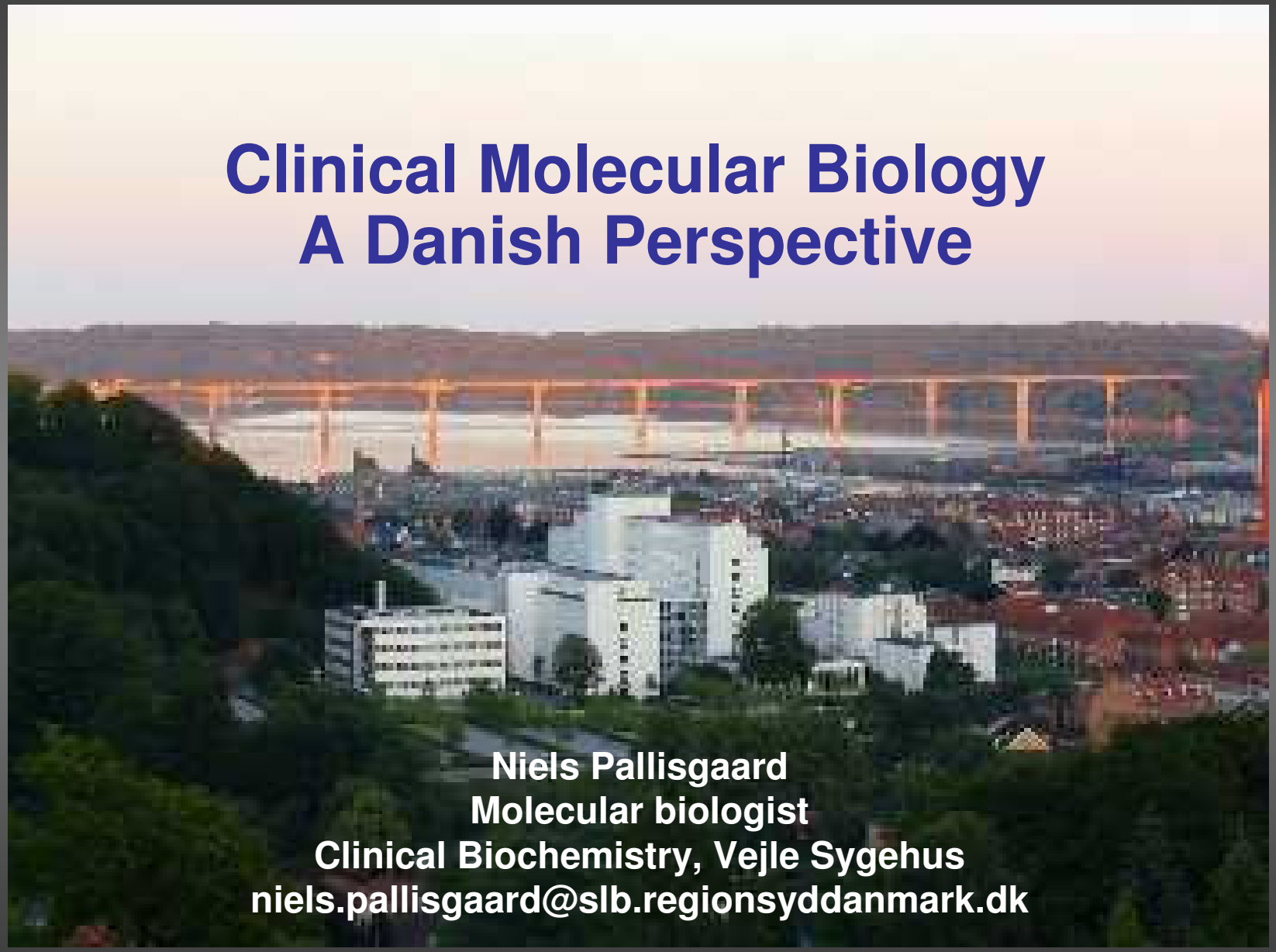
Clinical Molecular Biology
A Danish Perspective



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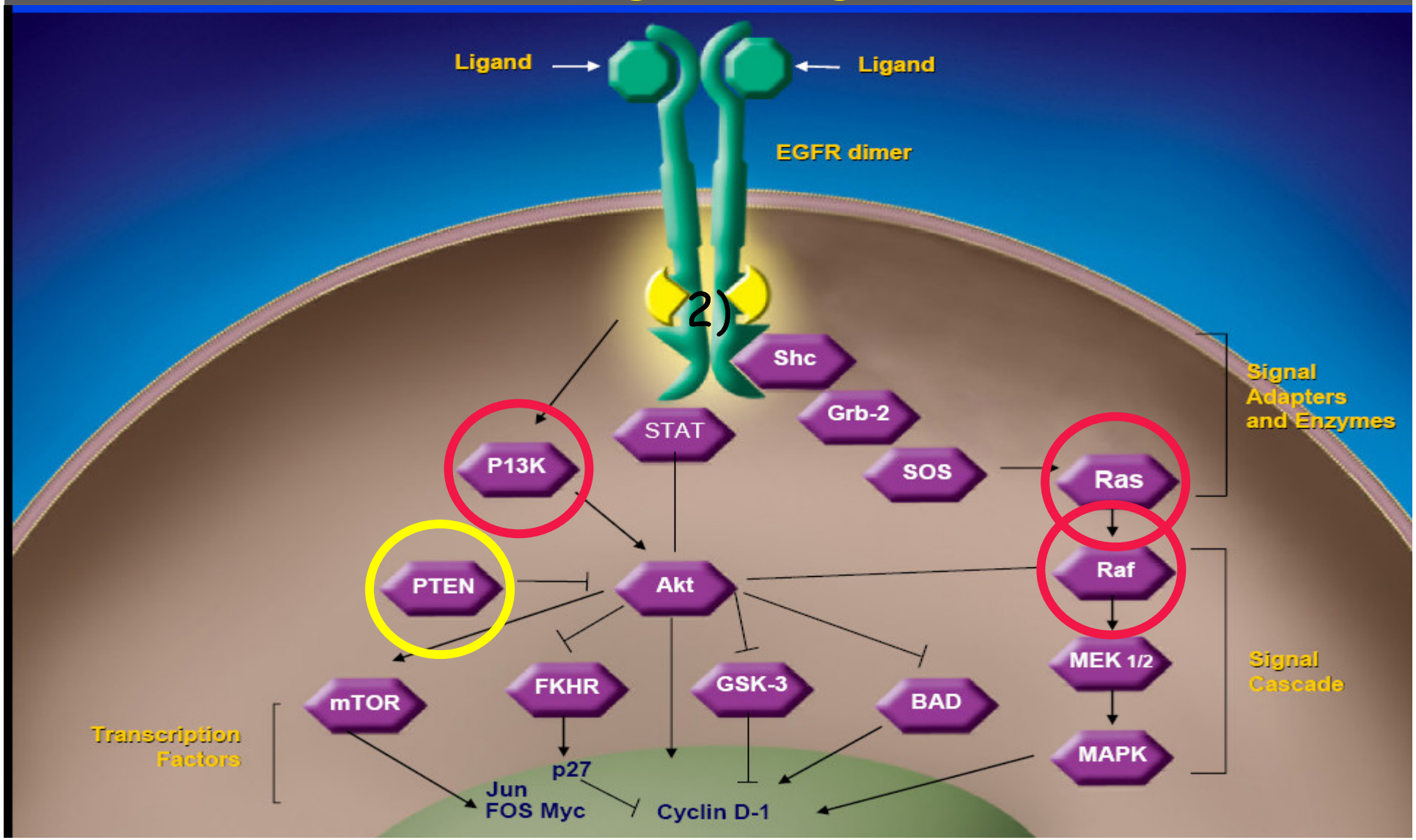
Here's my DNA sequence"

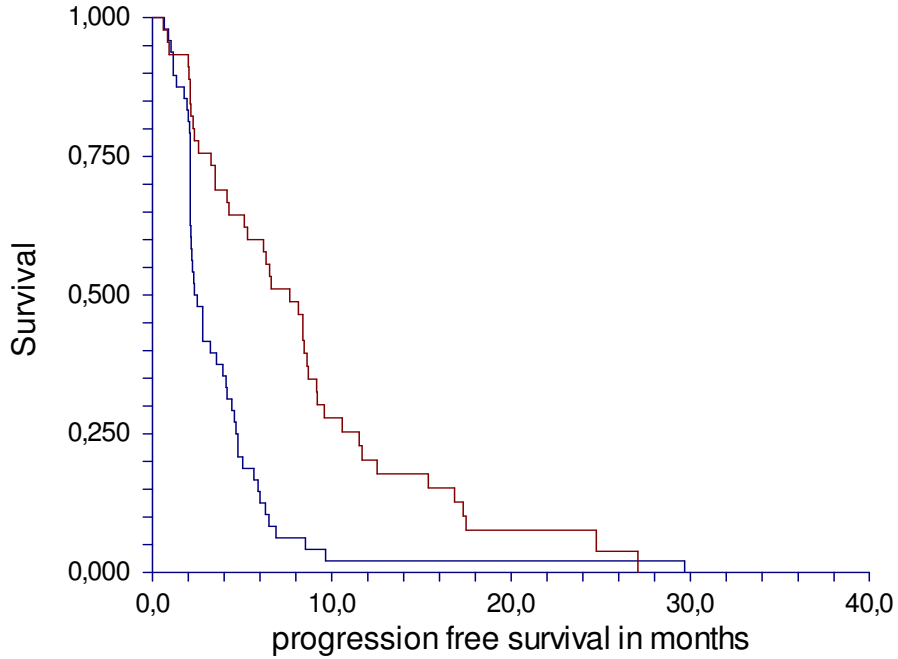
The Epidermal Growth Factor Signalling



EGFR related mutational status and association to clinical outcome of third-line cetuximab-irinotecan in metastatic colorectal cancer.

