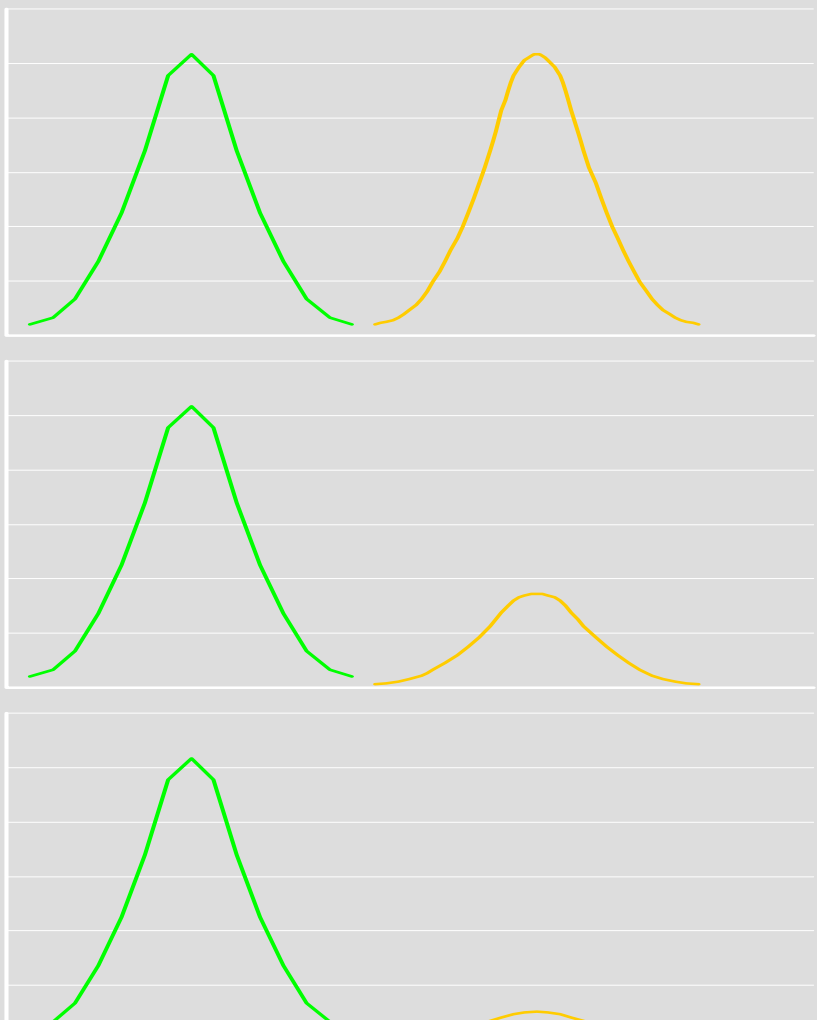
1 : 1



5 : 1



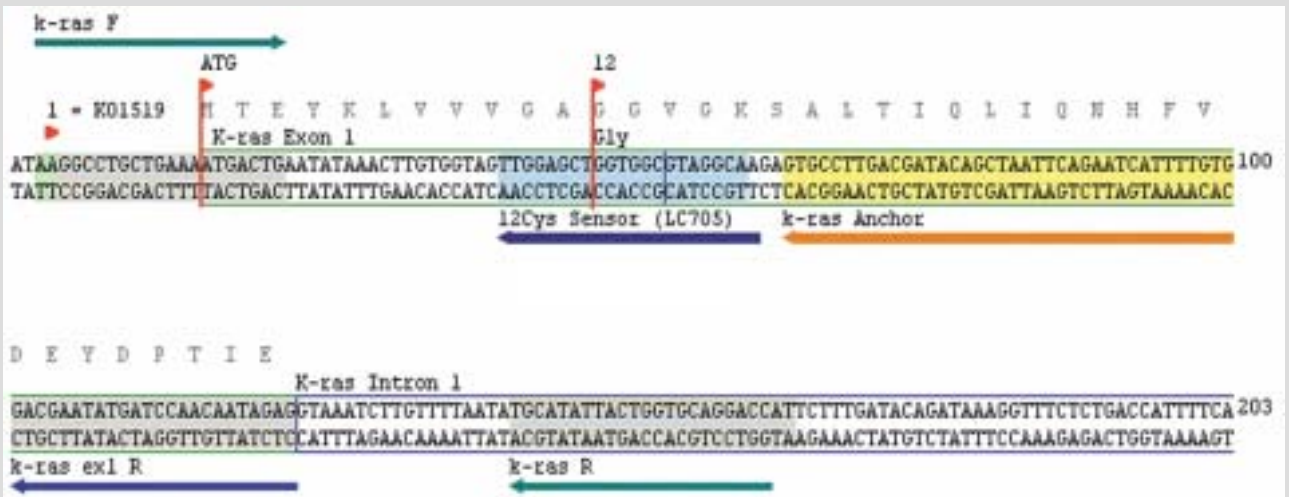
100 : 1



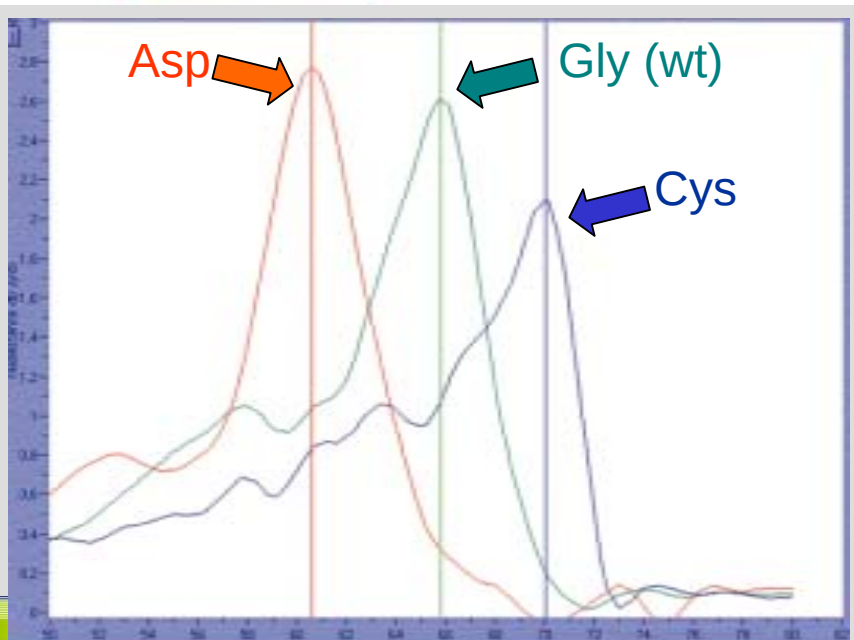


Example I : k-ras Codon Gly12

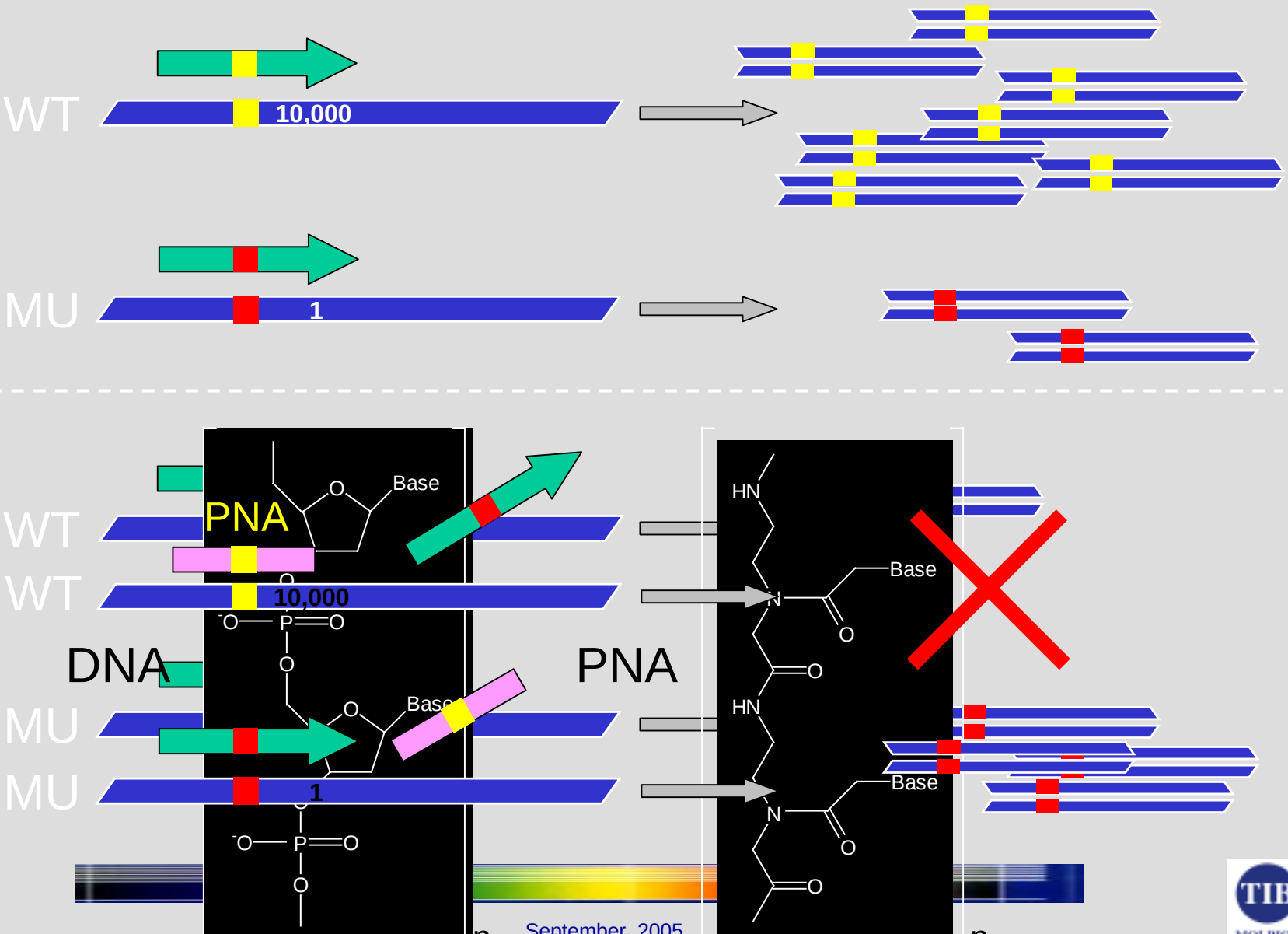
Several mutations
Ratio: 1:10,000



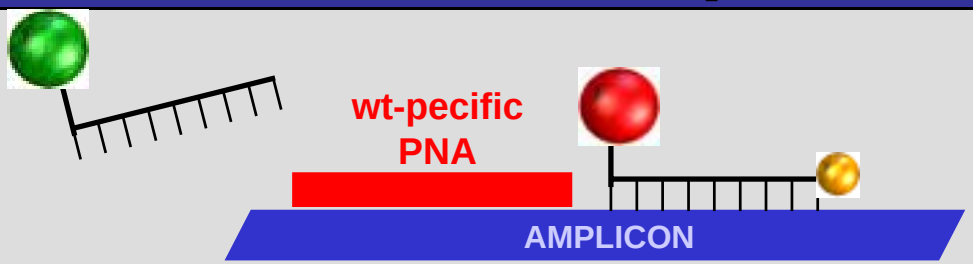
Melting on artificial targets

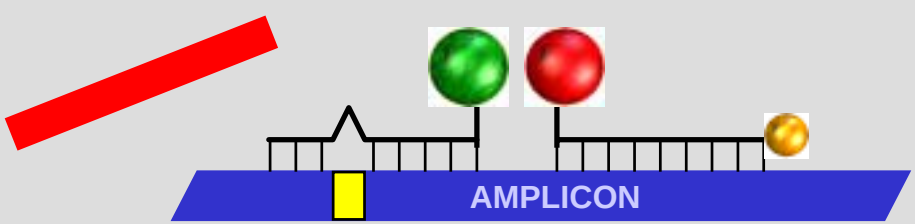


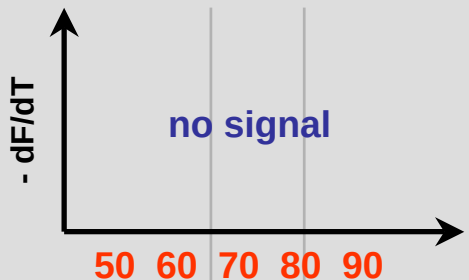
