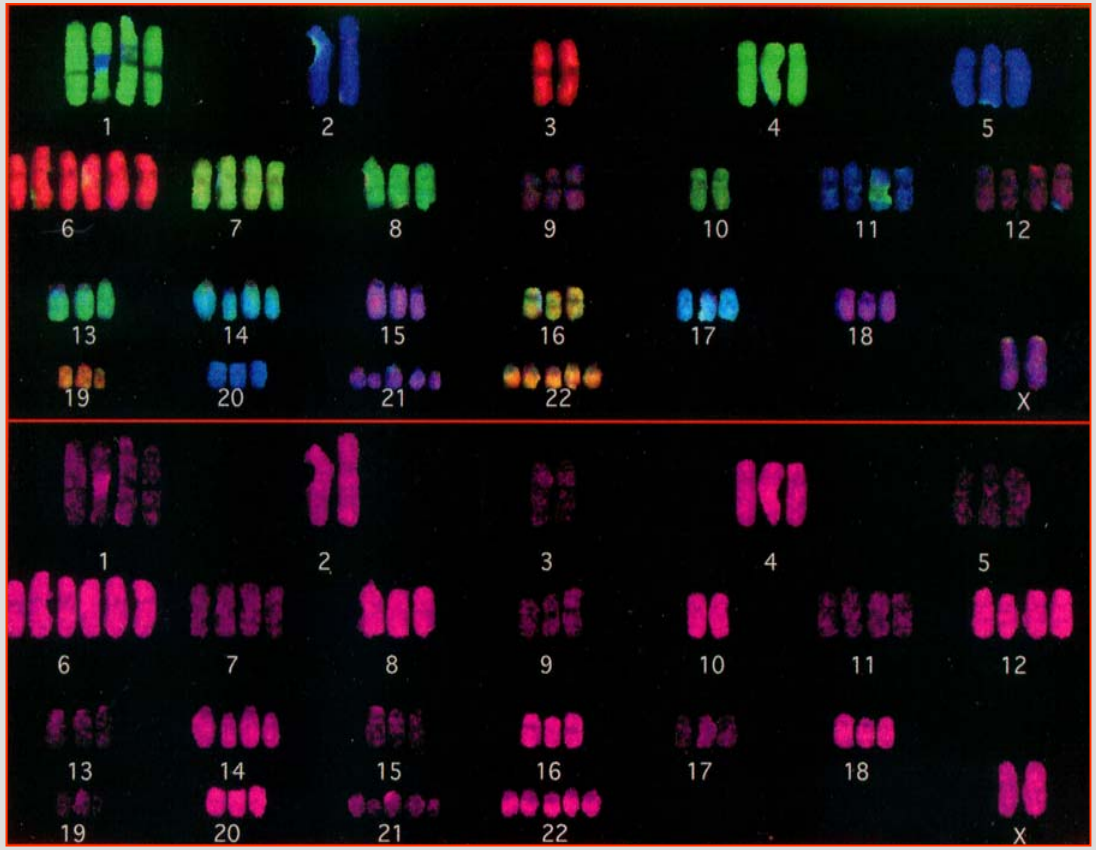
The Clamped-Probe-Assay : Detection & quantification of minor/minimal variants.

Olfert Landt qPCR symposium march 2005, Leipzig

qPCR means Real-Time PCR
Real-Time PCR is based on Fluorescence



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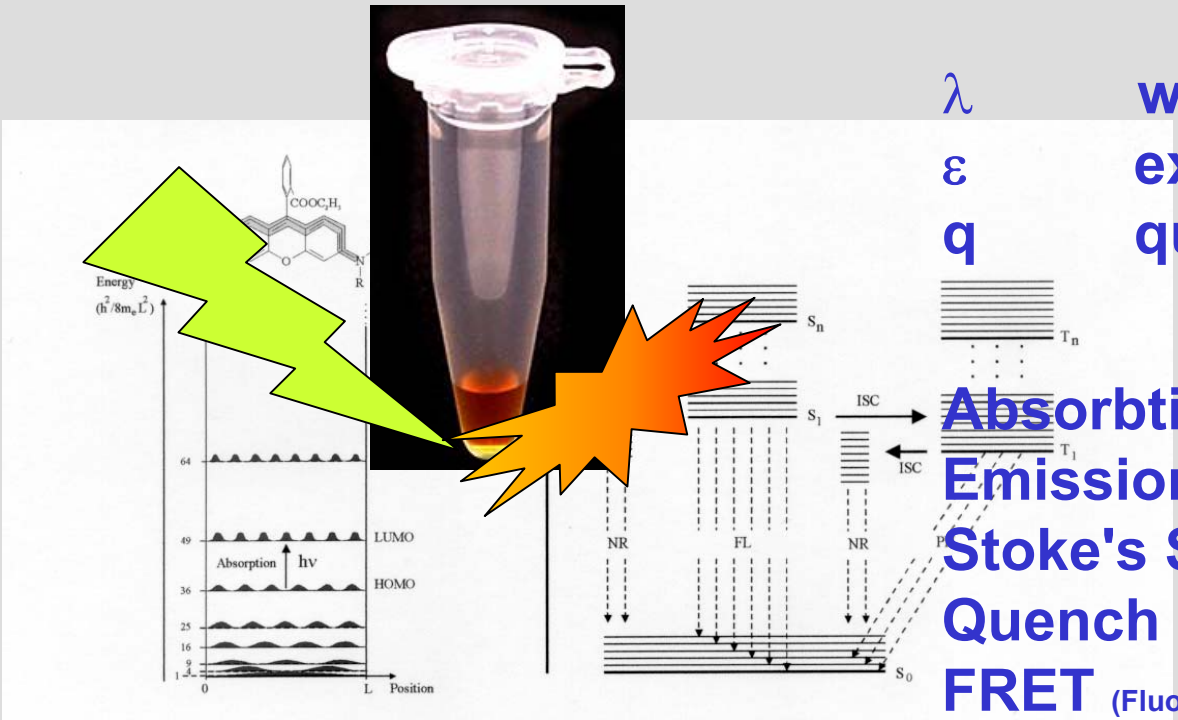
Real-Time PCR is based on Fluorescence



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

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

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

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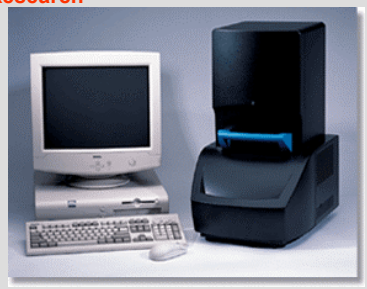
Real-Time-PCR Instruments

LightTyper
Roche Diagnostics

SmartCycler
Cepheid

SDS7700
Applied Biosystems

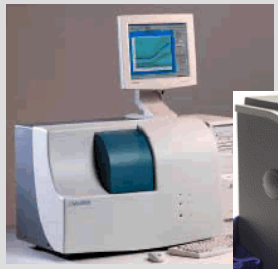
Opticon
MJ Research



iCycler
BioRad

SDS7000
Applied Biosystems

LightCycler
Roche Diagnostics



7900HT
Applied Biosystems

RotorGene
Corbett Research

Mx3000/4000
Stratagene

Superconvector
AlphaHelix

March 2005

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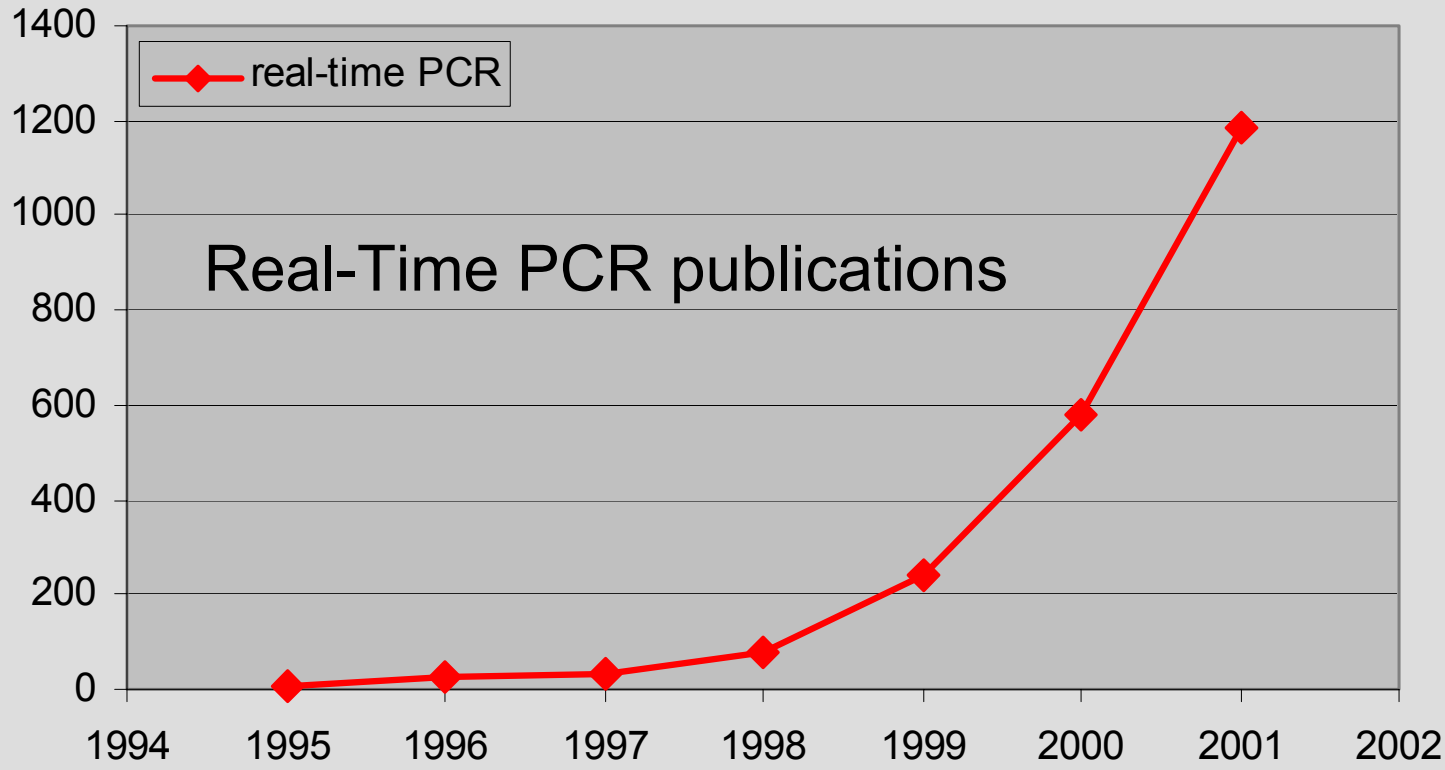
quantitative PCR itself is not very exciting