KLG Spindler, N Pallisgaard, J Lindebjerg, SK Frifeldt, A Jakobsen

(*BMC Cancer. 2011 Mar 25;11:107.*)



- Progression free survival in triple negative patients and patients with any mutations detected.
- The median progression free survival was significantly higher in patients with triple negative mutational status (Red: 7.7 months (5.1-8.6, 95%CI)) compared to patients with one or more mutations (Blue: 2.3 months (2.1-3.6, 95% CI) P<0.000).

New Targeted Treatment

- Same targeted treatment for different cancer types
- Targeted treatment alone does not cure the patients
- Respond will depend on patient mutation profile
- Clinical molecular biology need to develop assays to:
  - identify mutations activating target genes for therapy (e.g BRAF, cKIT)
  - predict treatment response (KRAS, BRAF, PIK3CA)
  - monitor treatment response (and progression)
  - identify resistance mutations during therapy (EGFR, KRAS, BRAF)

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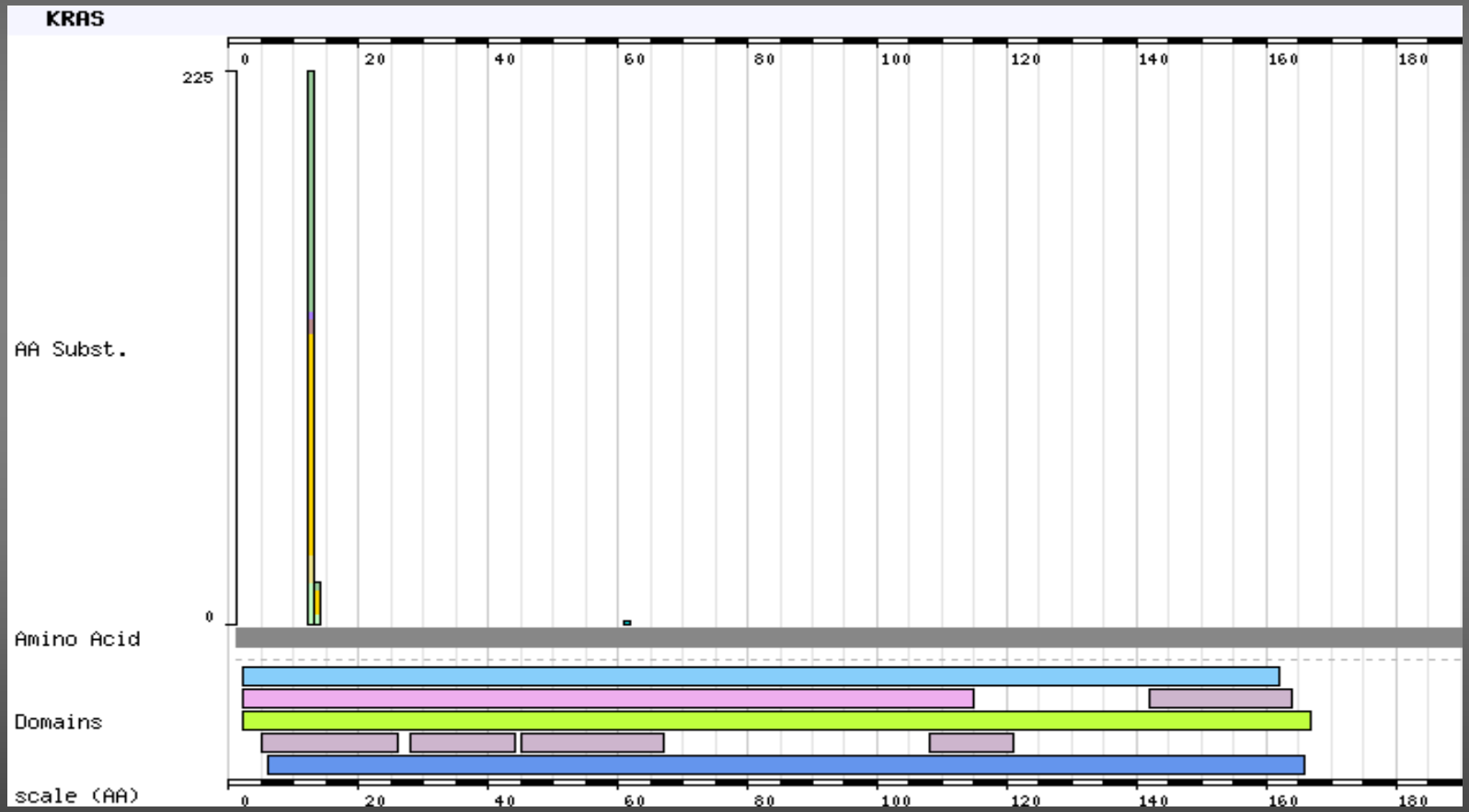
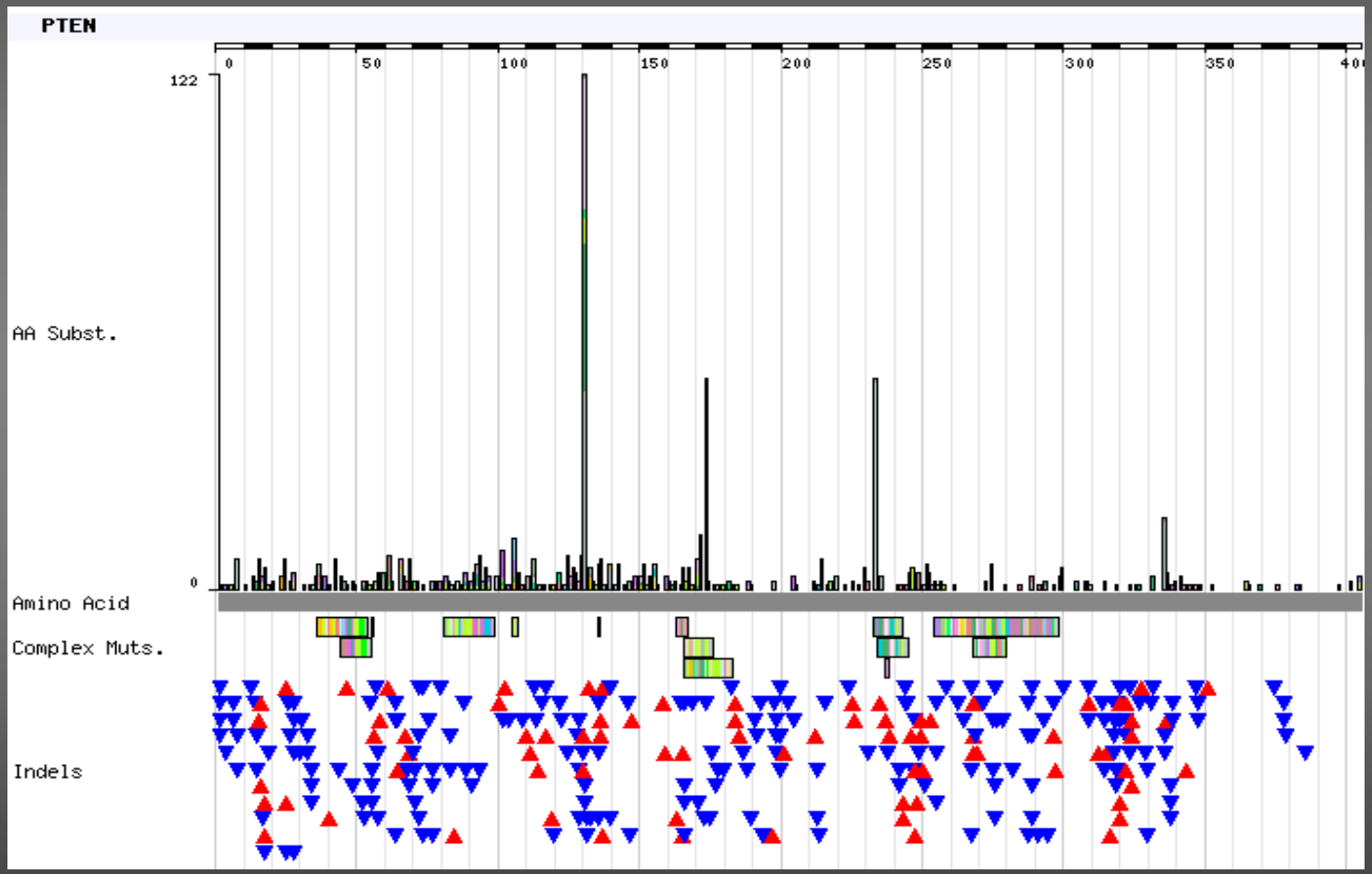
Gain of function mutations (*KRAS*)



www.sanger.ac.uk

Loss of function mutations (*PTEN*)