PNA/LNA-mediated PCR-clamping

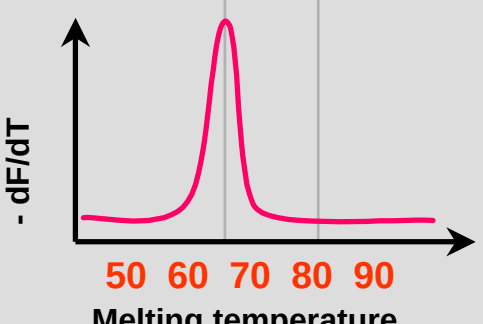


Molecular concepts: Clamped-Probe-Assay





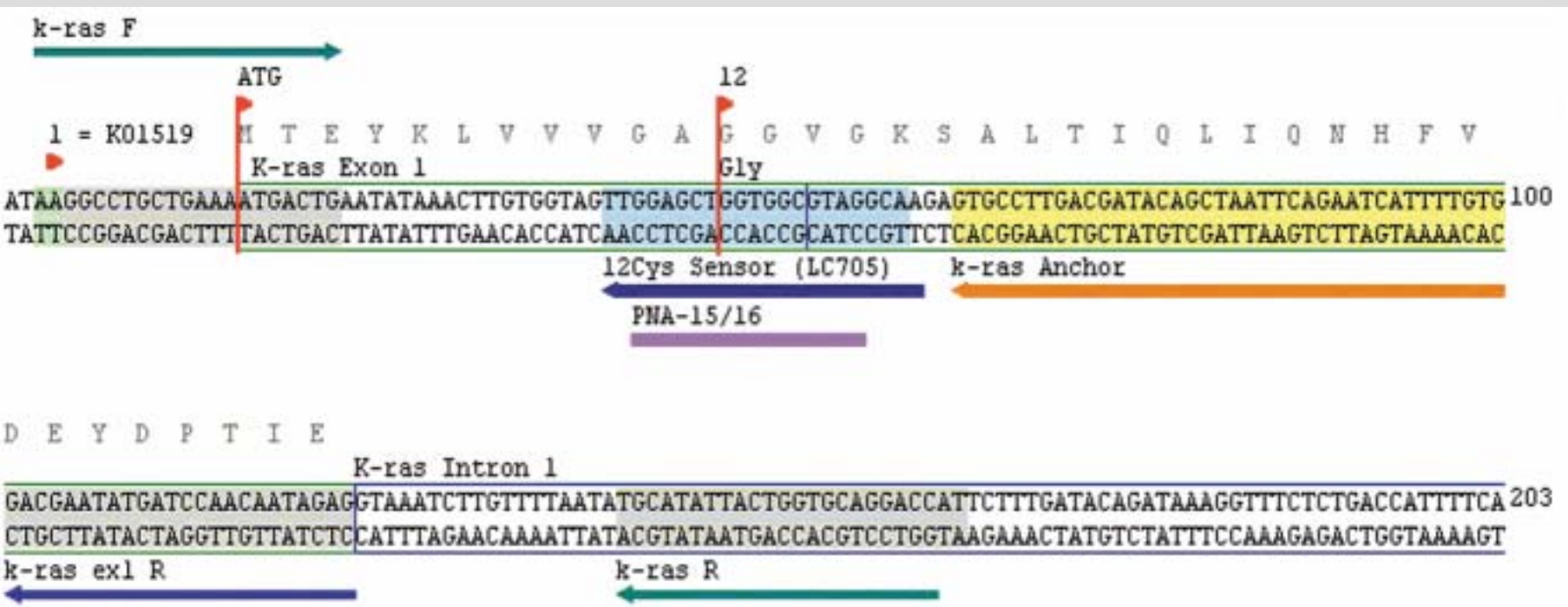




Possible applications :

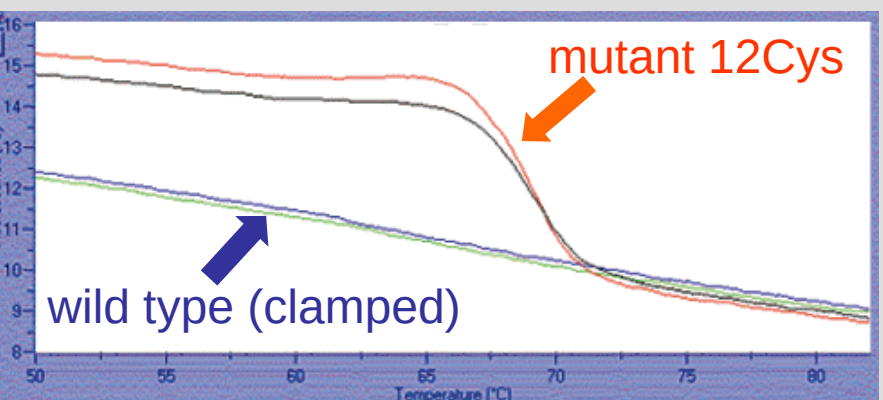
- detection of minor variants (e.g. k-ras codon 12,13)
- minimal residual diseases (MRD)
- developing resistances (STI-571 in abl exon 6 in CML, developing lamivudine resistance in HBV, ...)