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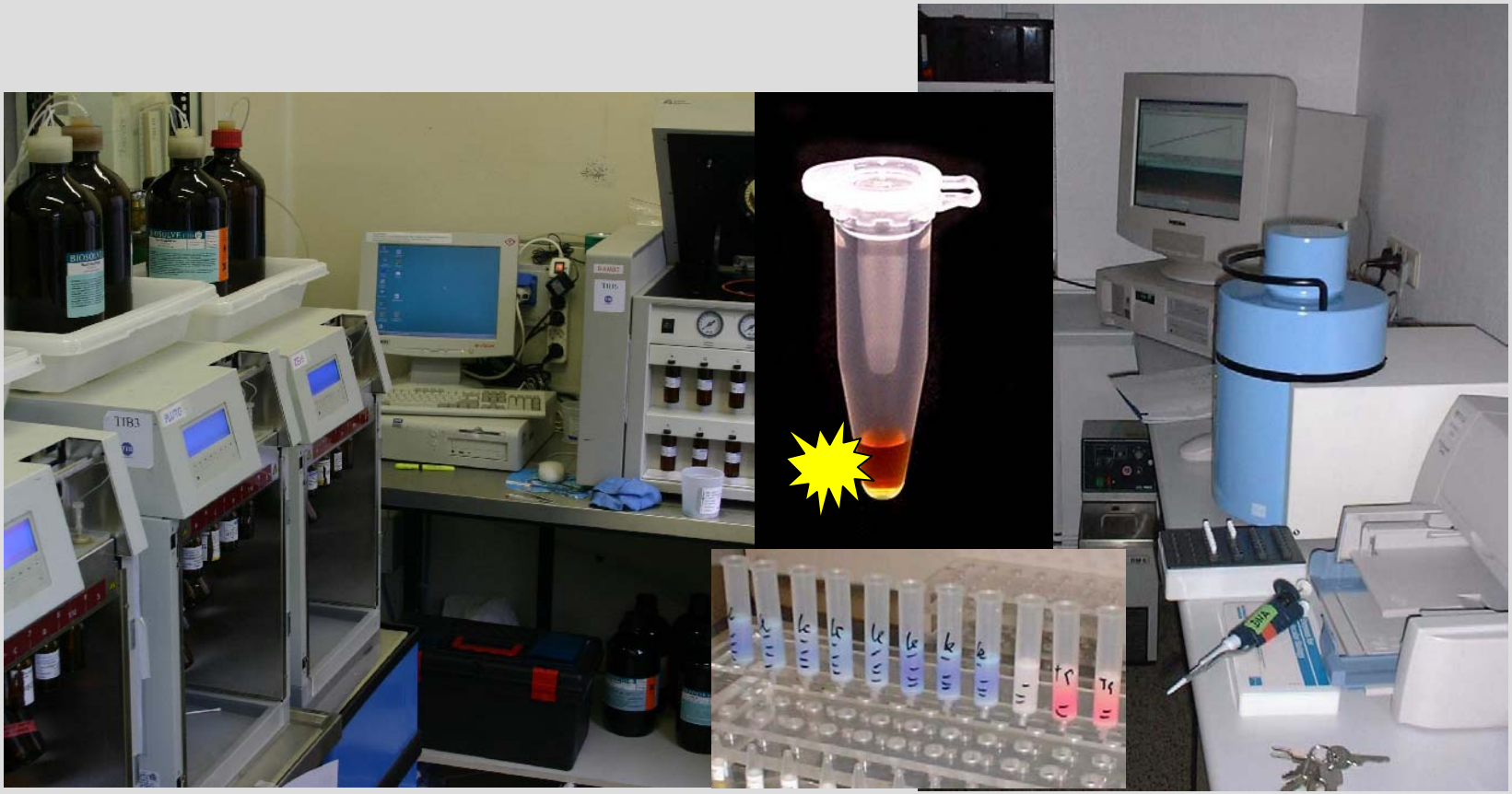
As a producer of fluorescent probes we are more interested in new (PCR) technologies



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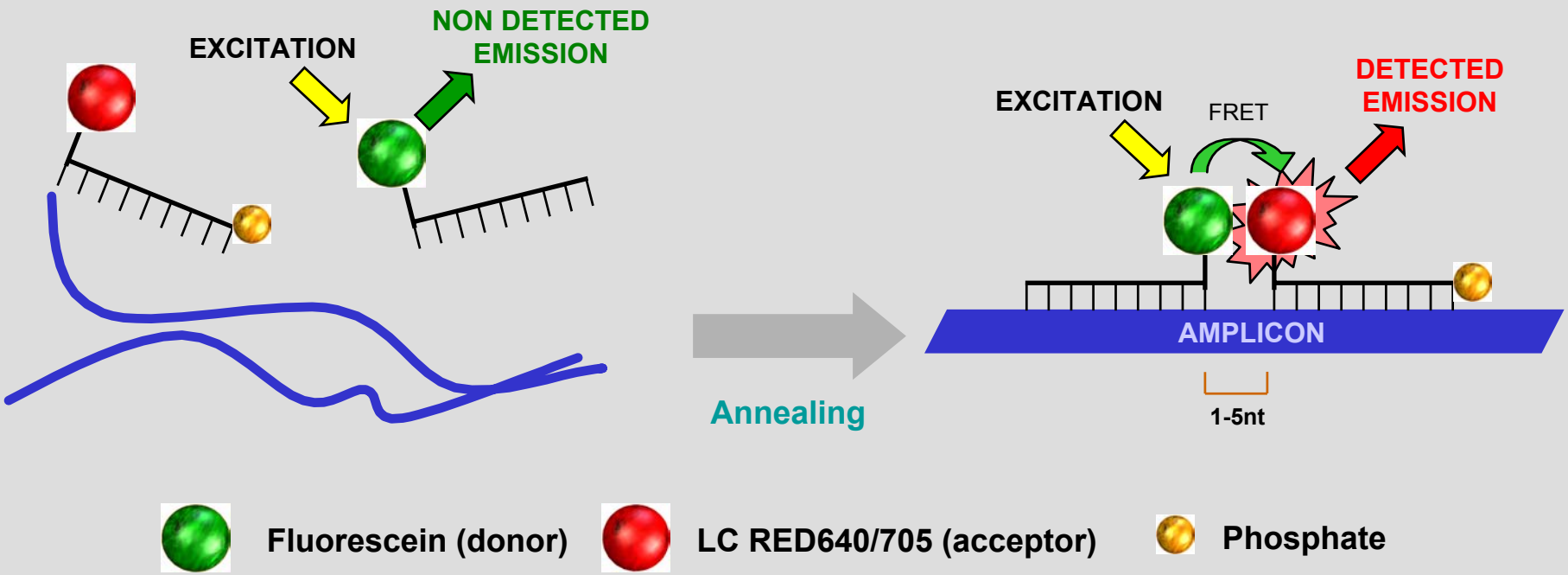
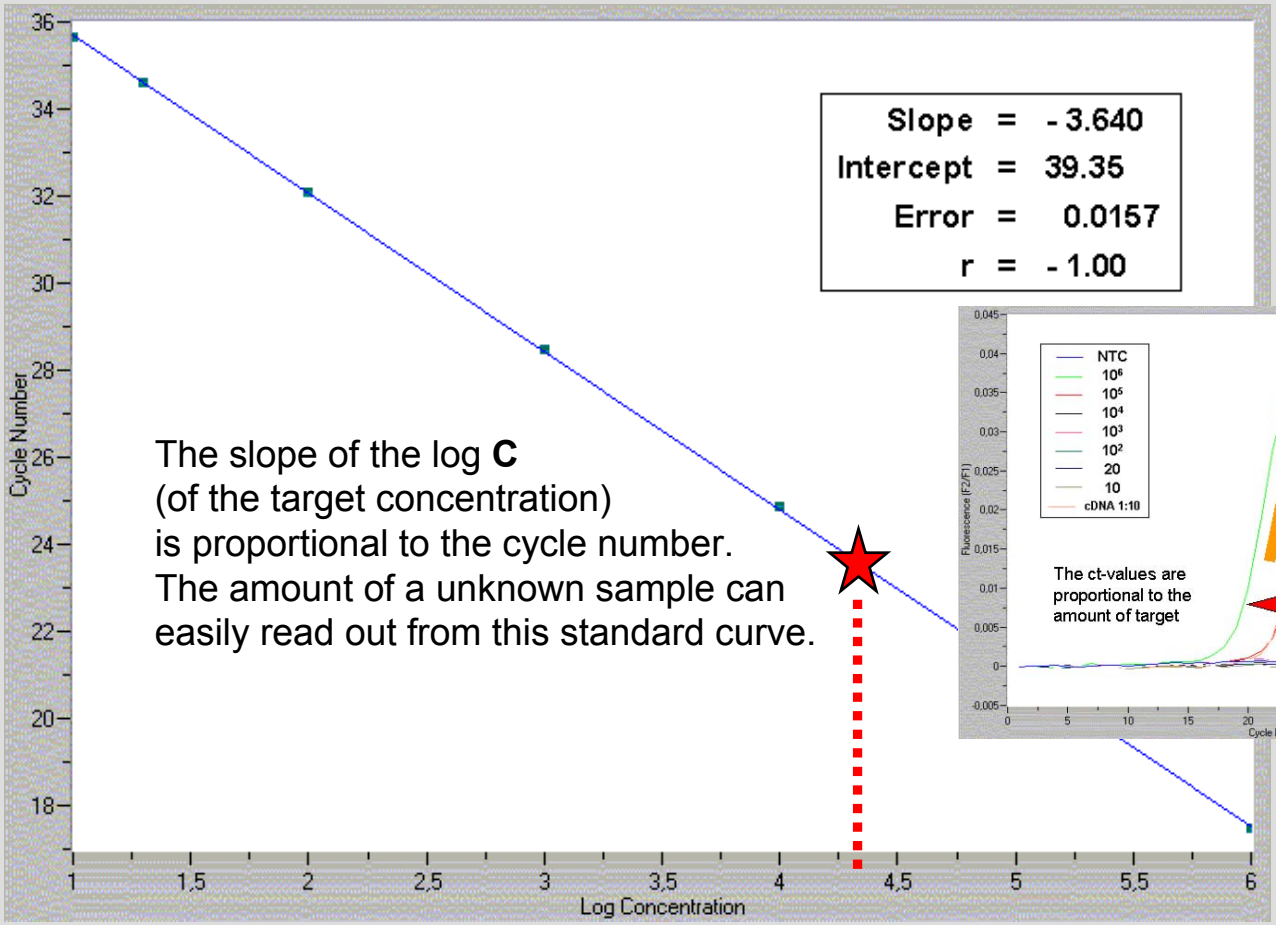
Molecular Concepts: Hybridization Probes