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PCR

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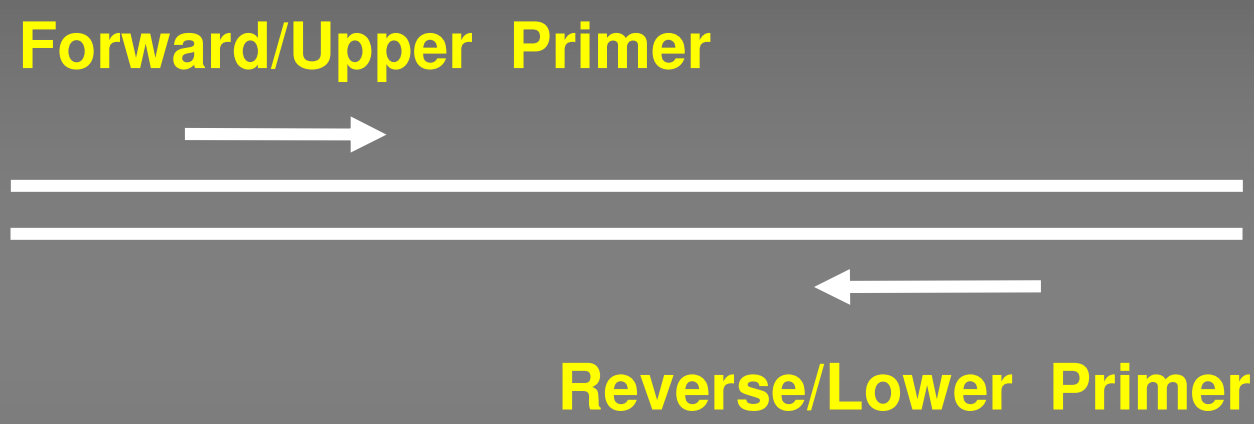
Polymerase Chain Reaction



PCR may be used to generate sufficient DNA of a region of interest for molecular analysis

KRAS mutation analyses

- Sequencing
- qPCR (ARMS amplification refractory mutation system)
- Pyro-sequencing
- Melting point analysis
- RFLP
- ARMS (Gel based)
- MLPA (Multiplex Ligation-dependent Probe Amplification)
- CSCE (Conformation-Sensitive Capillary Electrophoresis)
- Wave (HPLC)

KRAS: DxS versus Sequence in CRC

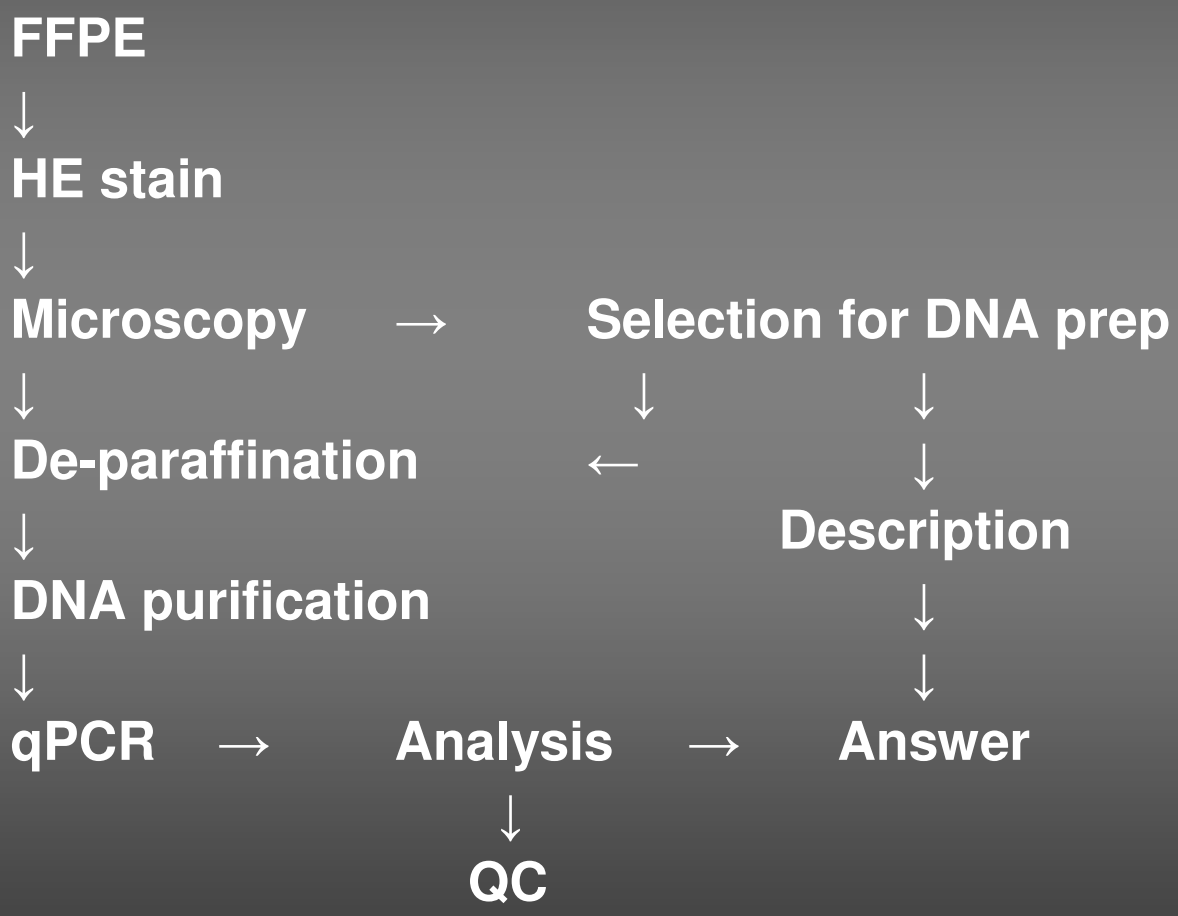
Primary tumor, paraffin (n=64)

	DxS	Seq
Gly12(wt)	43	46
Gly12Asp	6	5
Gly12Ala	3	0
Gly12Val	2	2
Gly12Ser	2	1
Gly12Arg	3	2
Gly12Cys	1	1
Gly13Asp	4	3
Gly13Cys	0	1
Mutations	21	15
Total	64	61

Sensitivity of PCR Methods Detection of (point) Mutations

• ARMS gel	1- 5%
• ARMS qPCR	0.1- 0.01%
• SNP qPCR	1- 5%
• Pyro-sequencing	1- 5%
• HRM	1- 5%
• Sanger sequencing	25%
• Next Generation sequencing	1- 0.01%
• dPCR (digital PCR)	1- 0.1%
• Other	?

Work flow *KRAS* mutation analysis



Material for *KRAS* mutation analysis

Examples of sample types:

- FFPE (formalin fixed paraffin embedded)
- Glass slides (e.g. HE stain)
- Frozen tissue (e.g. in TissueTech)
- Fresh (e.g. in RNAlater media)
- Needle biopsies
- Plasma

Mutation specific qPCR

Amplification Refractory Mutation System-Quantitative PCR

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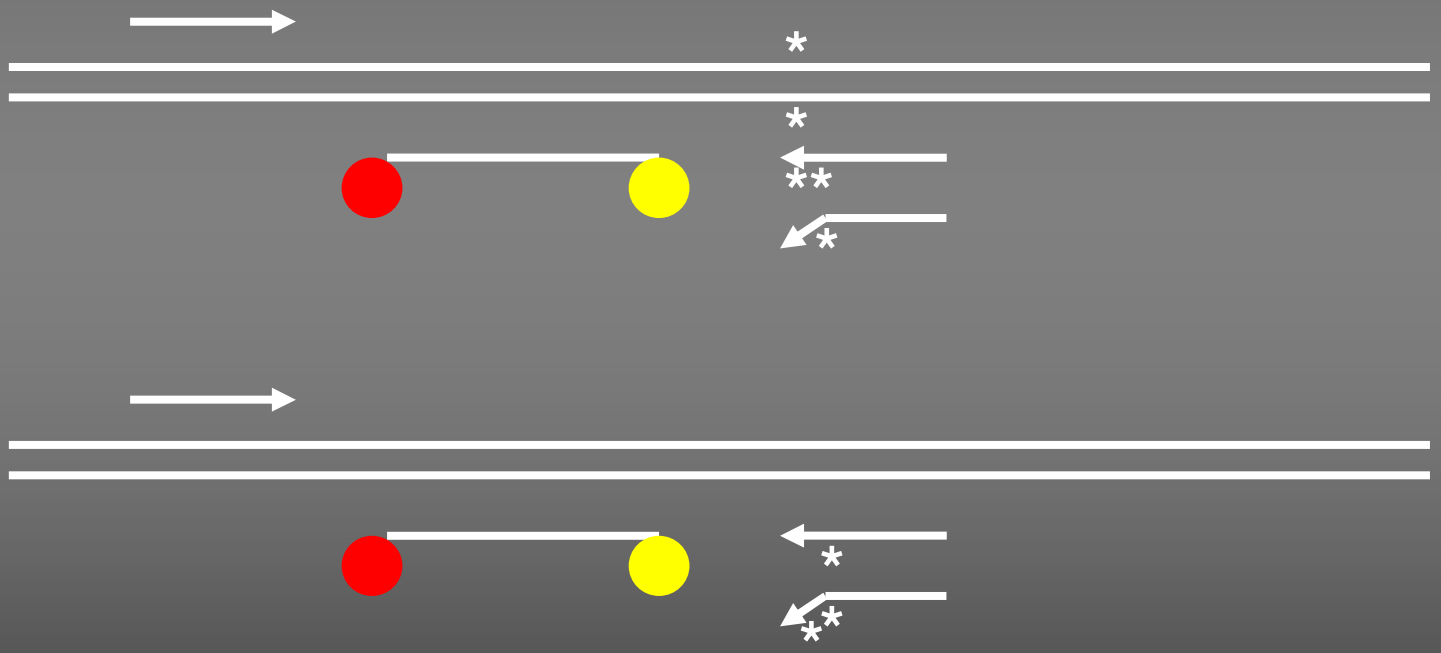
ARMS-qPCR