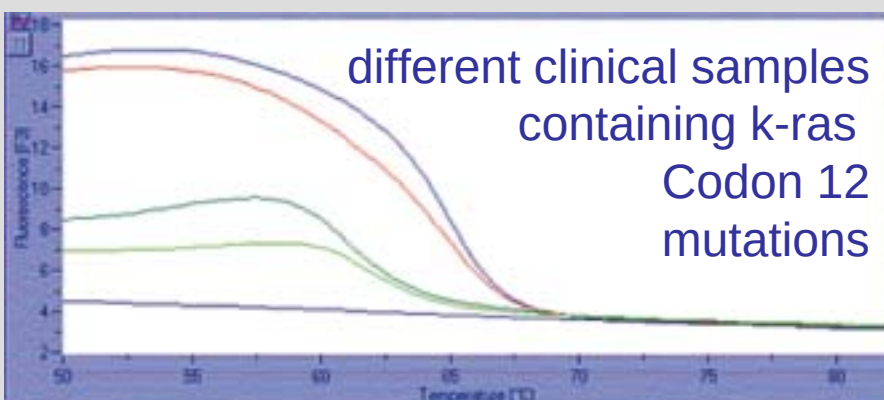
Example I : k-ras Codon 12

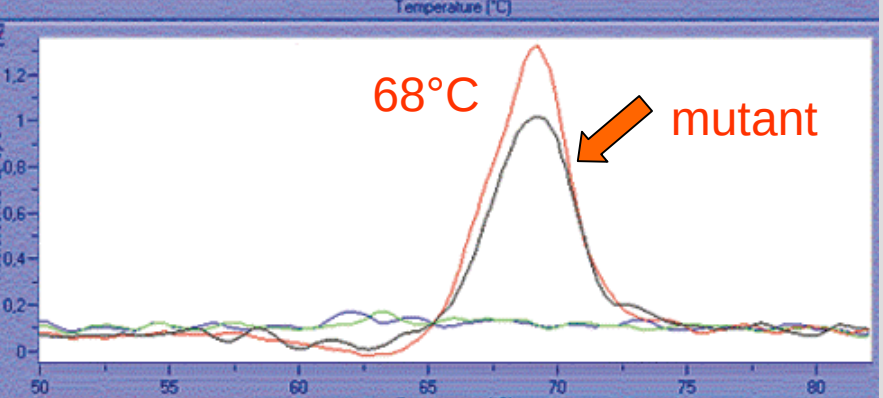


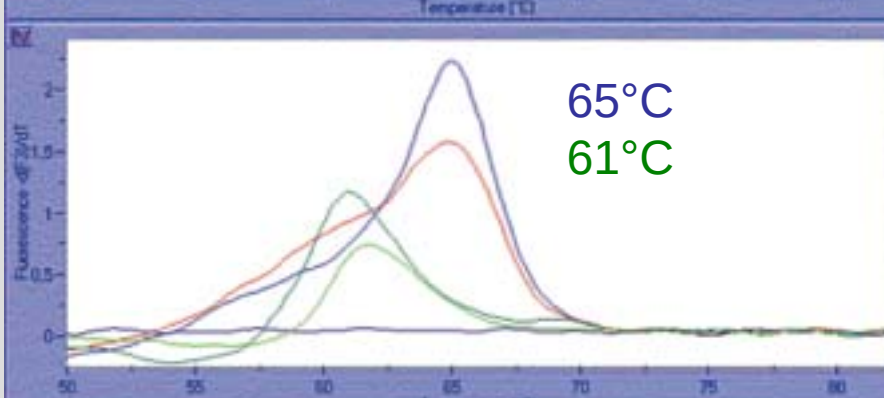
Melting curves from different patients

Melting curve (fluorescence vs. temperature)







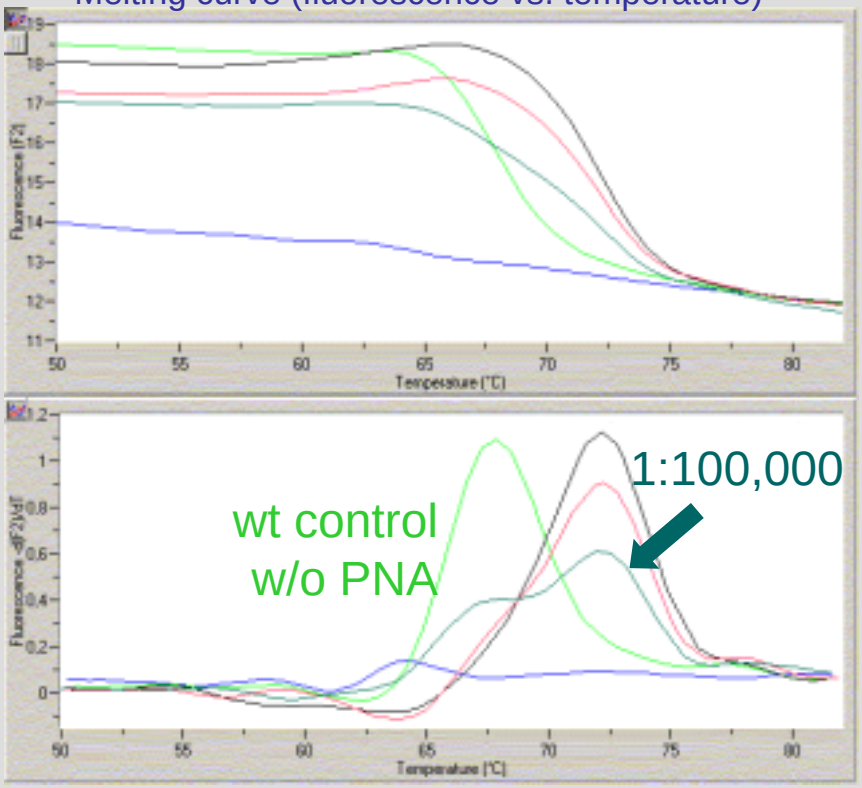


Melting curve (dFl/dT vs. temperature)



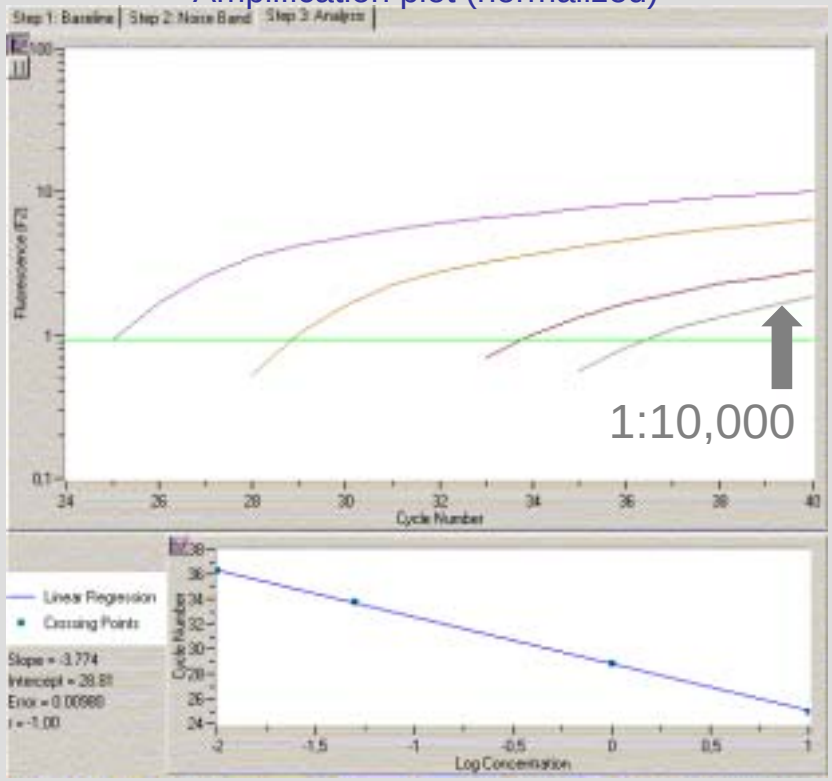
Dilution row of mutant in wt DNA

Melting curve (fluorescence vs. temperature)