Principle: adjacent hybridisation and FRET

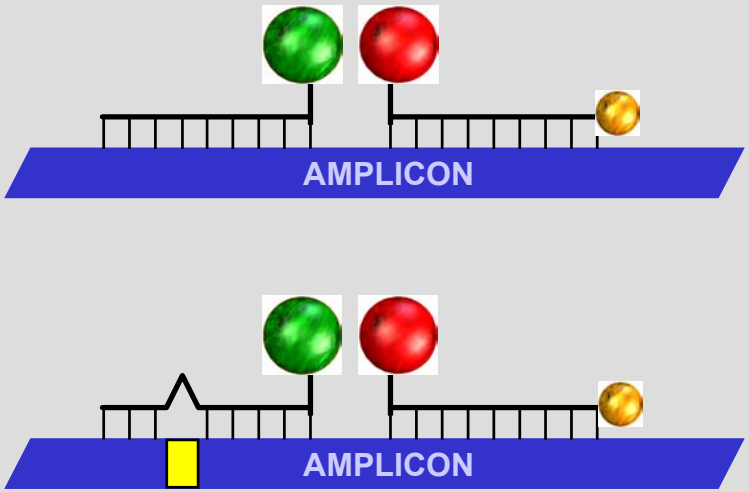


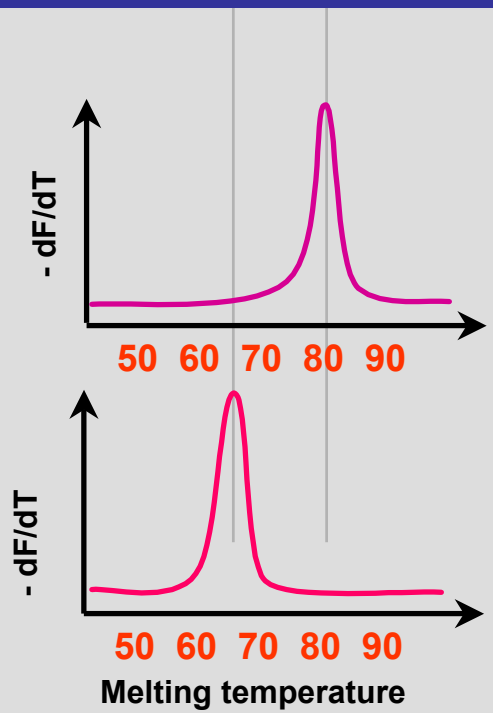
How quantification works

The probes monitor the actual amount of PCR-product



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

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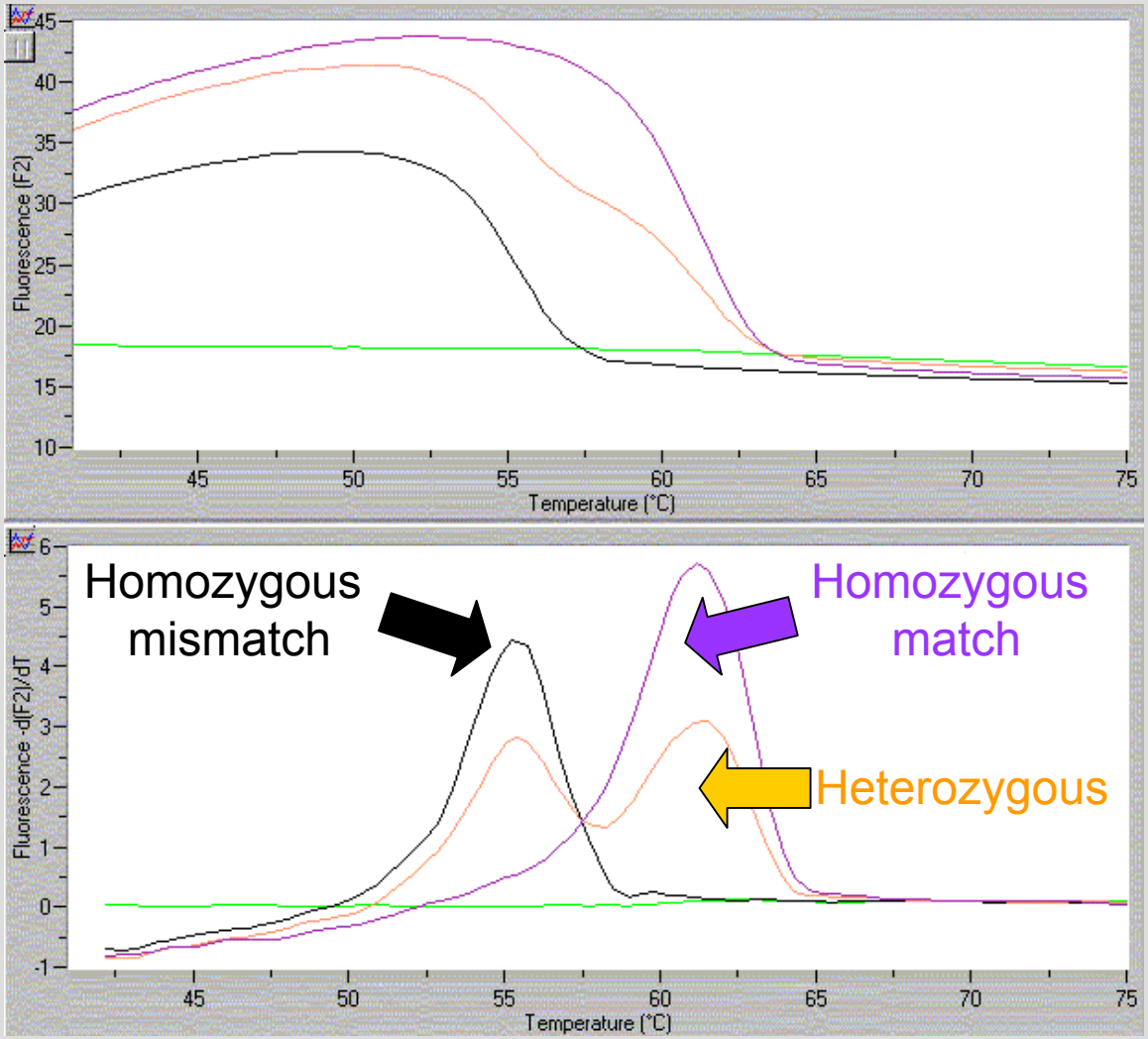
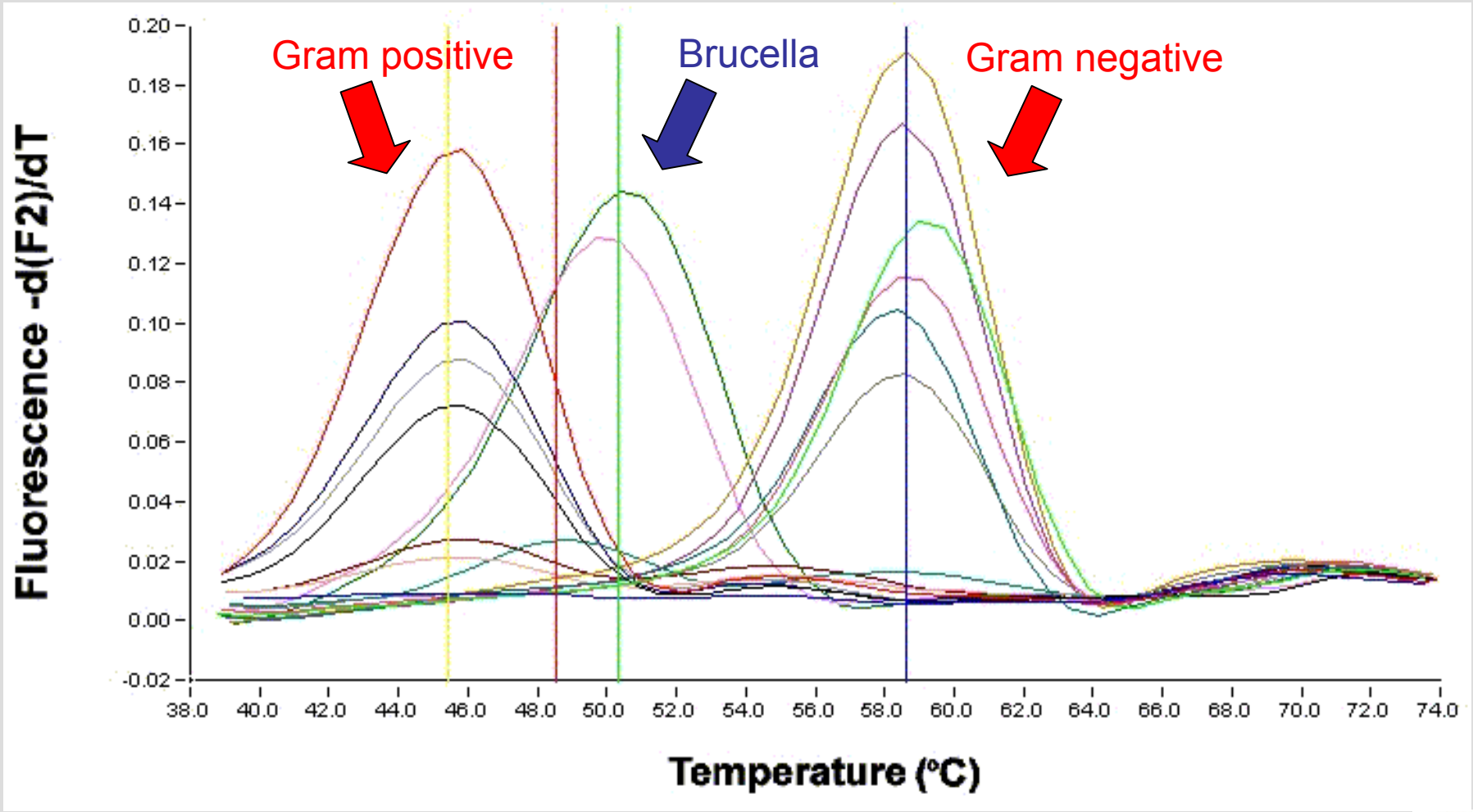
Example : hemachromatosis (HFE)