ARMS qPCR



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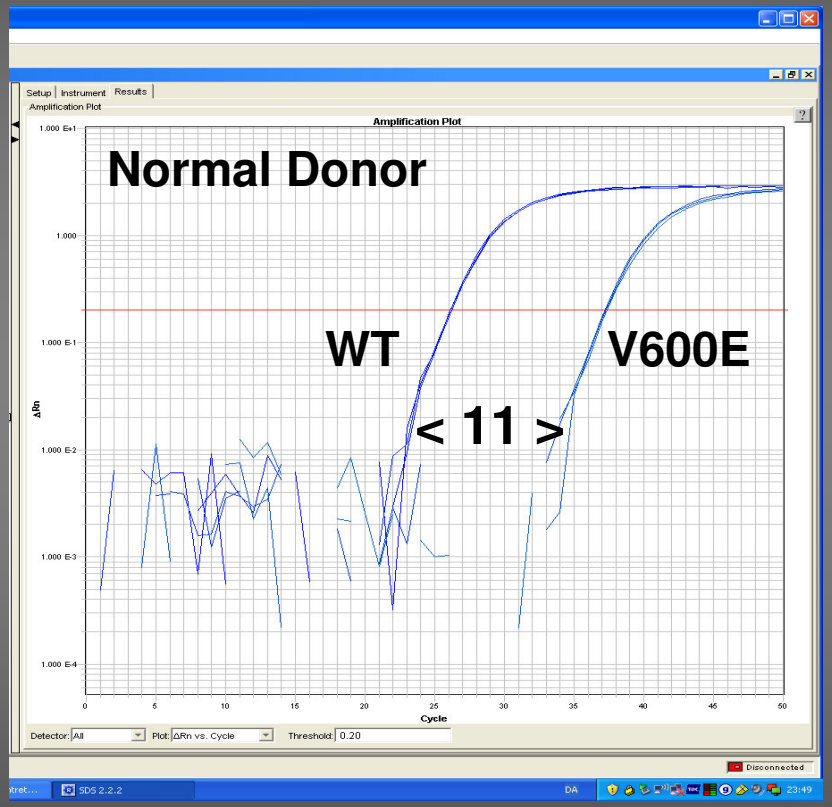
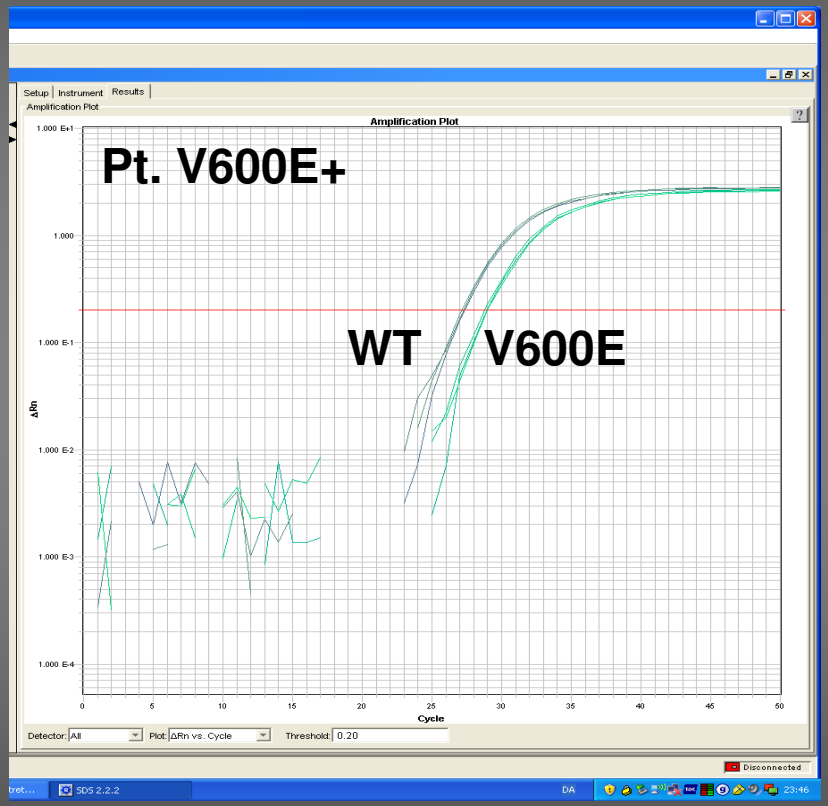
BRAF ARMS-qPCR WT / V600E





Standard ARMS qPCR - Specificity: 1:2000

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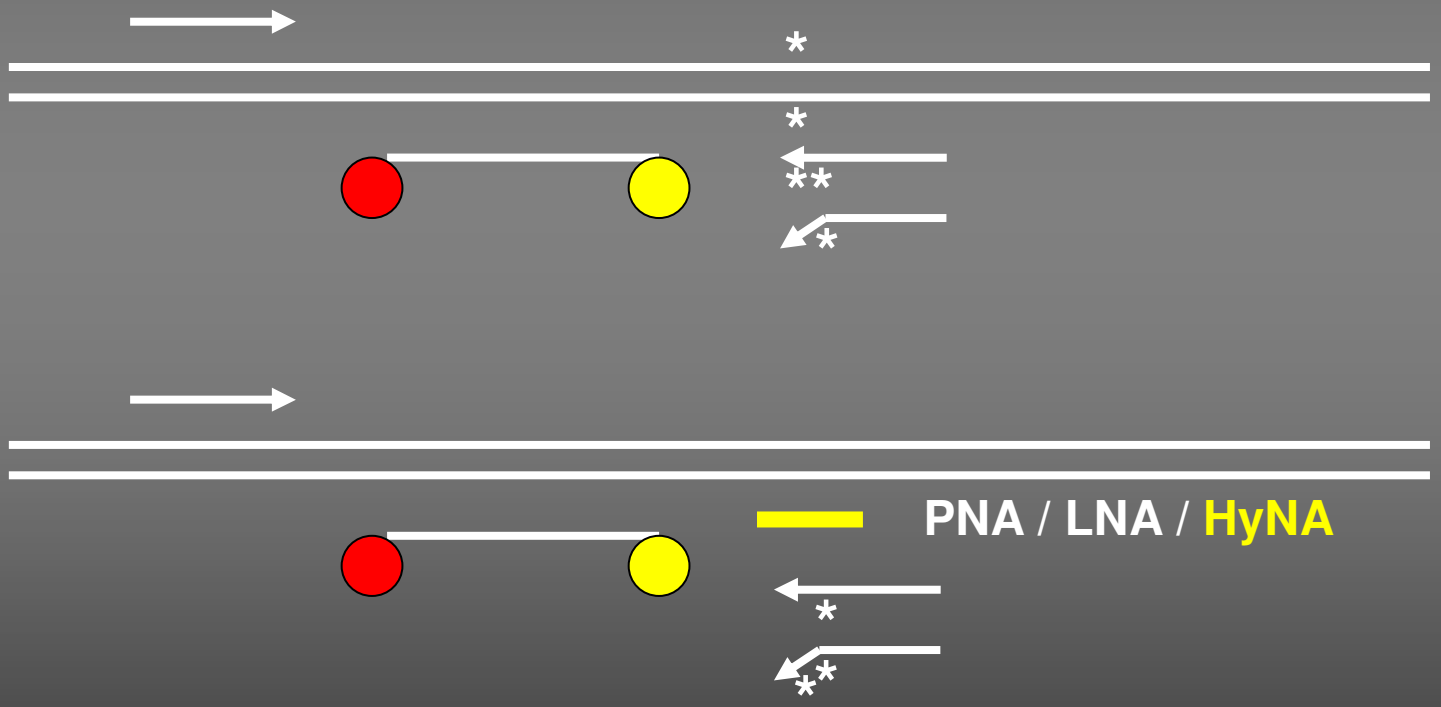
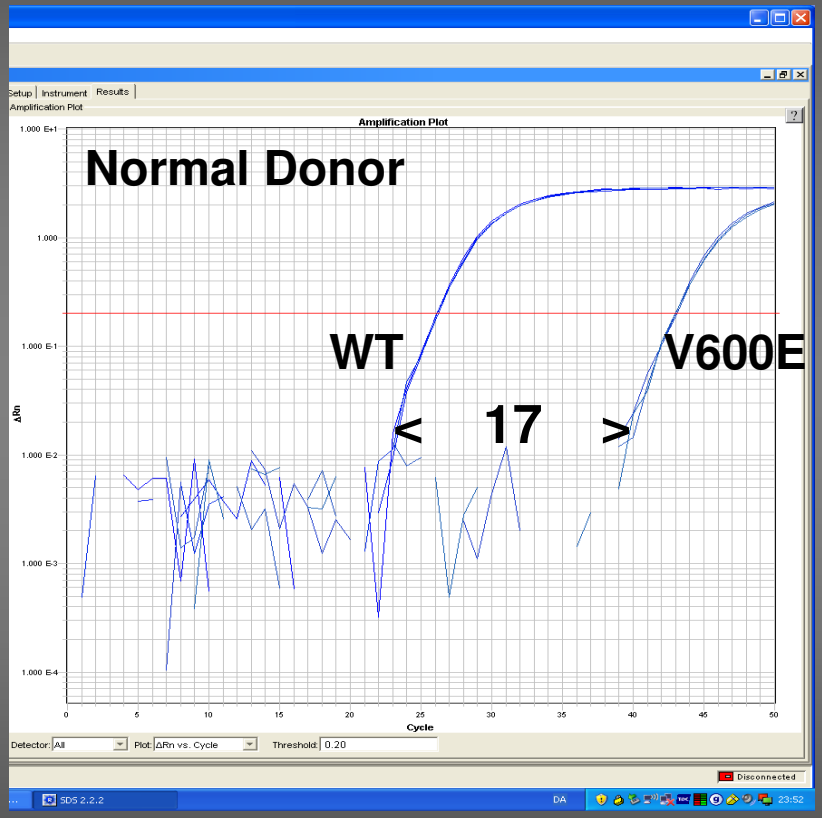
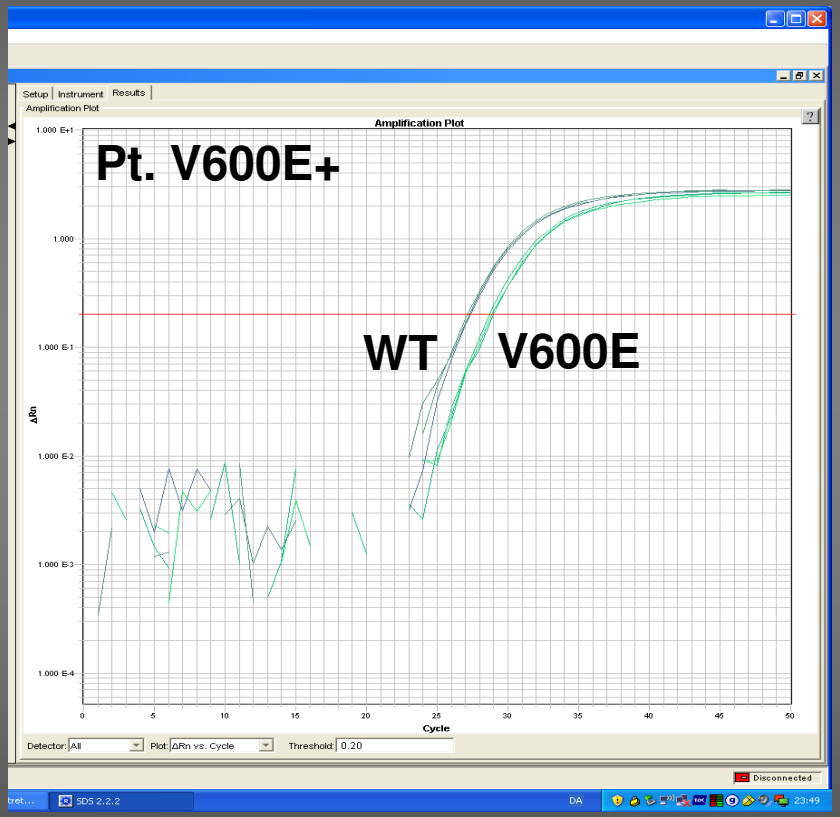
ARMS qPCR with block