Melting curve (dFl/dT vs. temperature)

Amplification plot (normalized)



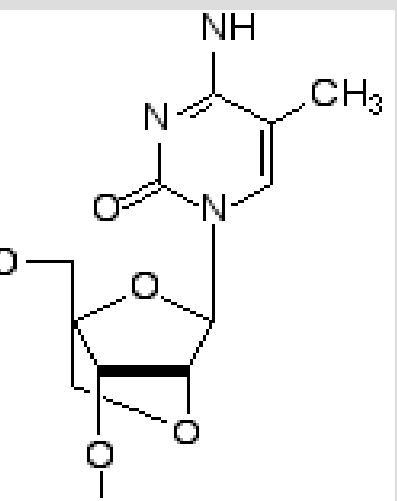
Rapid detection of K-ras mutations in bile by peptide nucleic acid-mediated PCR clamping and melting curve analysis: comparison with restriction fragment length polymorphism analysis. Chen CY, Shiesh SC, Wu SJ. Clin Chem. 2004 Mar;50(3):481-9. Epub 2004 Jan 12

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





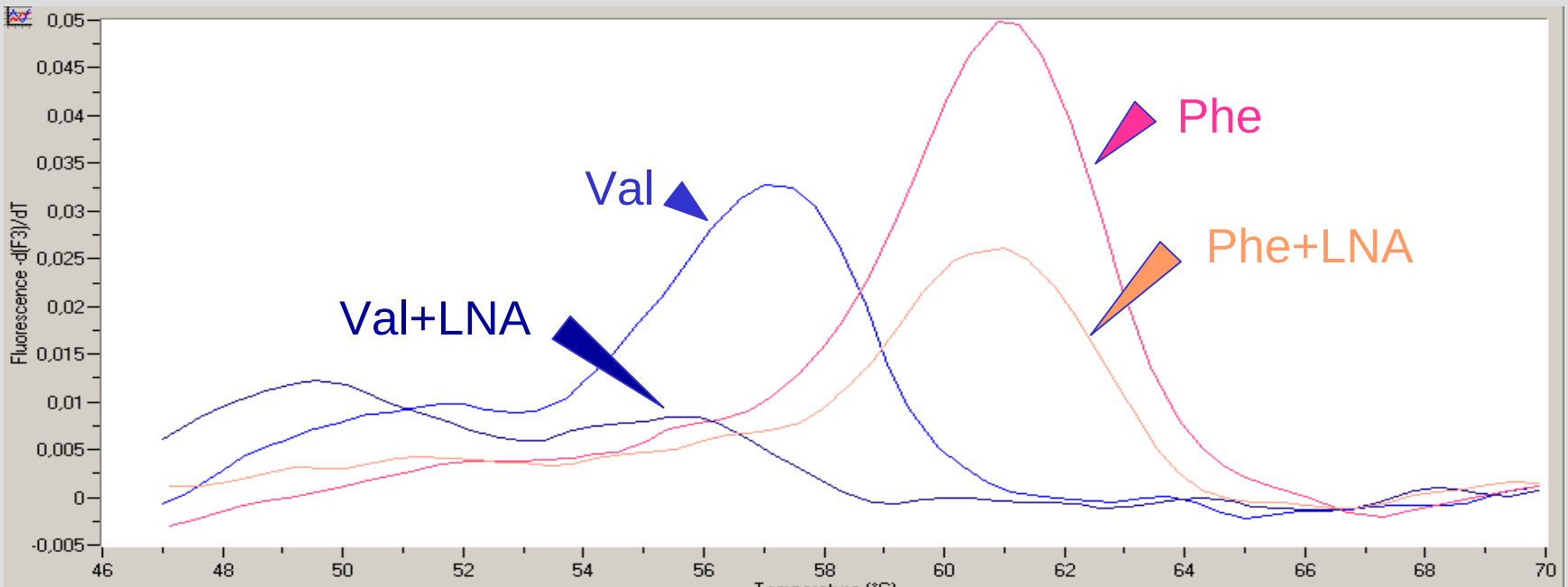
Clamping probes consisting of Locked-Nucleic Acid (LNA) work even well



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 µM§	
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 surpression significantly (working concentration is 20-fold lower).