Example : Identification of bacteria



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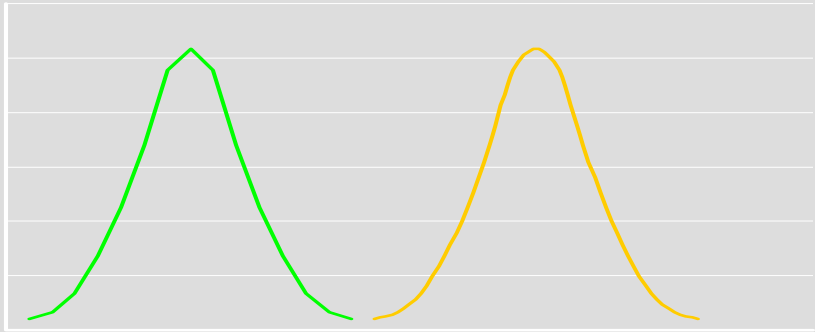
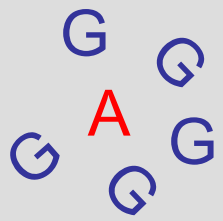
Problem : Low-abundance (geno)types

G A

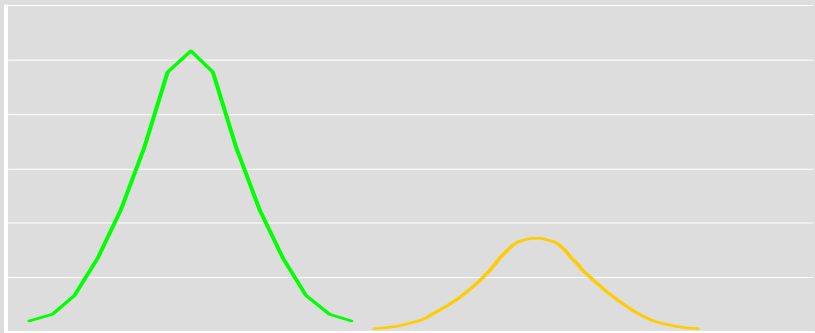
G A

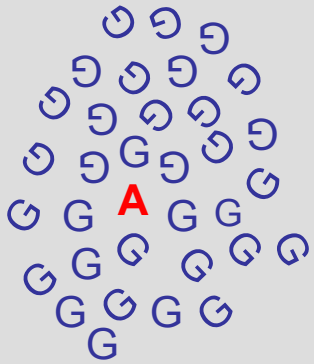
1 : 1



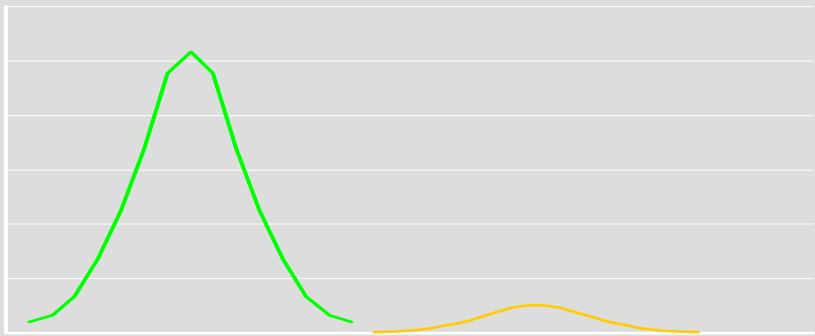


5 : 1





100 : 1



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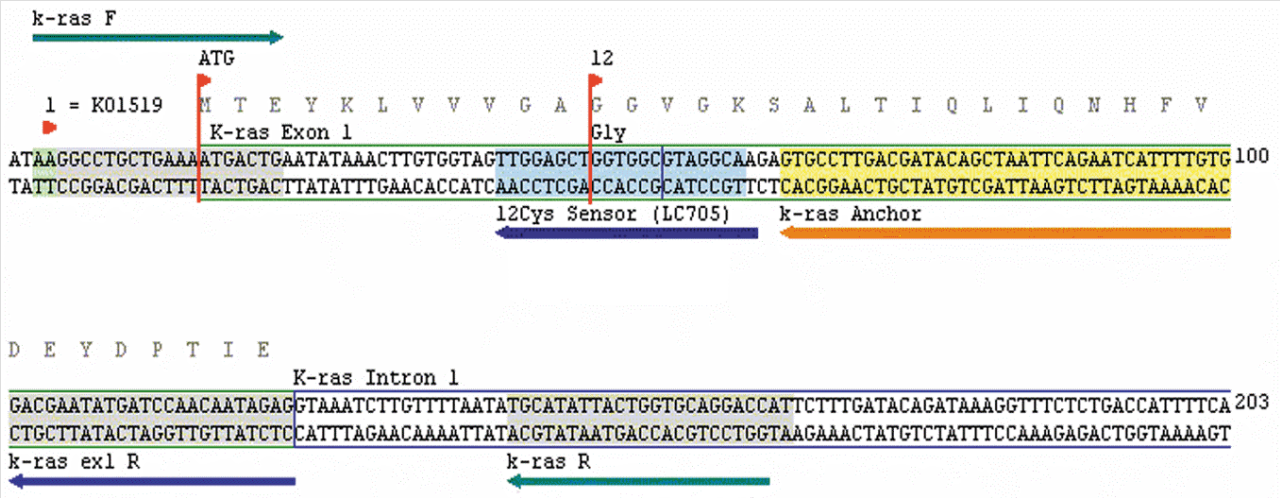
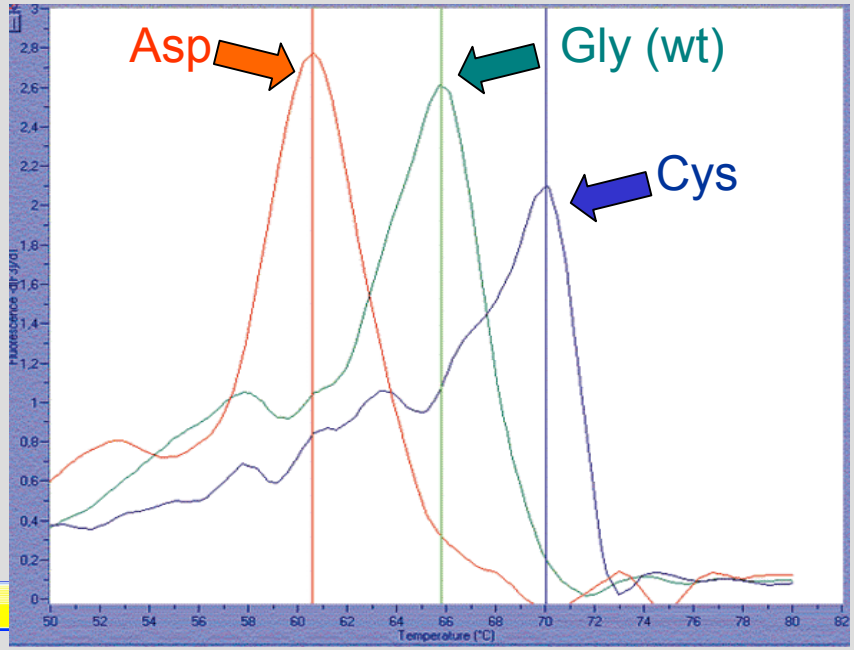
Example : k-ras Codon 12