BRAF ARMS-qPCR WT / V600E





Modified ARMS qPCR - Specificity: 1:100.000

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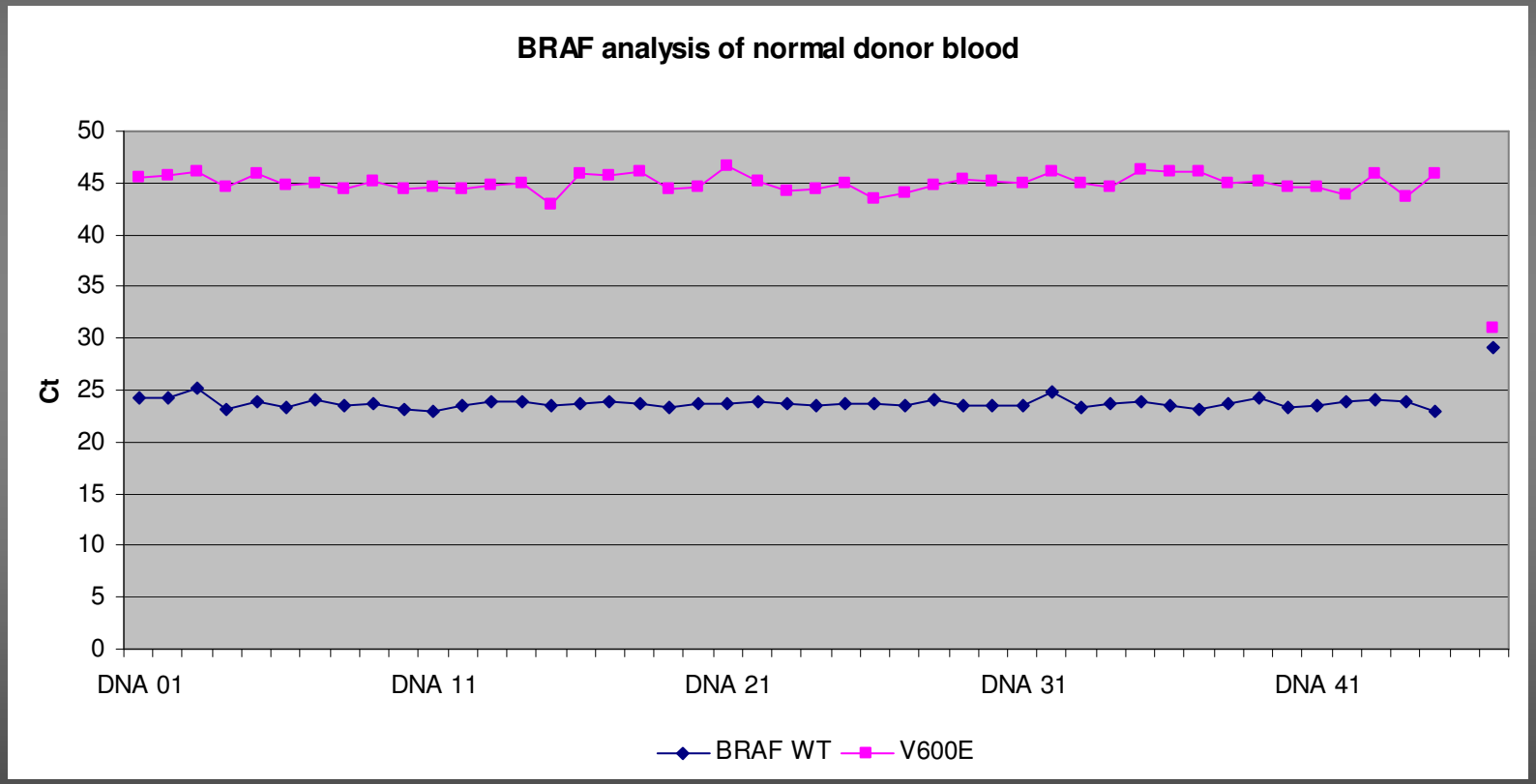
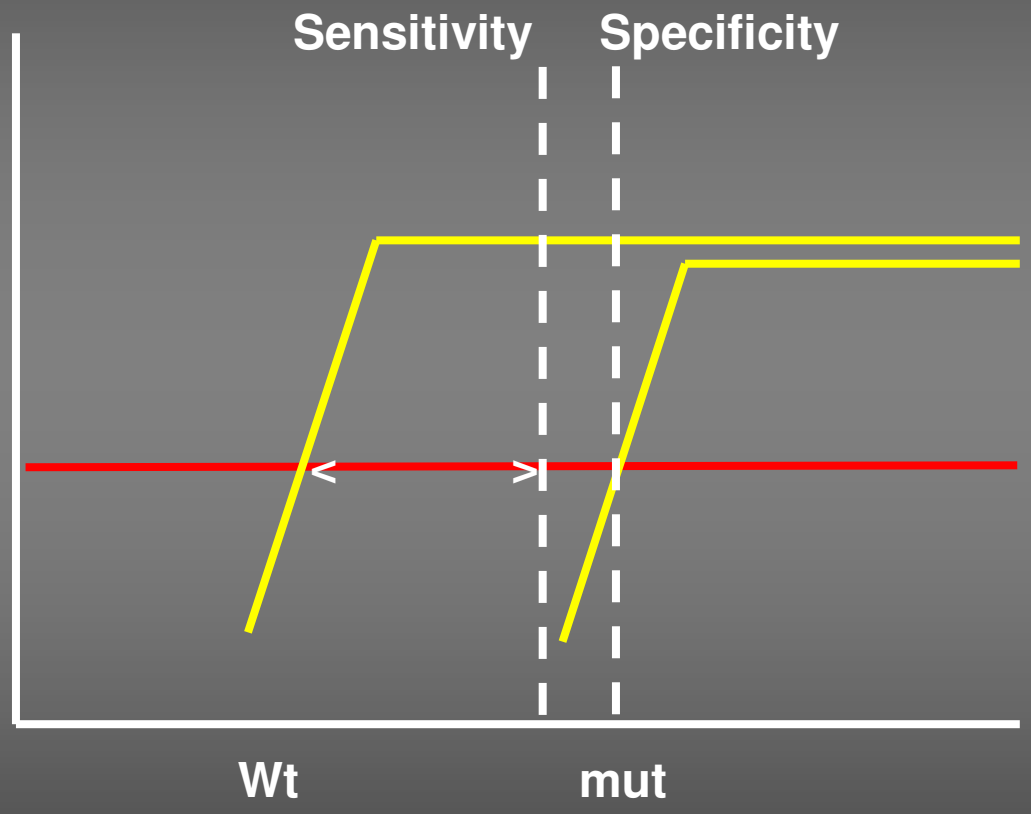
Validation of in house *BRAF* V600E Specificity on normal donor DNA



n=46

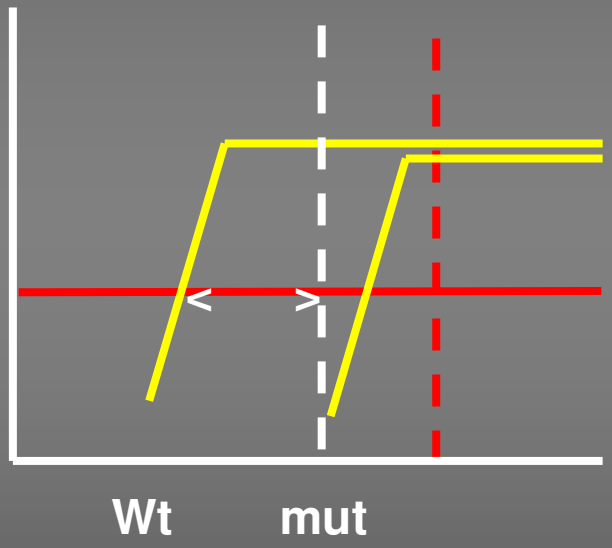
qPCR Sensitivity



Sensitivity may be defined as 10 fold less (3.5 PCR cycles) than assay specificity