Example II : JAK2 mRNA or gen. V617F



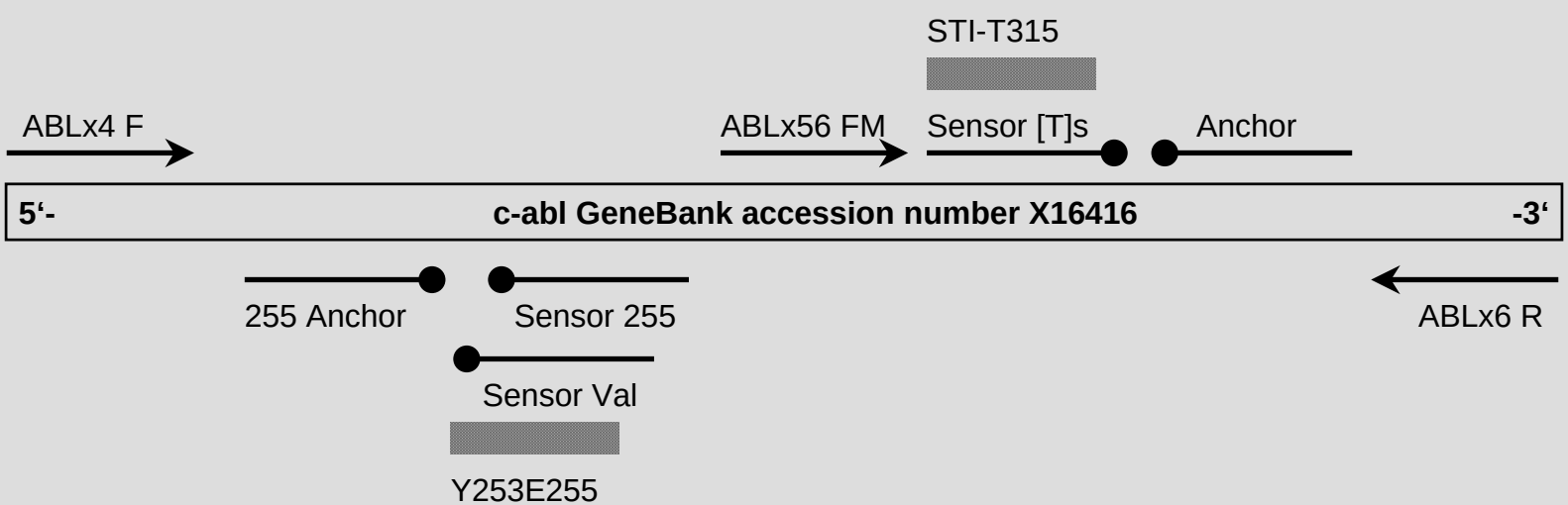




Example III : Leukemia (CML) STI resistance

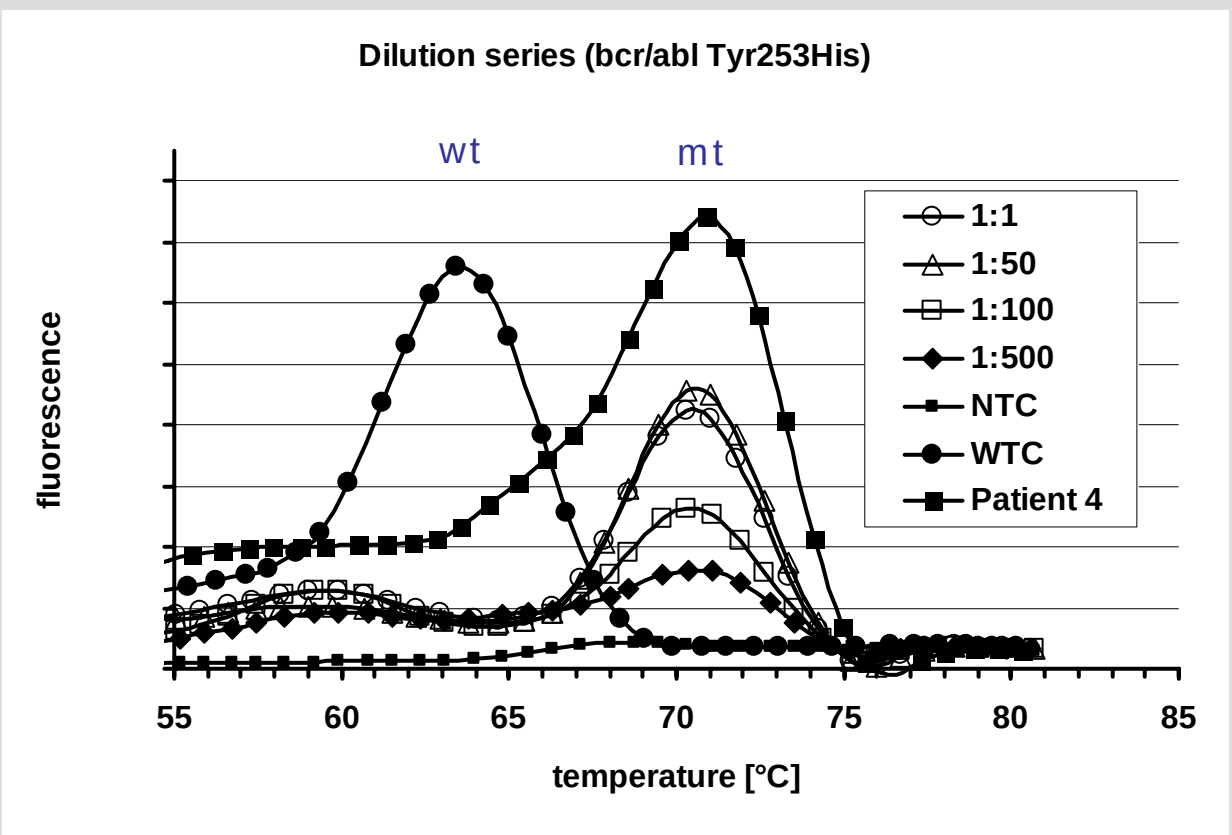
The M315T mutation in ABL exon 6 and mutations at Y253 and E255 in ABL exon 4 are responsible for the resistance of bcrABL-clones in CML.

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

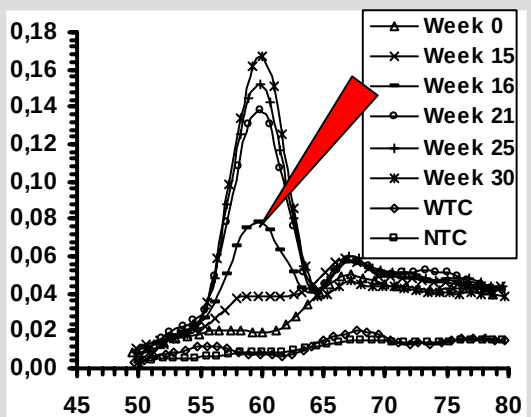


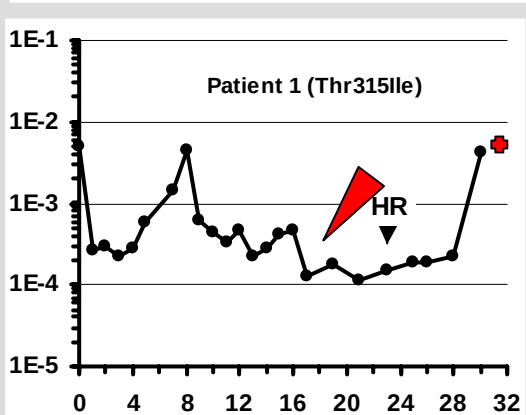
Preexistence and evolution of imatinib mesylate-resistant clones in chronic myelogenous leukemia detected by a PNA-based PCR clamping technique. Kreuzer KA, Le Coutre P, Landt O, Na IK, Schwarz M, Schultheis K, Hochhaus A, Dörken B. Ann Hematol. 2003 Apr 12

Example III : Leukemia (CML) STI resistance



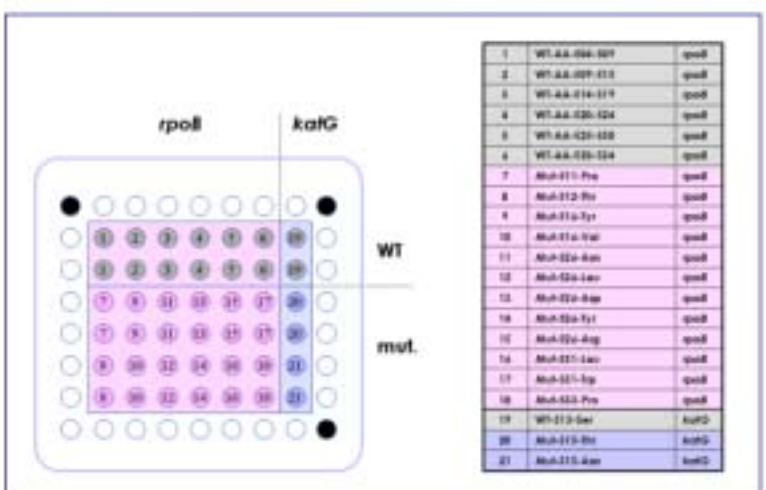
Serial dilutions of mutant in wildtype, wildtype control and patient sample.





Patient history monitored with the CPA assay.

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

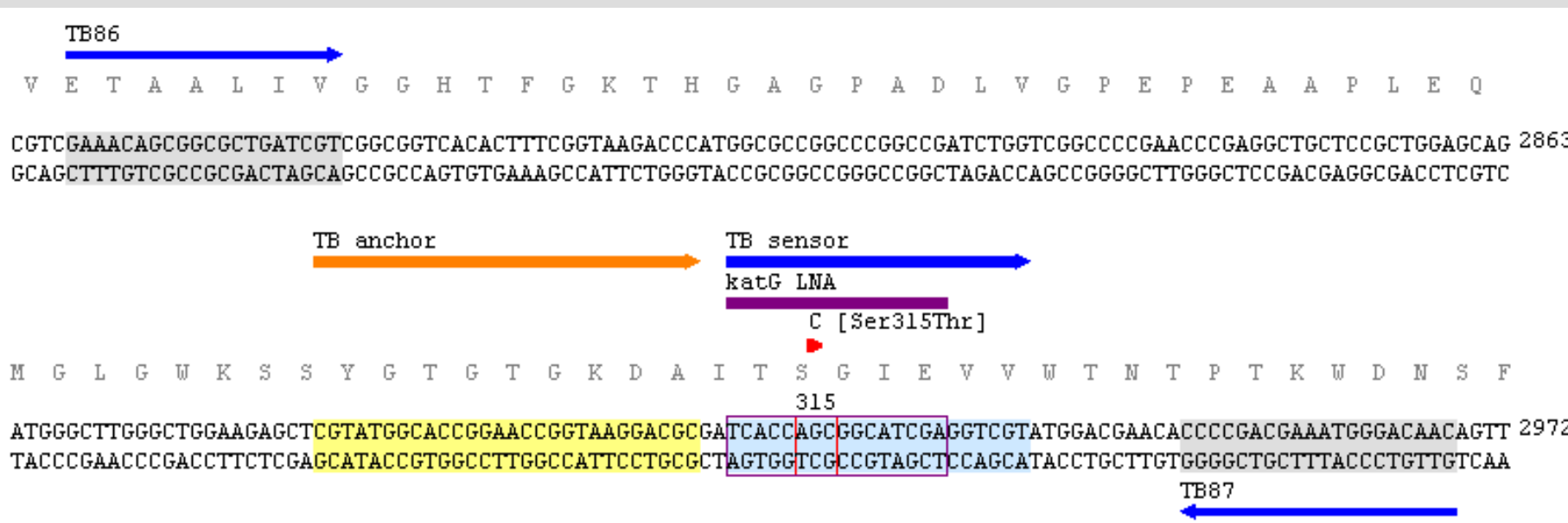


- identification of multiresistant strains (MRS) in 2.5 hrs
- *rpoB* gen: 6 wt probes (compl. 81bp region)
  12 SNPs associated with RIF ^{res}
- *katG* gen: 315-Ser wt probe
  2 SNPs associated with INH ^{res}
- additional capture probes upon request (e.g. *inhA* gen etc.)