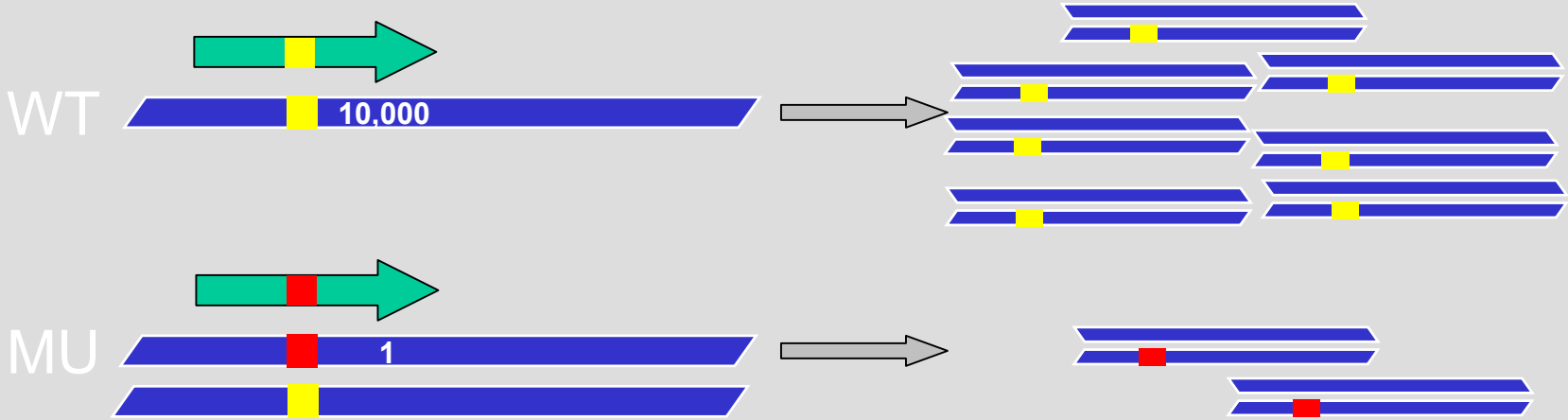
- Several mutations
- Ratio: 1:10,000

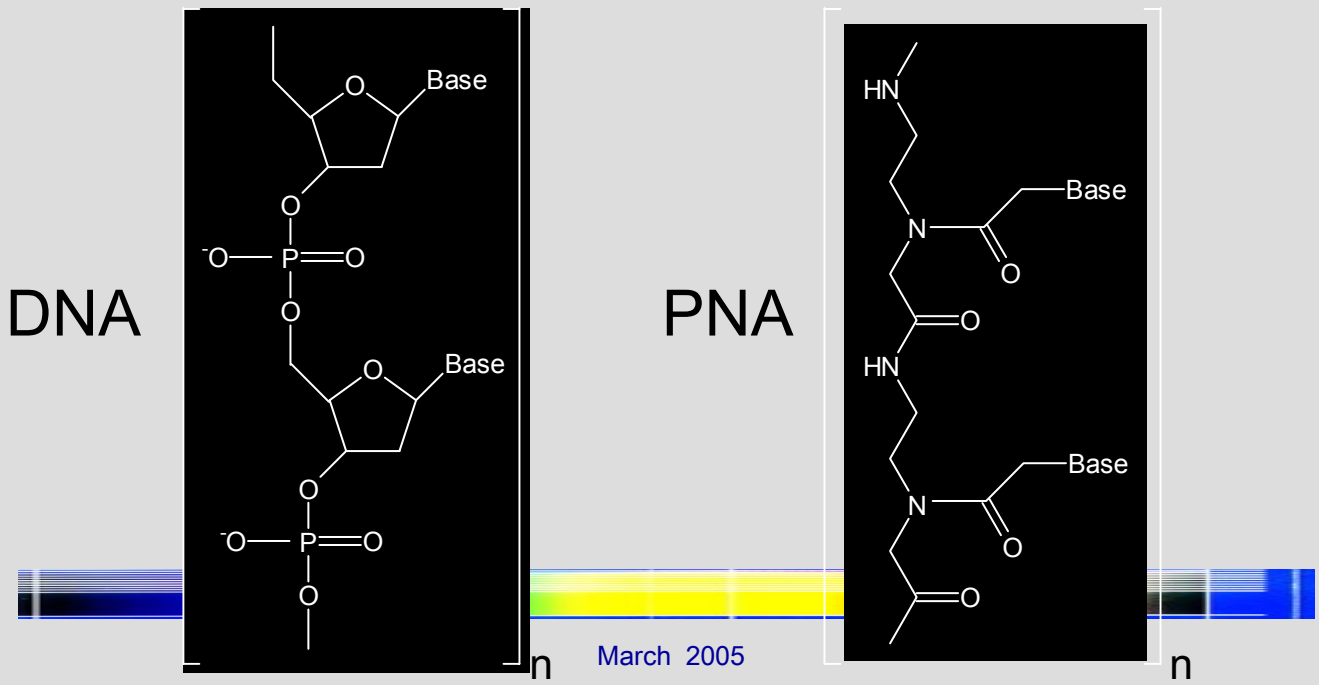


Melting on artificial targets



PNA/LNA-mediated PCR-clamping





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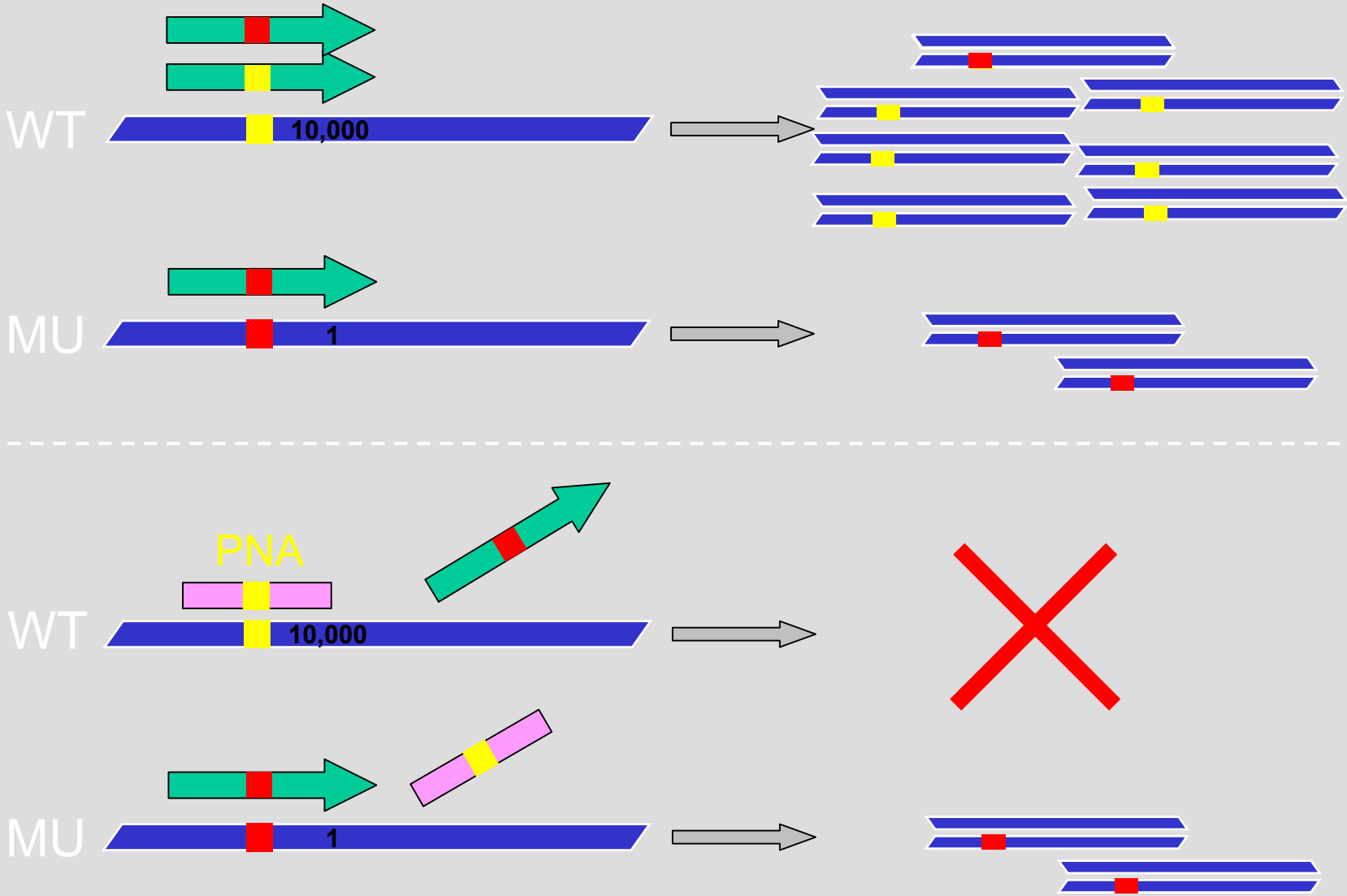
PNA/LNA-mediated PCR-clamping



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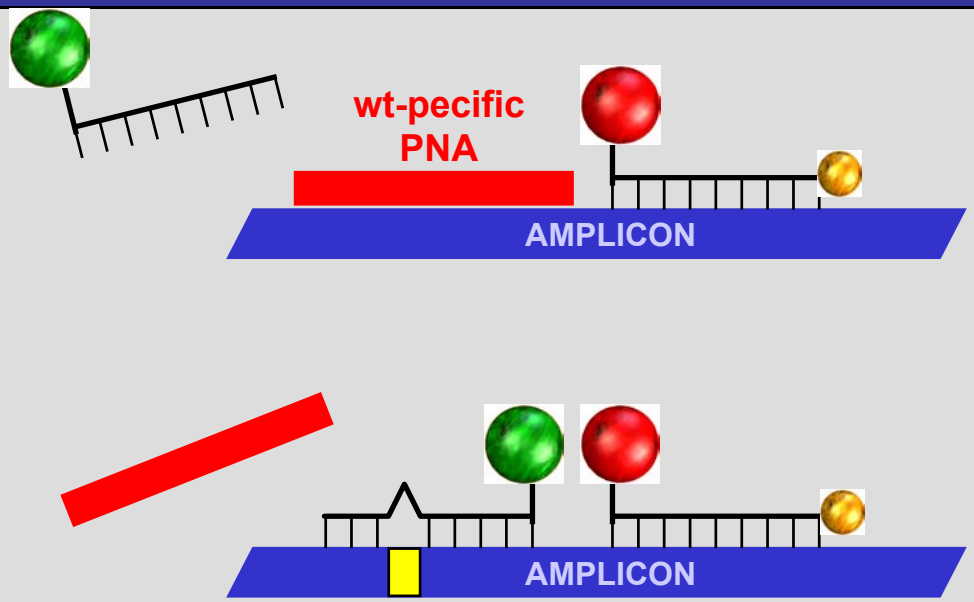
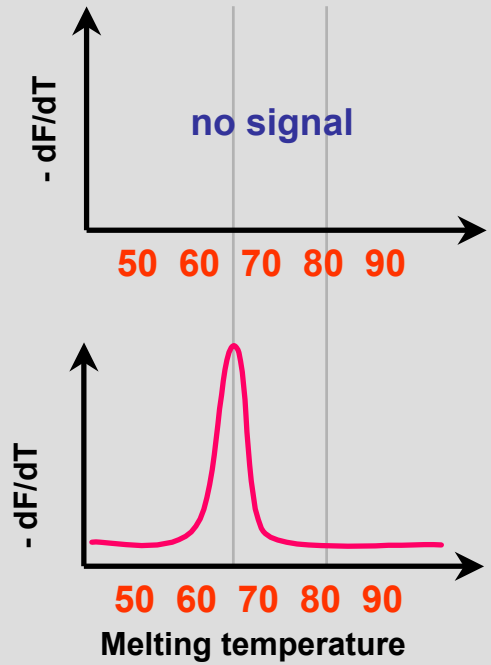
Molecular concepts: Clamped-Probe-Assay