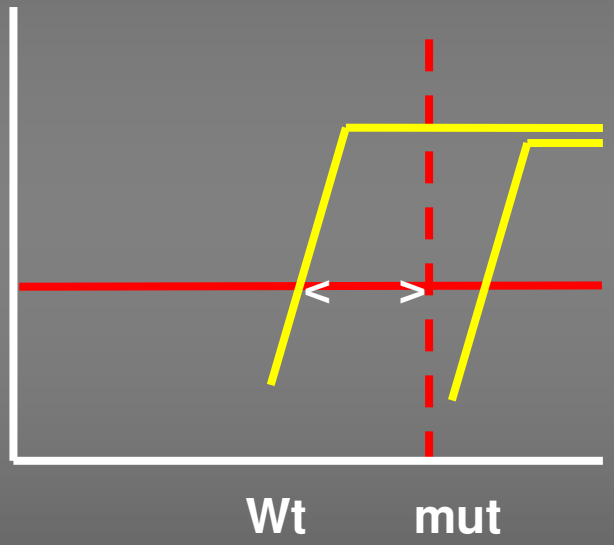
qPCR Specificity/Sensitivity

Normal Donor HIGH DNA conc.



At high DNA conc. sensitivity should be 10 fold less (3.5 PCR cycles) than assay specificity (white dotted line).

Normal Donor LOW DNA conc.



At low DNA conc. the Y-intercept (red dotted line) should be used in calculating the sensitivity.

PCR

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Proper Controls Required

PCR generated Positive controls for *KRAS* mutations

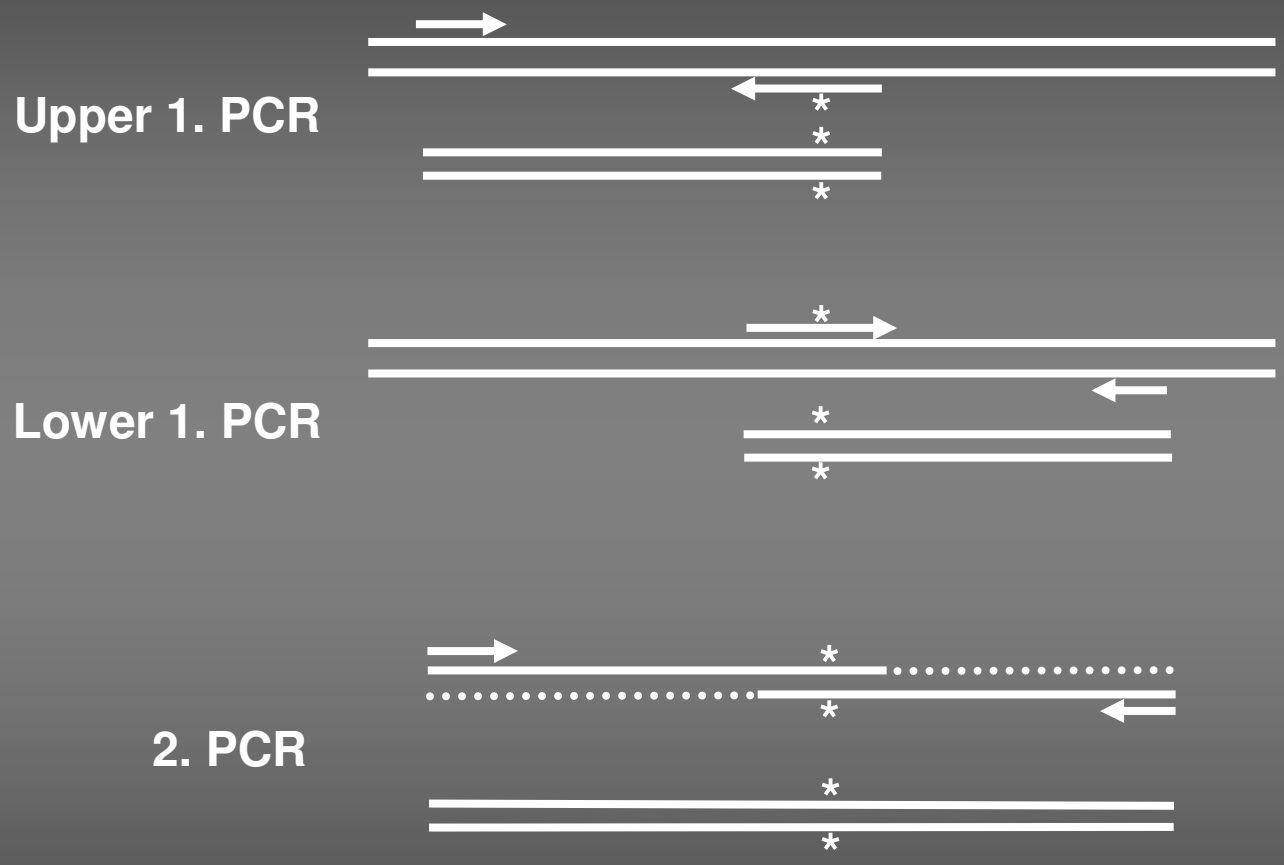
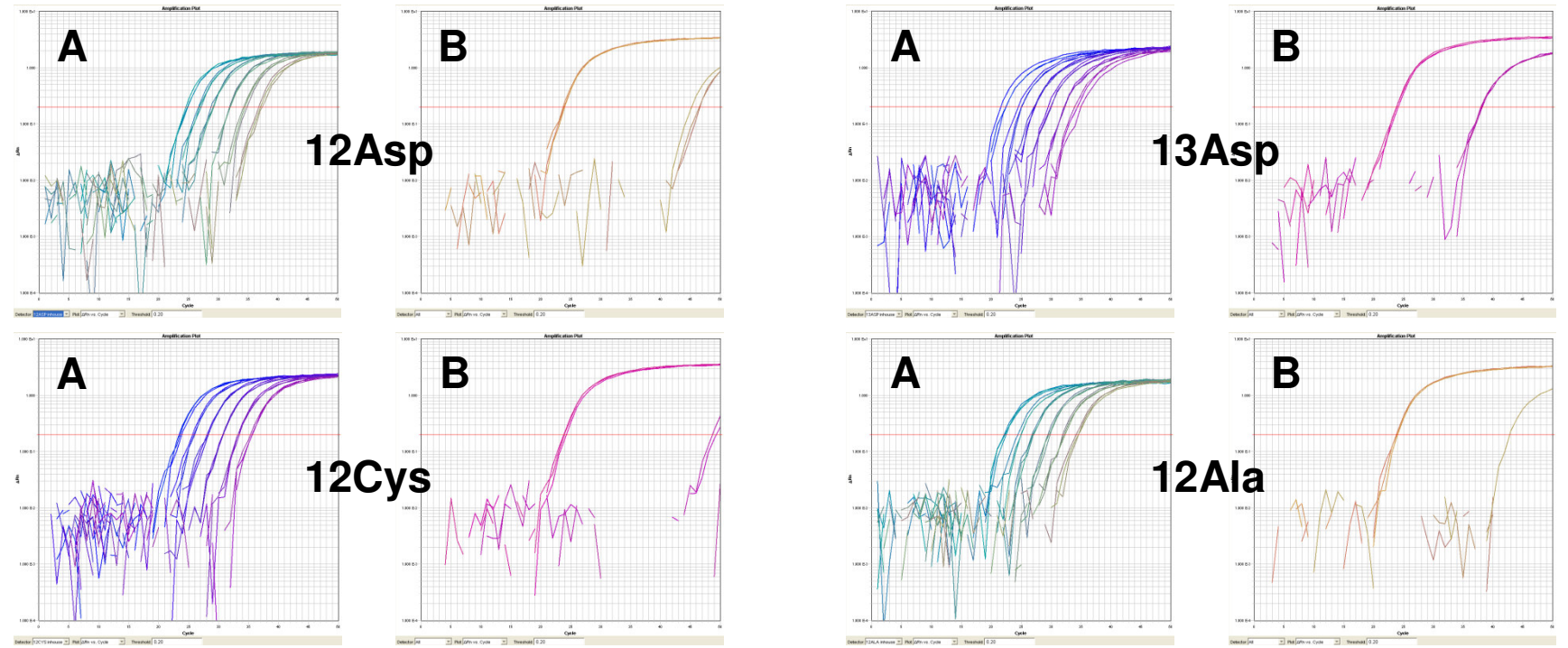


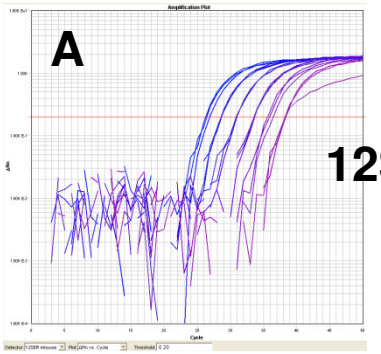
Diagram of method to produce a DNA fragment with a specific *KRAS* mutation.