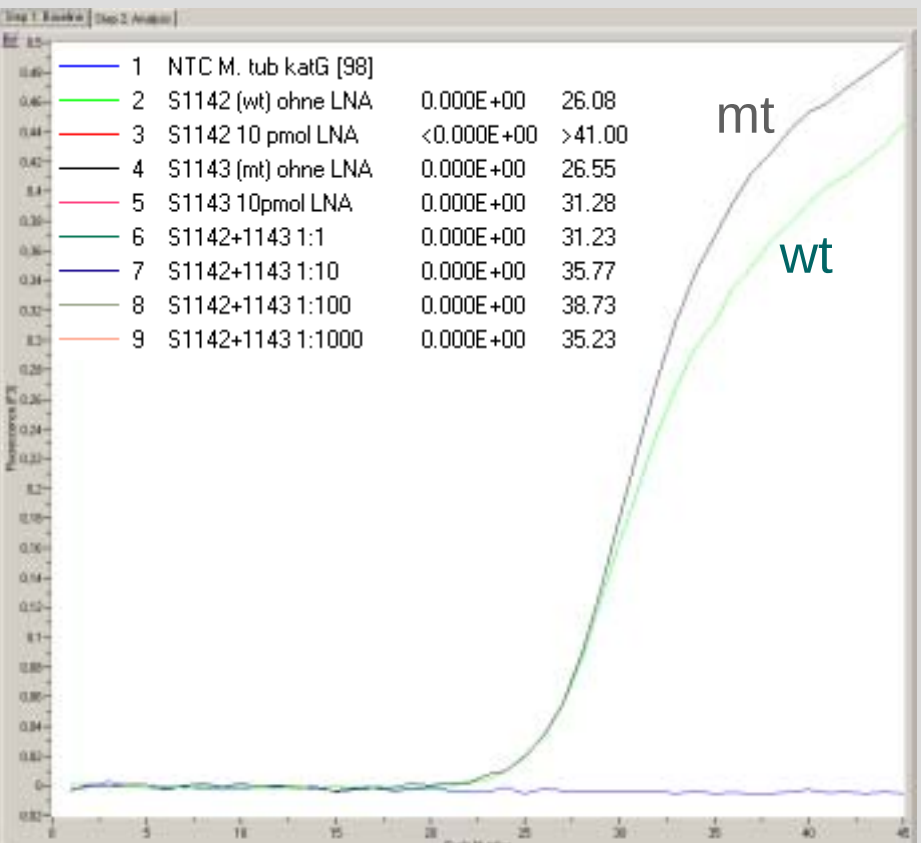


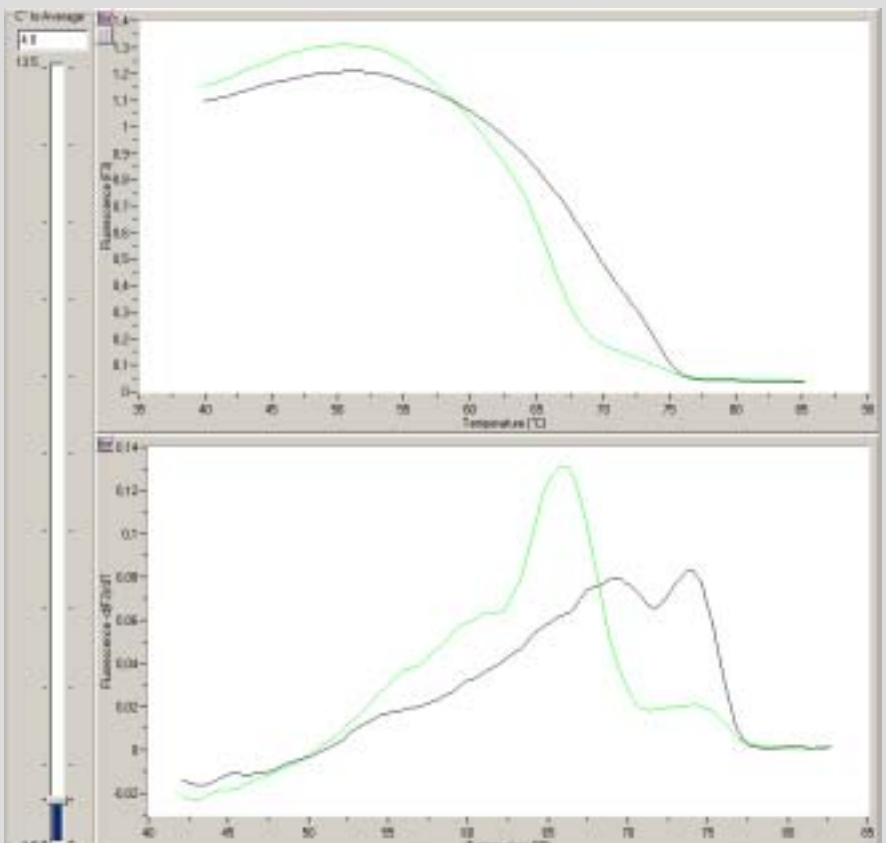
- 1 „duplex" amplification
- 45 min protocol (excl. PCR)
- optional combination with quantitative Real-Time PCR