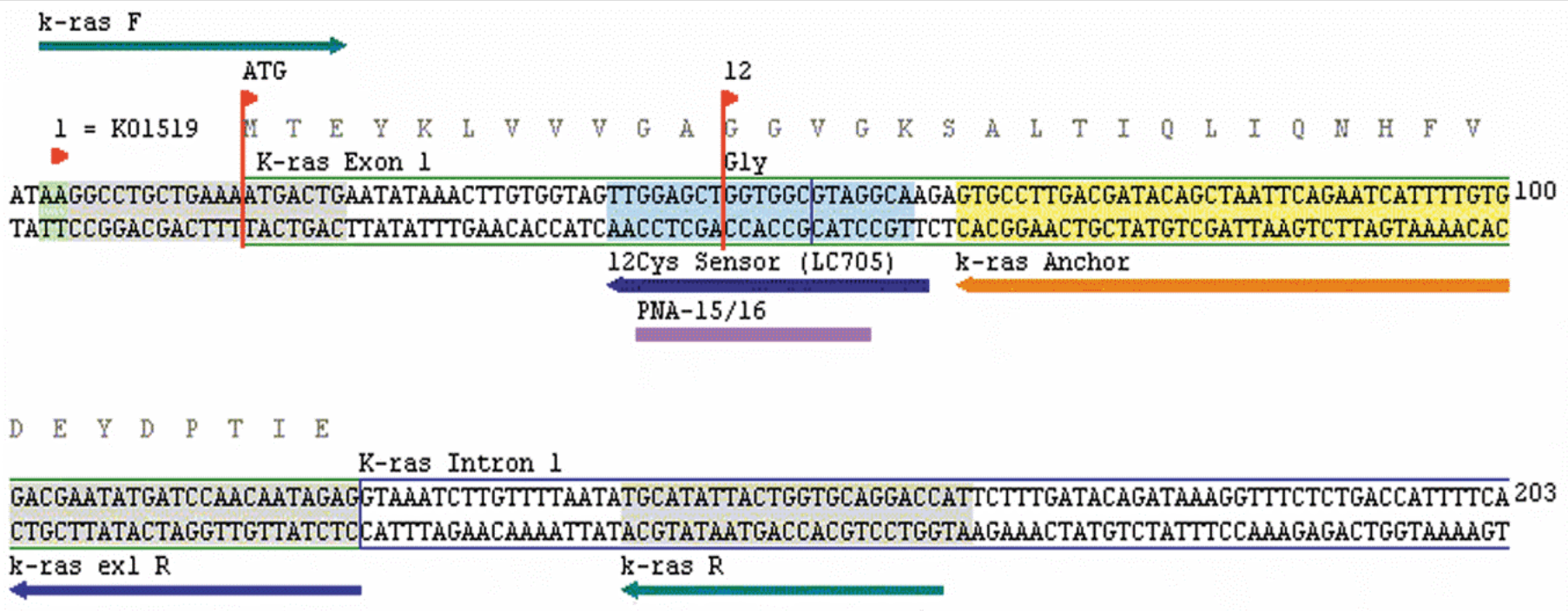




Possible applications :

- 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, ...)

Clamped-Probe-Assay: k-ras Codon 12



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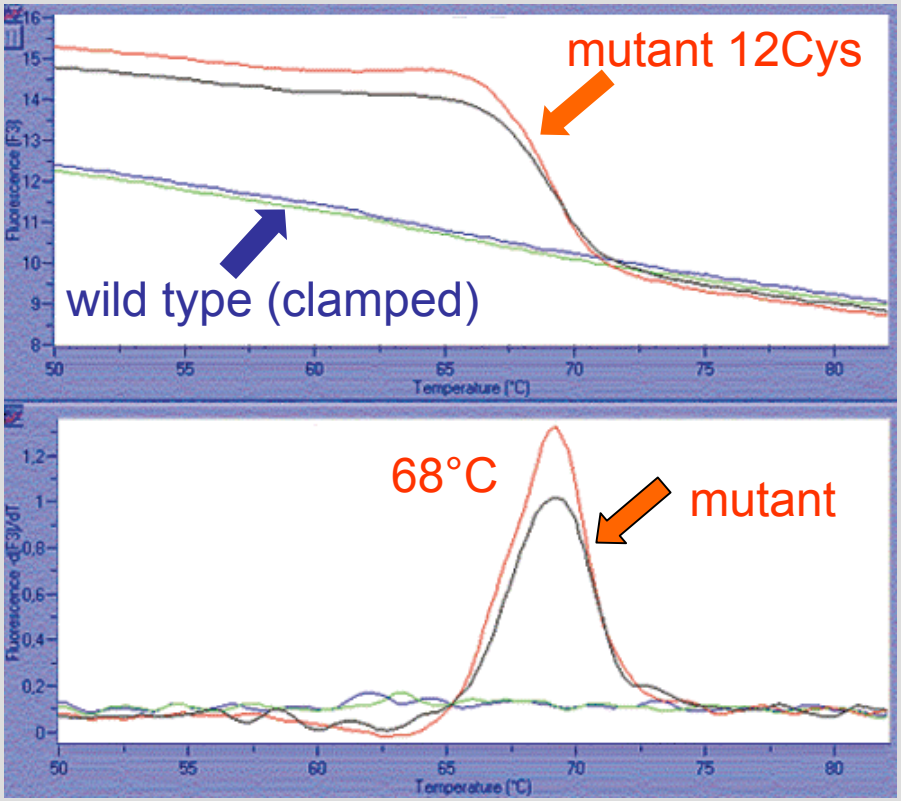
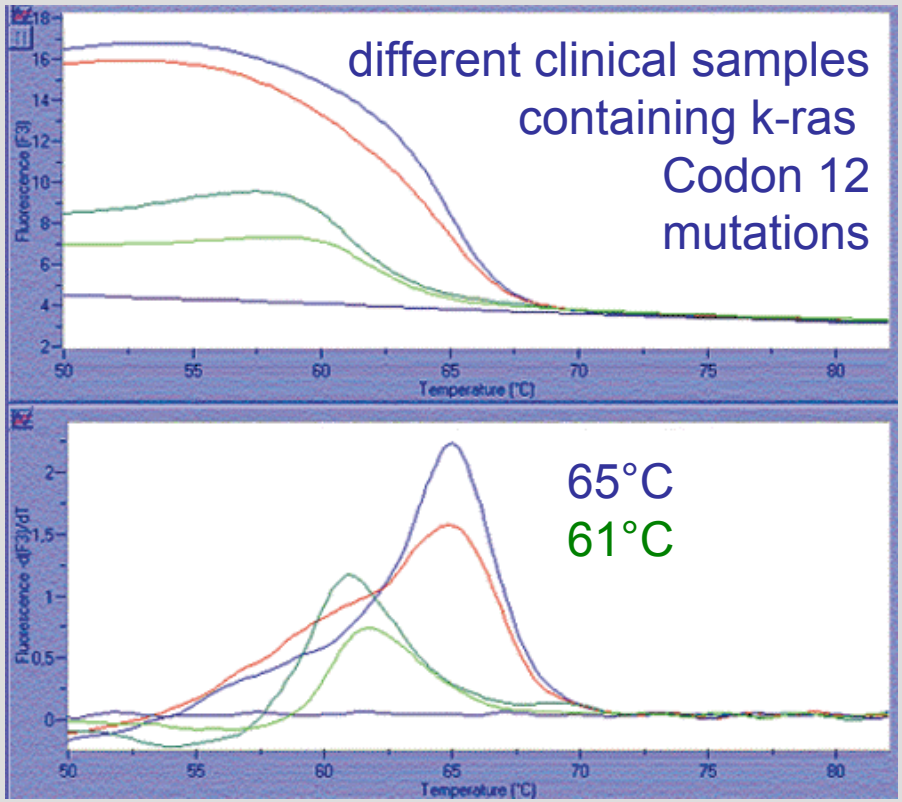
Melting curves from different patients

Melting curve (fluorescence vs. temperature)





Melting curve (dFl/dT vs. temperature)