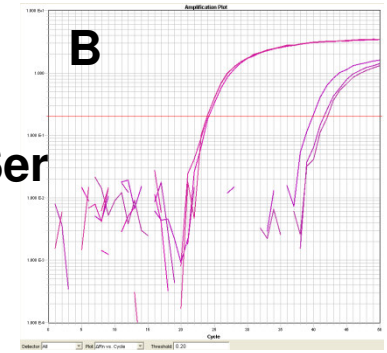
KRAS Standard curves and specificity 1



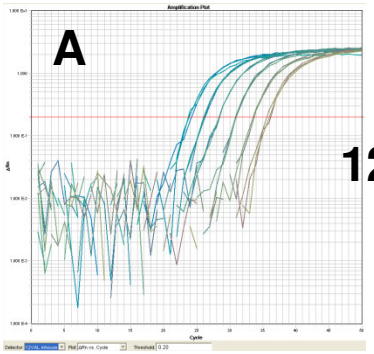
A; Standard curves generated by spiking 5 fold dilutions of *KRAS* mutated PCR fragment into 100 ng normal donor DNA.
B; Specificity of the in house *KRAS* assays: 100 ng normal donor DNA was analysed with *gCYC* and indicated *KRAS* mutation. From the obtained Ct's (Cycle threshold = PCR Cycle) the specificity of the assays for the different mutations was calculated.

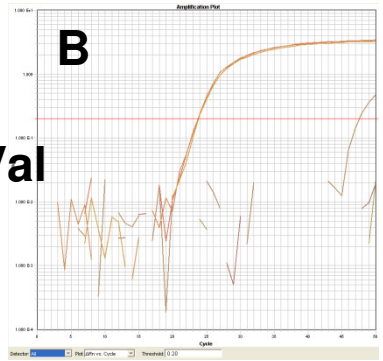
KRAS Standard curves and specificity 2



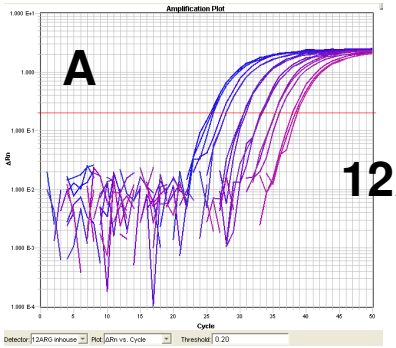


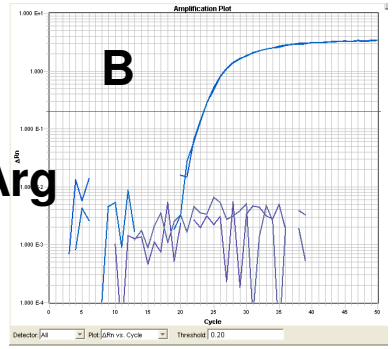
12Ser





12Val





12Arg

A; Standard curves generated by spiking 5 fold dilutions of *KRAS* mutated PCR fragment into 100 ng normal donor DNA.
B; Specificity of the in house *KRAS* assays: 100 ng normal donor DNA was analysed with *gCYC* and indicated *KRAS* mutation. From the obtained Ct's (Cycle threshold = PCR Cycle) the specificity of the assays for the different mutations was calculated.

Validation of in house *KRAS* qPCR

	KRAS DxS	*KRAS* In house
Ovarian Cancer *KRAS* mutated	44	44
Ovarian Cancer *KRAS* wild type	231	231

n=275

Steffensen et al, Int J Gynecol Cancer. (2011) 9:1592

PCR

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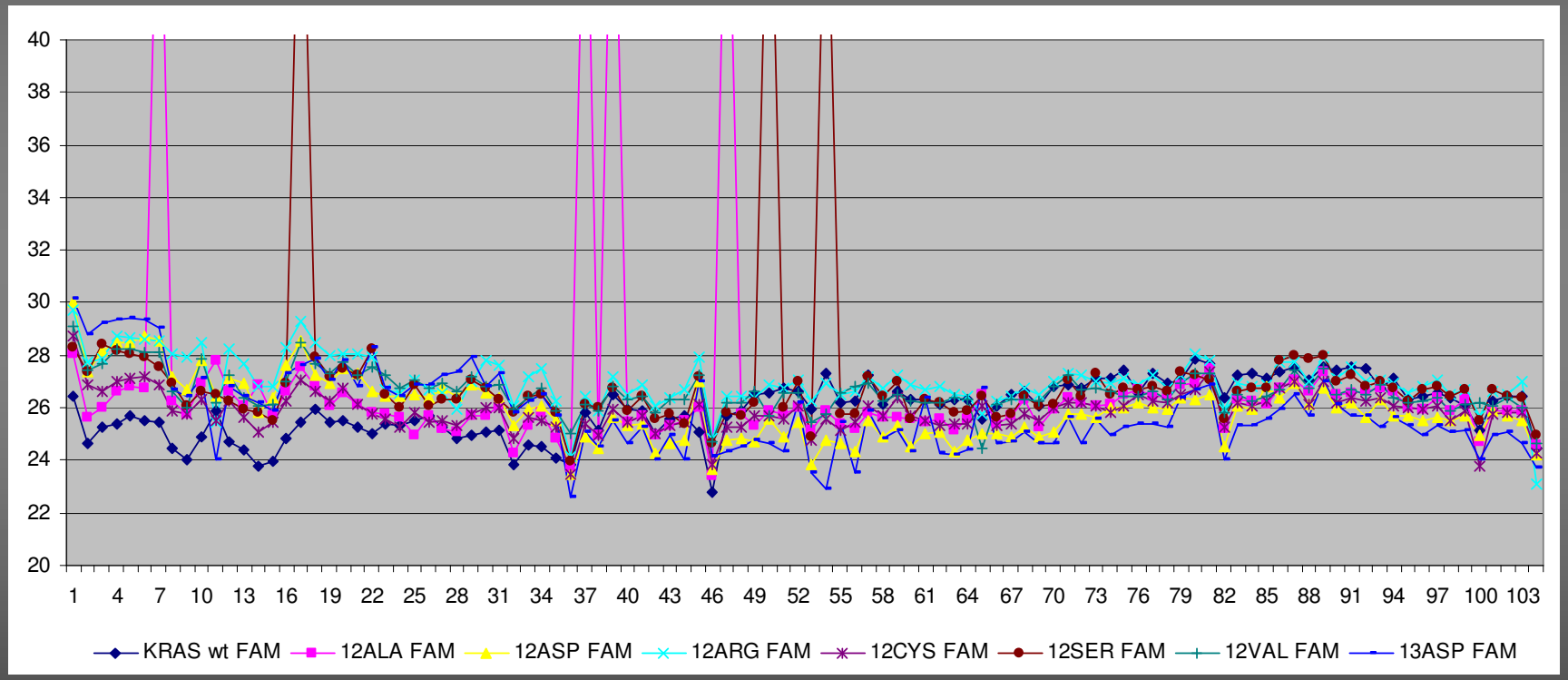
Potential Contamination Risk

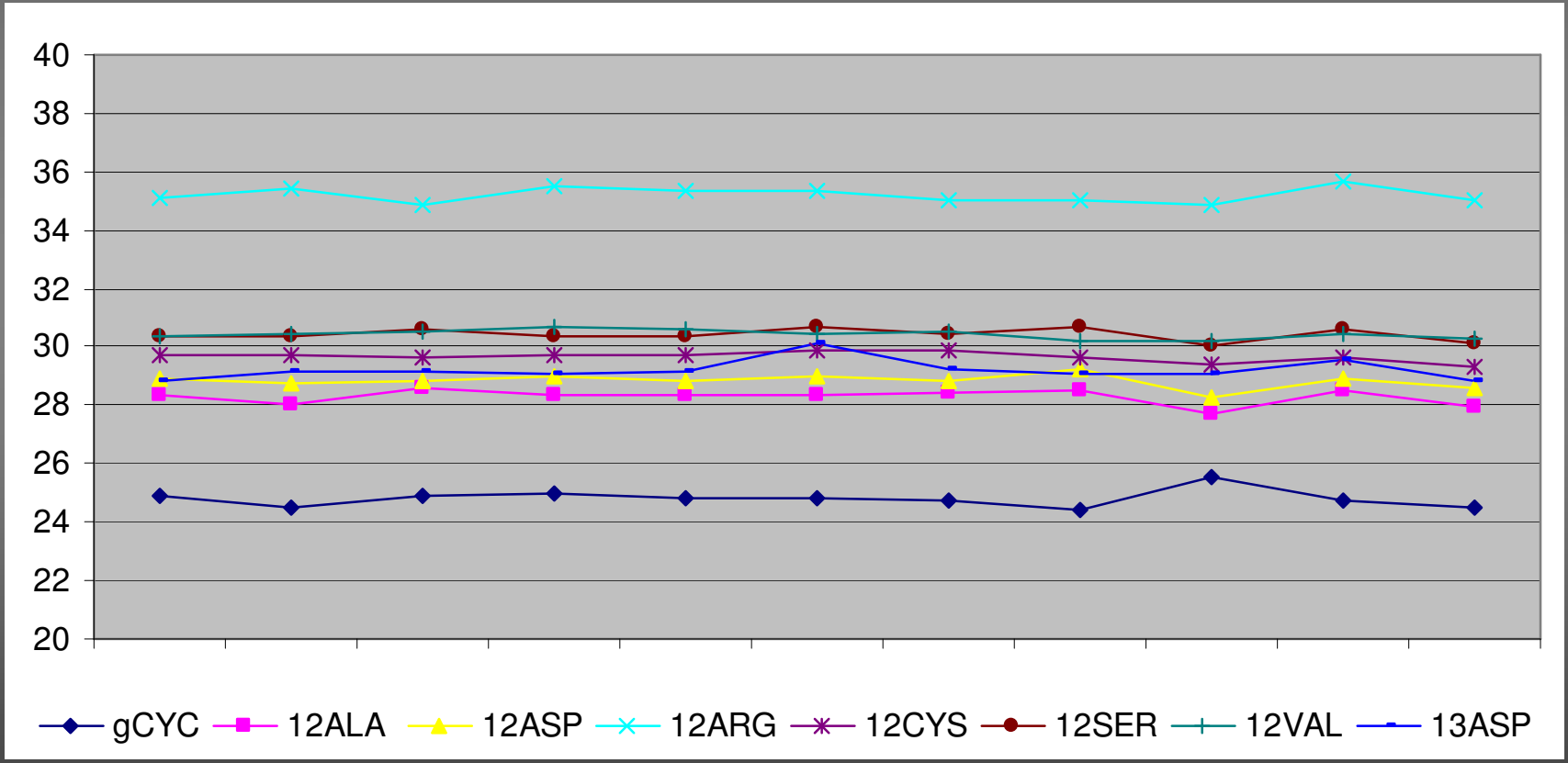
Controls and QC

- Replicates
  - qPCR in duplicates (diagnostic setting)
  - qPCR in triplicates (MRD setting)
- Controls (mandatory in each analysis)
  - negative H2O + wt sample (e.g. normal donor) + positive sample (e.g. cell line)
- QC
  - internal (day to day)
  - external (national and/or international, every 6 - 12 months)