Example IV: Mycobacteria katG –Design

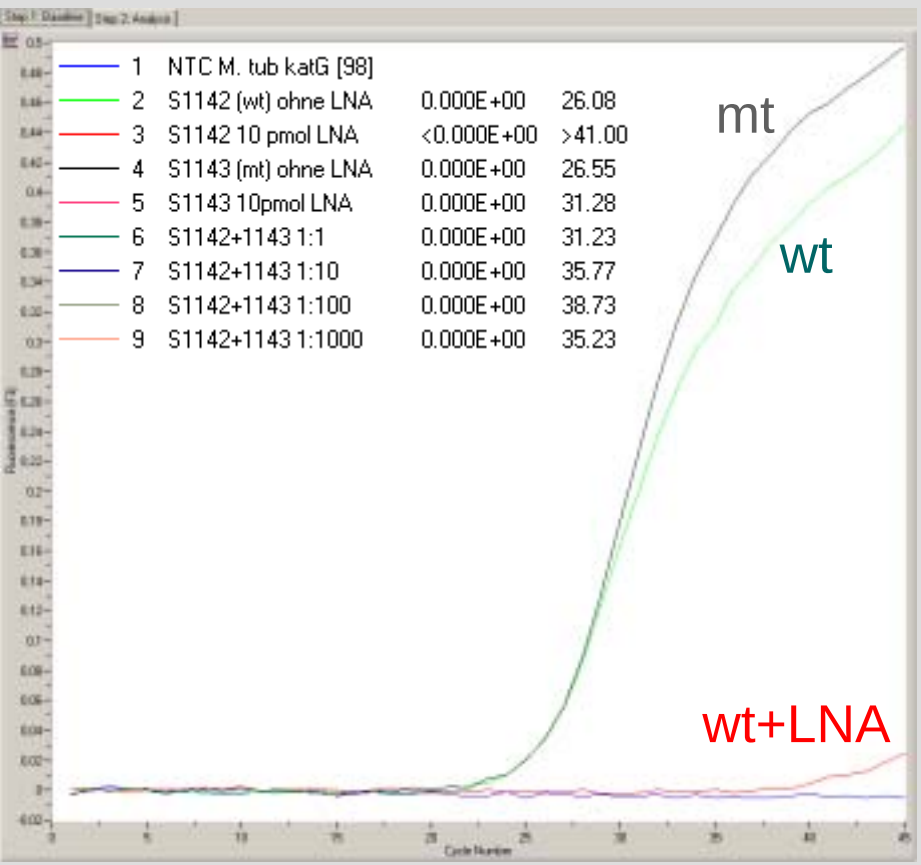


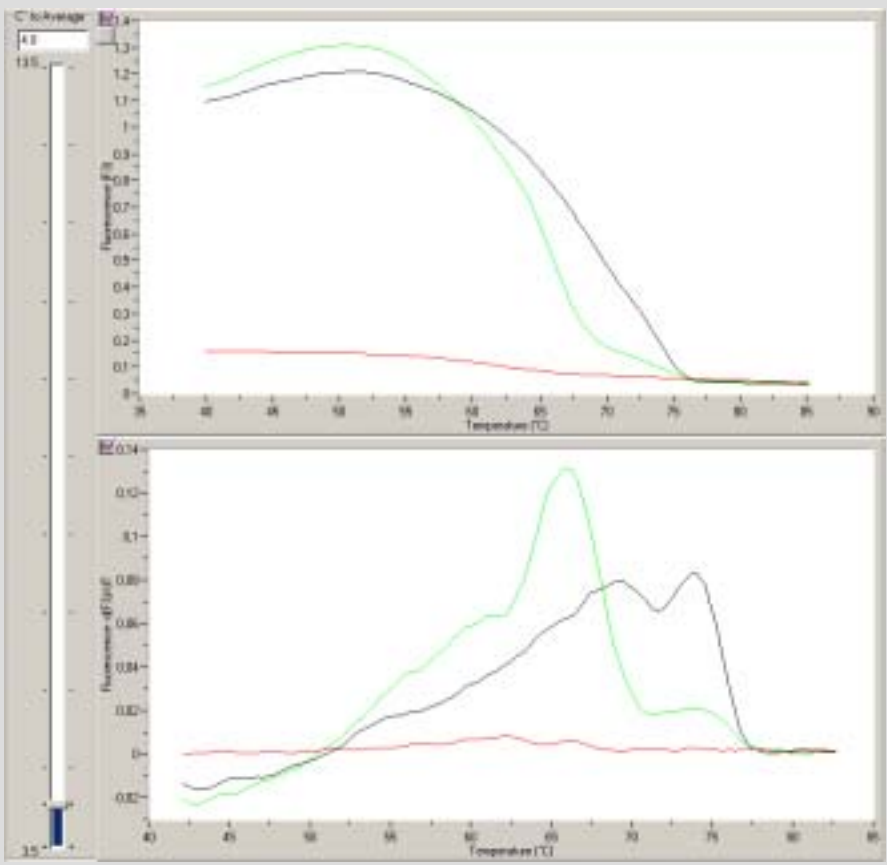
Amplification – Melting Analysis wt/mt



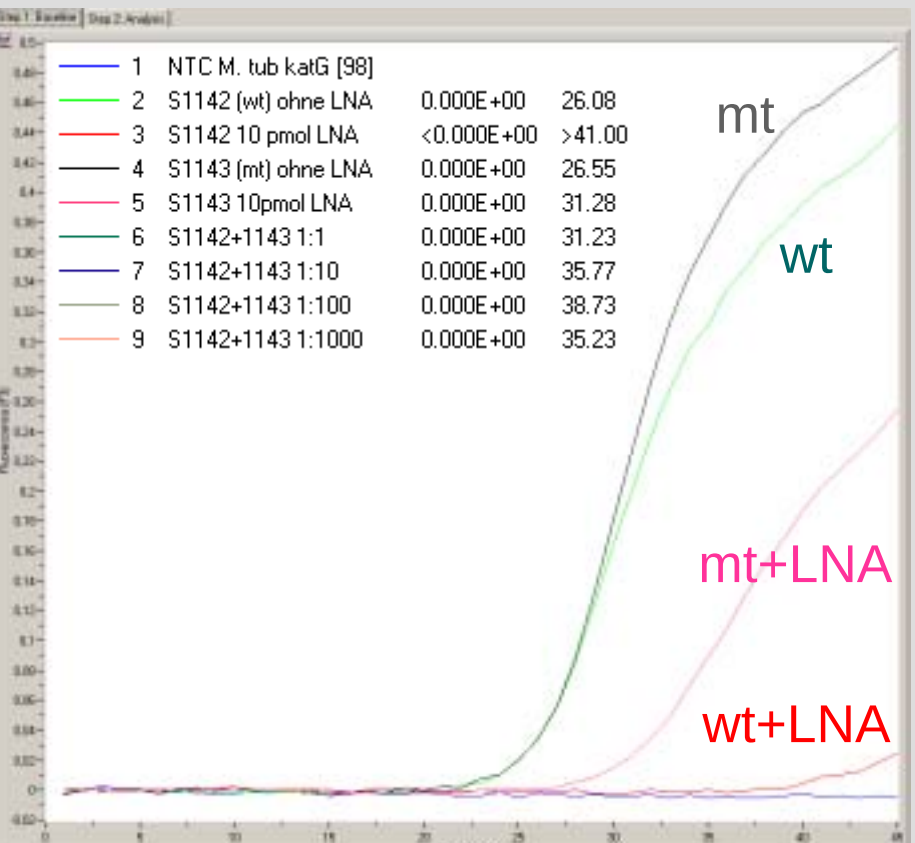


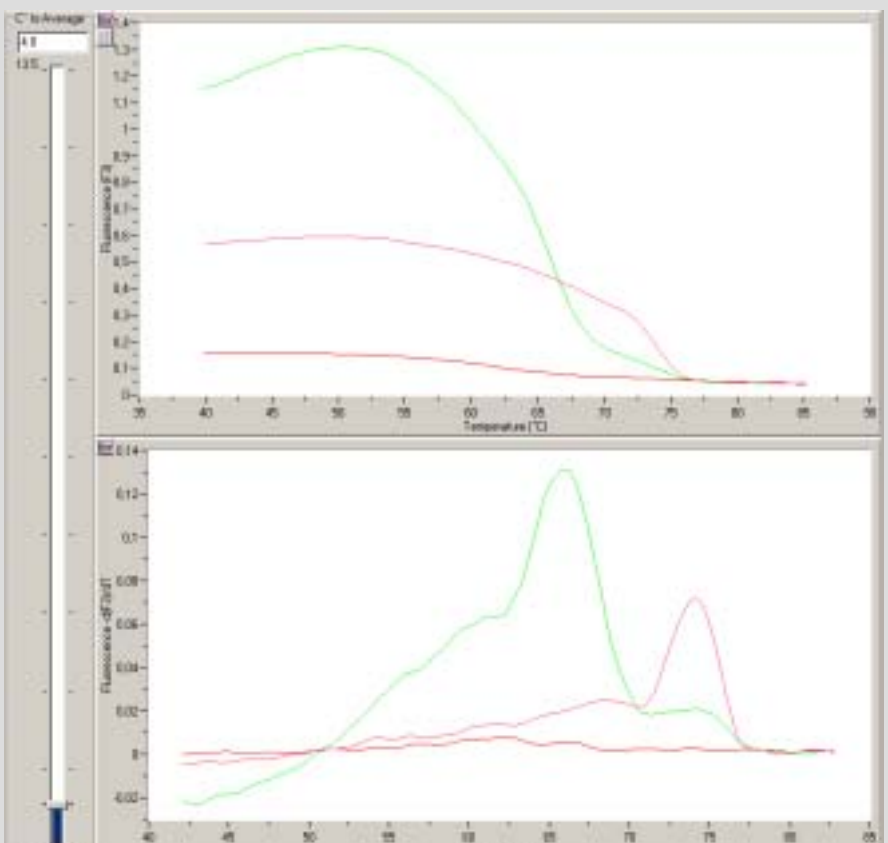
Amplification – Melting Analysis wt/mt



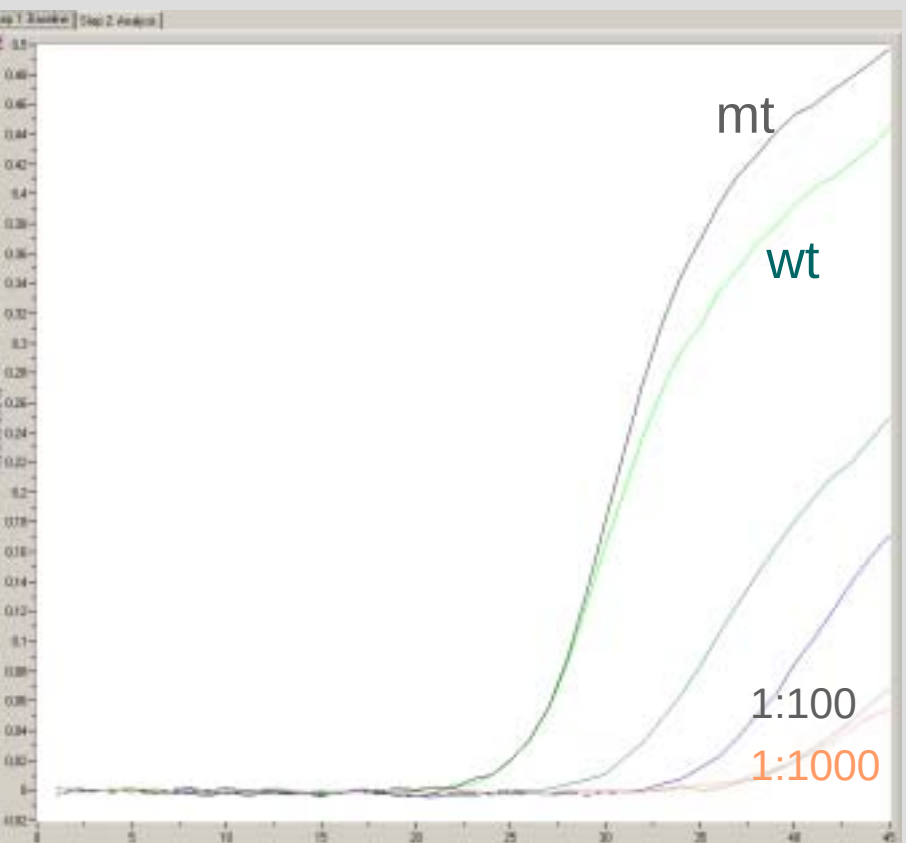


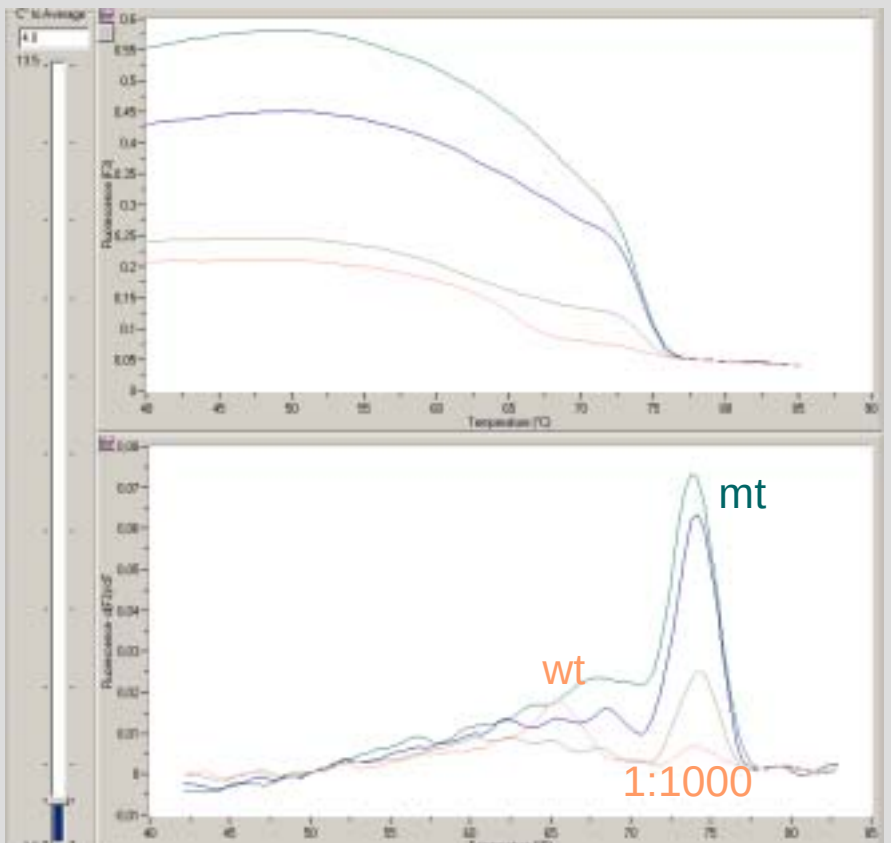
Amplification – Melting Analysis wt/mt



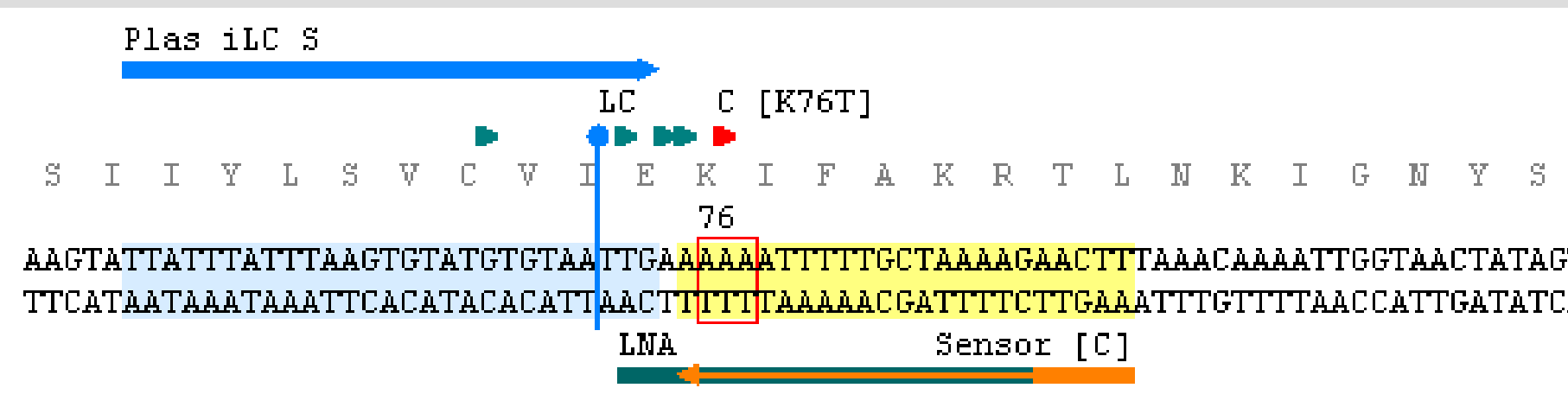