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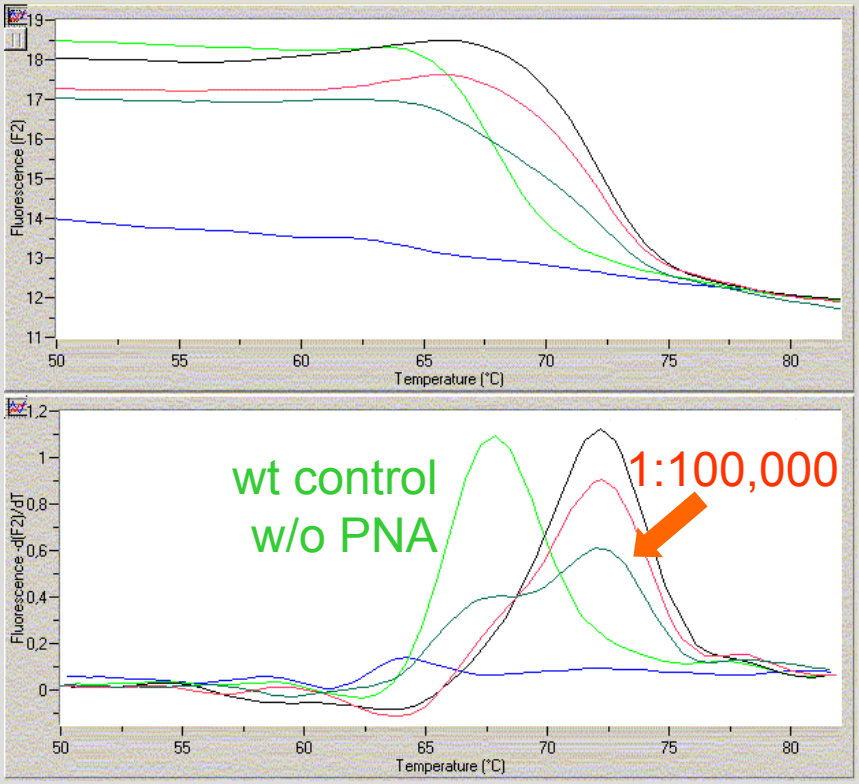
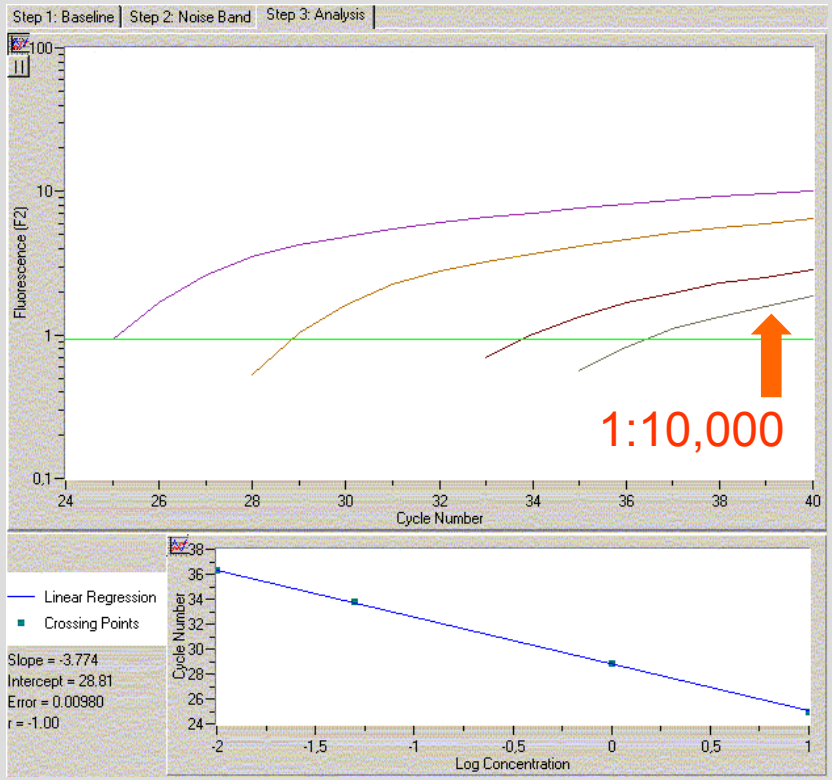
Dilution row of mutant in wt DNA

Melting curve (fluorescence vs. temperature)

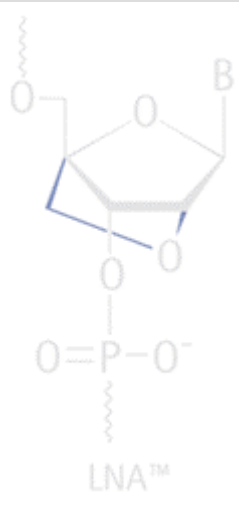


Amplification plot (normalized)



Melting curve (dFl/dT vs. temperature)

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



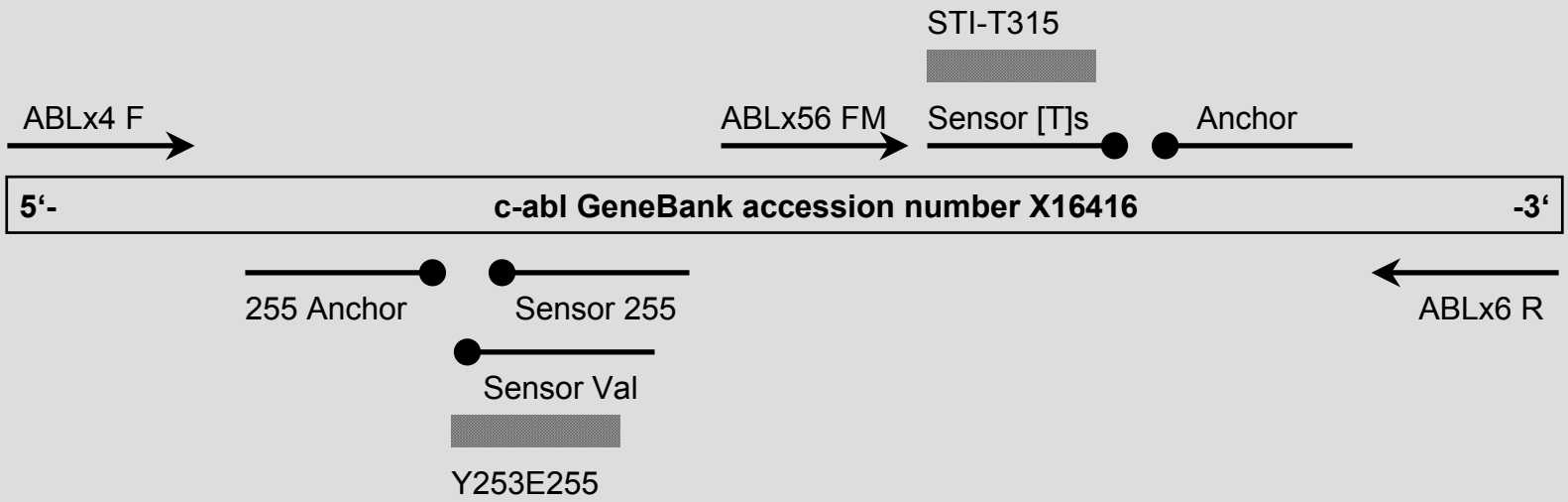
Nr.	Chemistry of the clamping probe	Concentration of probe still suppressing the 12 Gly-variant	Concentration of probe suppressing the 12 Cys-variant (1 mismatch)
1	PNA 15mer 12Gly	2,8 µM*/3 µM§	
2	PNA 17mer 12Gly	1,0 µM	
3	LNA 17mer 12Gly	0,1 µM	
4	5'-NH LNA 17mer	0,05 µM	0,2 µM
5	5'-MB LNA 17mer	0,02 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 (the working concentrion is 20-fold lower).

Future targets for the CPA assay: CML

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



Pre-existence and evolution of imatinib mesylate resistant clones in chronic myelogenous leukemia monitored using PNA-clamped hybridization probes. Kreuzer et al., (2002) submitted

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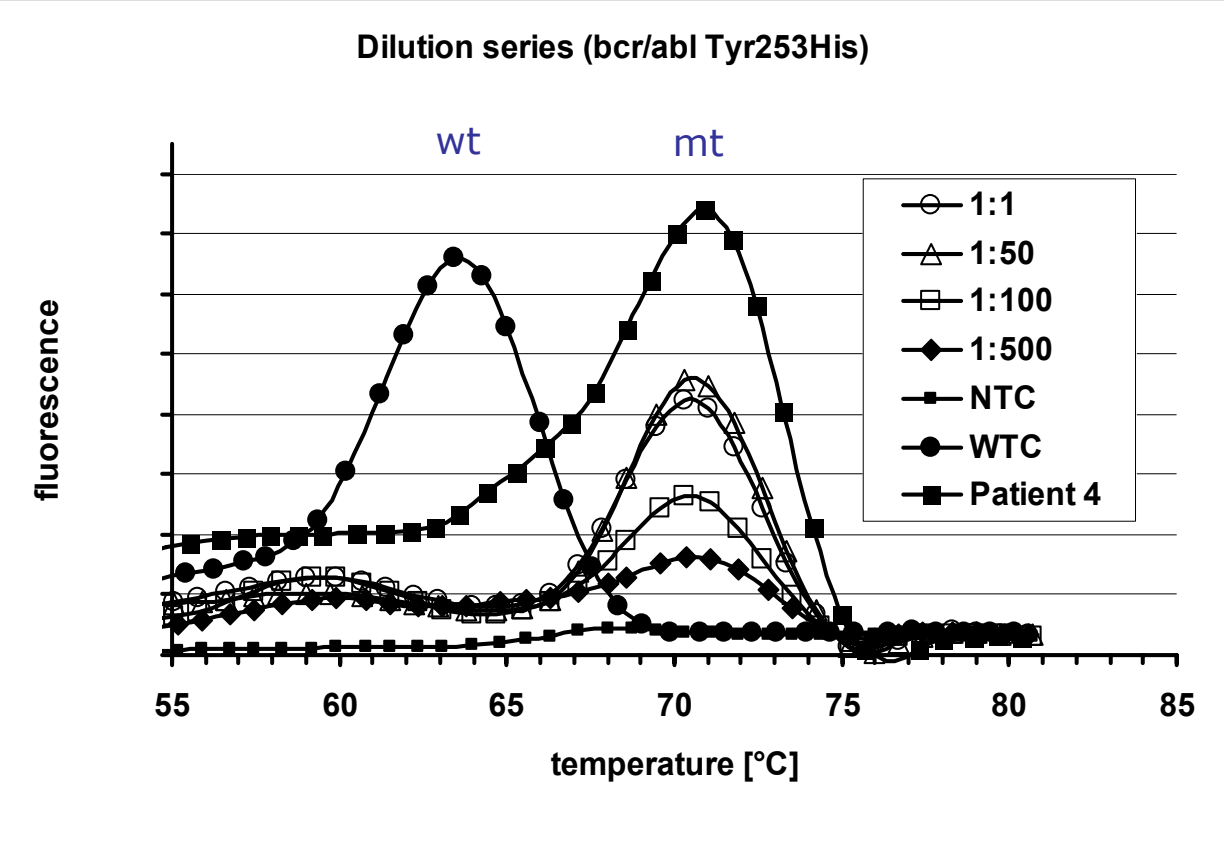
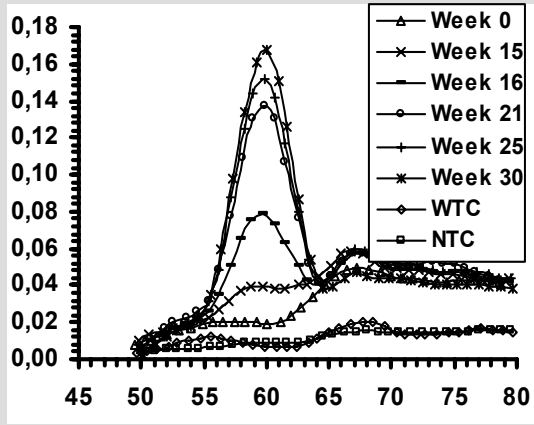
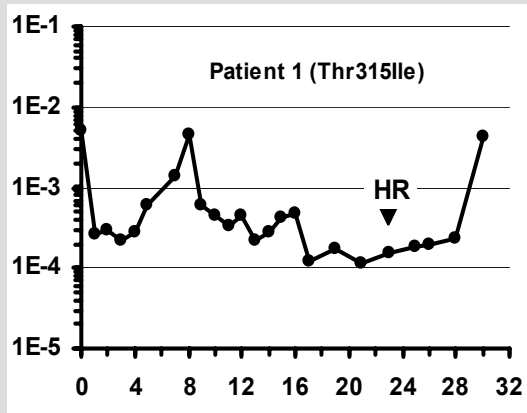
Future targets for the CPA assay:CML







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

Patient history monitored with the CPA assay.

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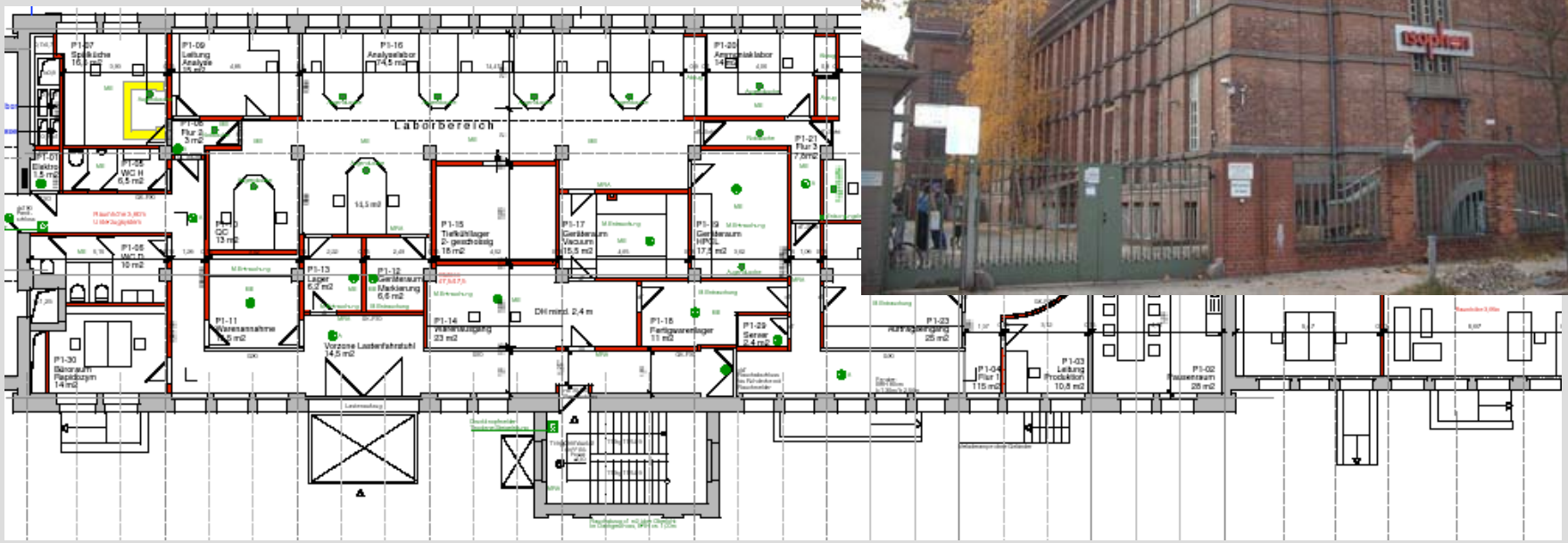
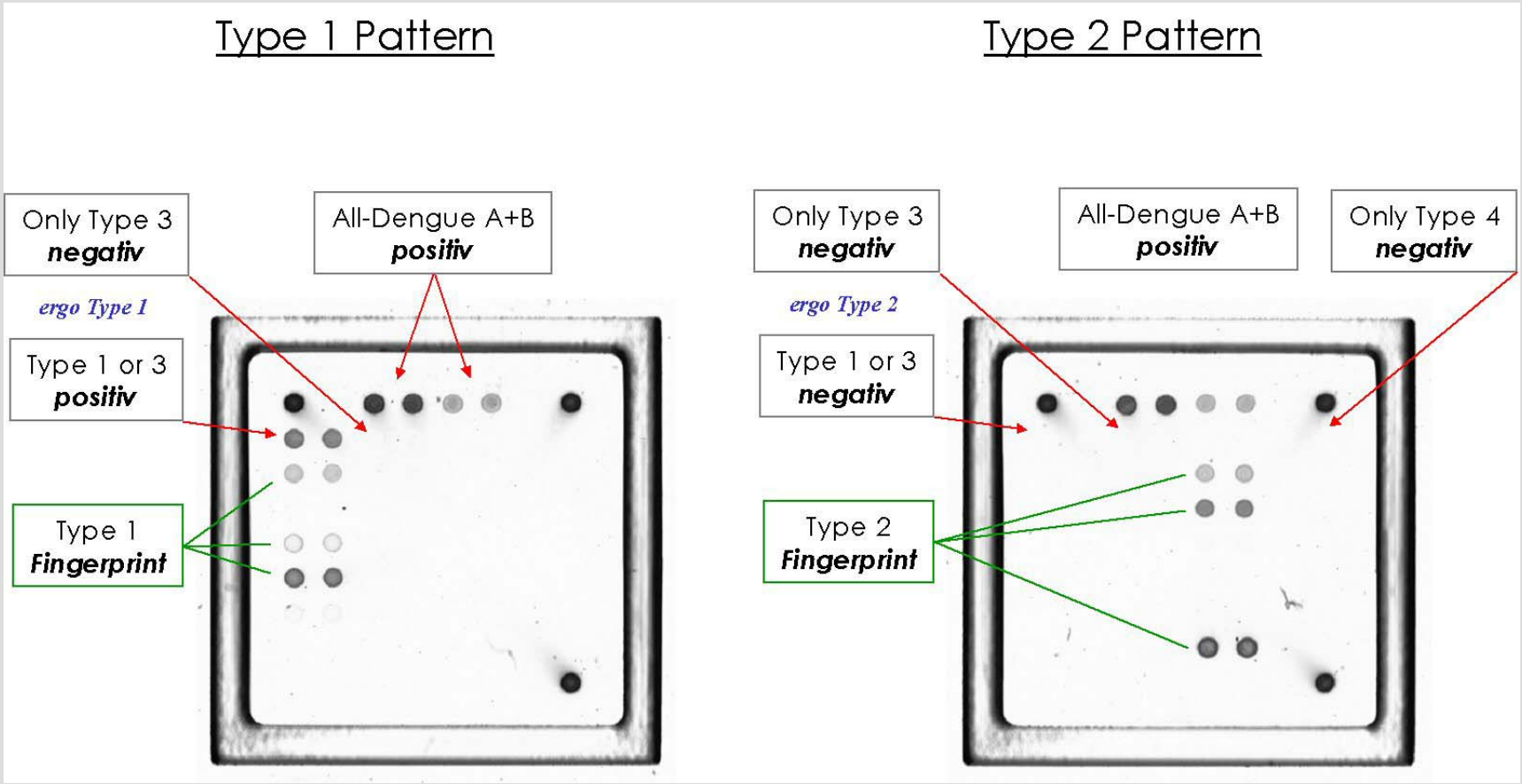
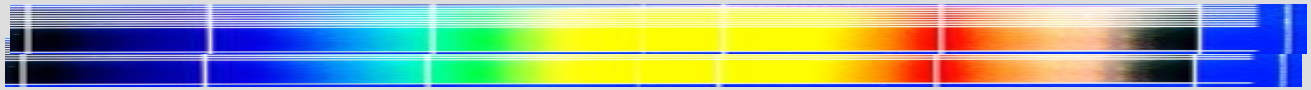
2005 : TIB moves into our new building

TIB MOLBIOL (oligo synthesis)
GenExpress (cloning services)
Chipron (low-cost/low-density array)
Chemicell (magnetic beads)
emp biotech (fluorescent reagents)



New technologies ... for example chips





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Precise quantification
is a function of standards