Amplification – Melting Analysis wt/mt





Expl. V: Plasmodium chloroquine resistance



Use of a locked-nucleic-acid oligomer in the clamped-probe assay for detection of a minority Pfcrt 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.

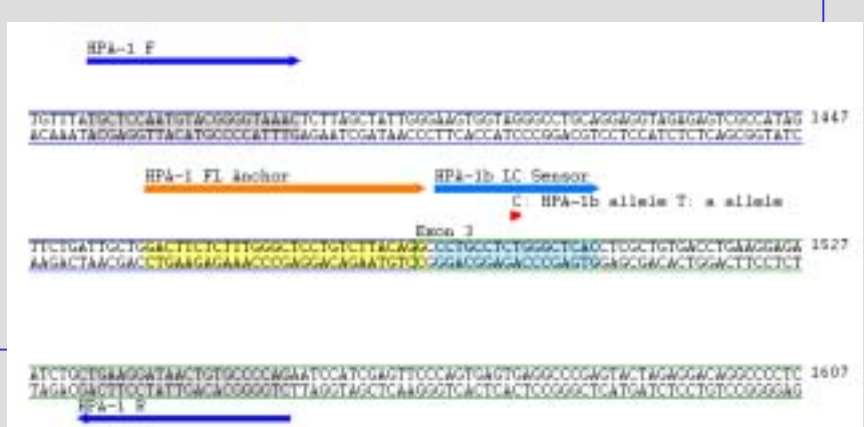
Example VI – current projects – HPA-1

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)



TIB MOLBIOL



Locked Nucleic Acid

opens new perspective