QC of *KRAS* assay over time: DxS kit control DNA

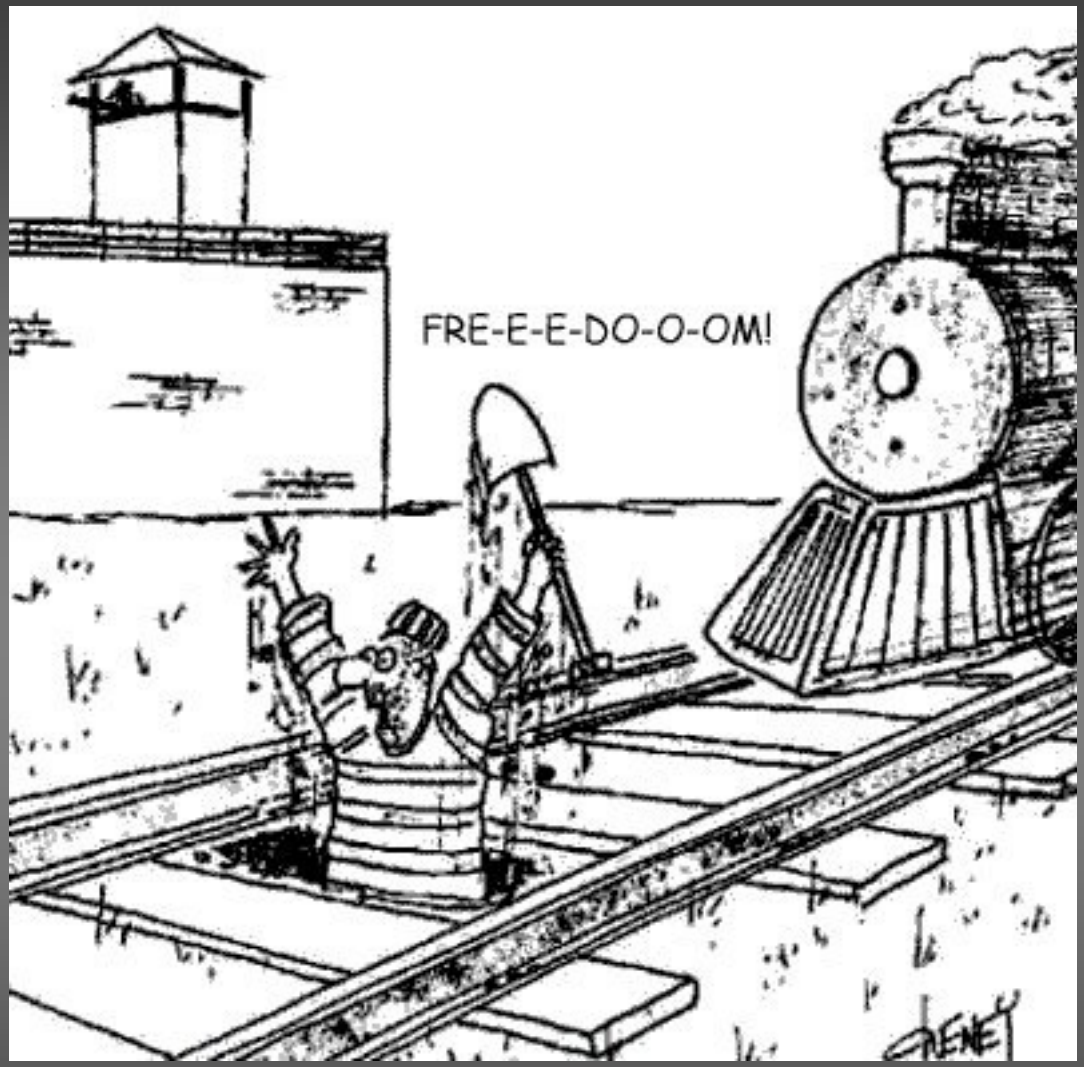


QC of in house *KRAS* assay over time: *KRAS* Mutated PCR fragments spiked into normal donor DNA



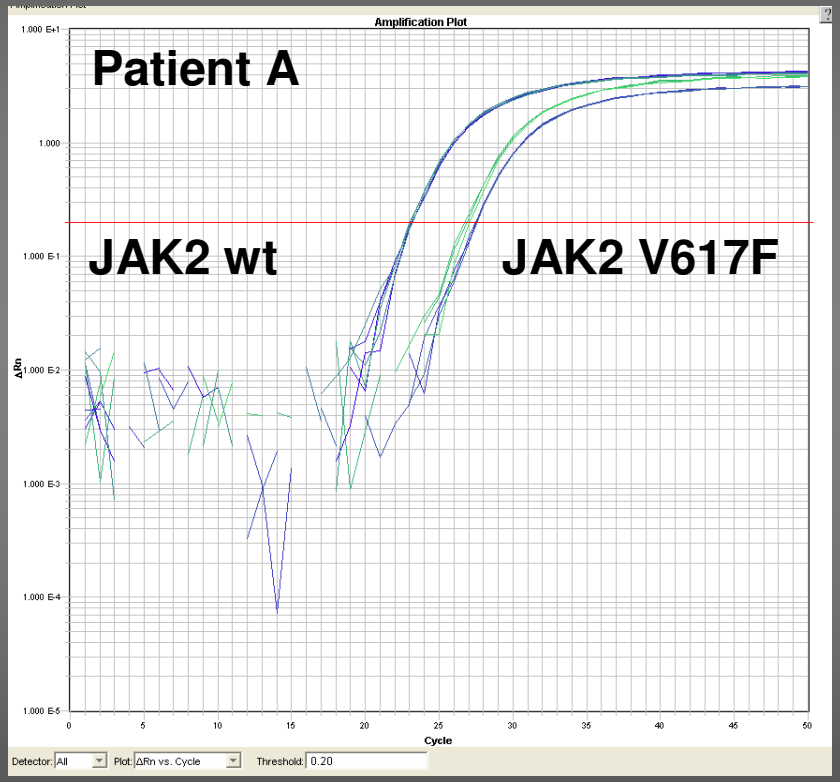
KRAS Quality Control in CRC

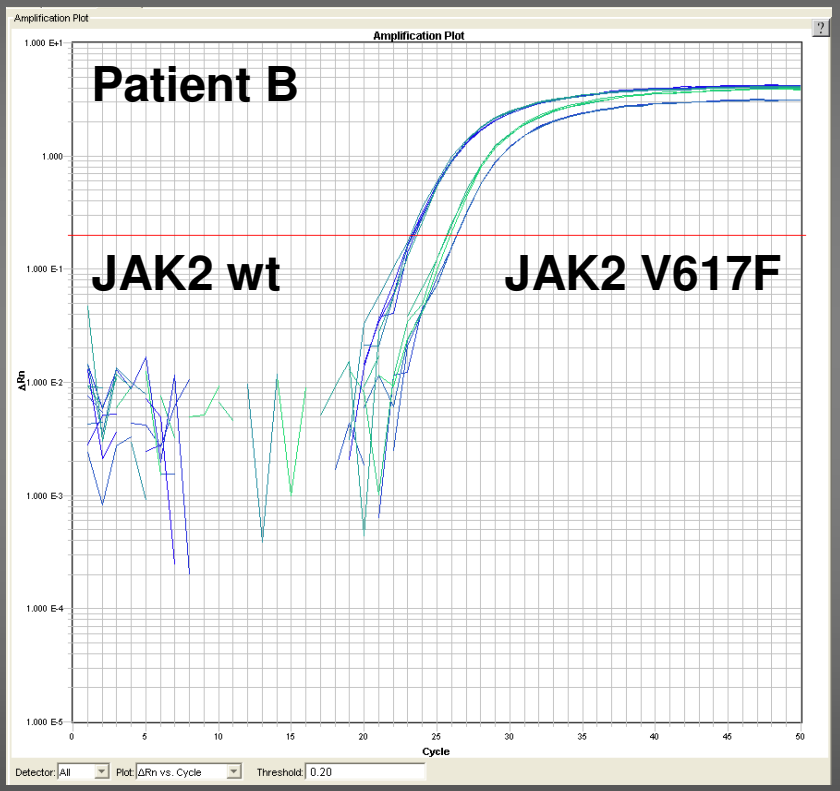
- Analysis sensitivity of negative samples should always be calculated
- Negative samples with a sensitivity >5% should be conferred with pathologist to conclude on negativity
- In CRC samples should be micro-dissected if adenoma is present
- If synchronous tumors both should be analyzed
- Control reactions should be plotted (e.g. in Excel) to monitor analysis stability
- Lab should participate in international a validation/quality control program(s) e.g. ESP
- Lab should run >250 samples per year in order to gain/keep sufficient experience and for the analysis to be economical



If everything seems to be going well, you have obviously overlooked something

Primer-mix Batch Variation

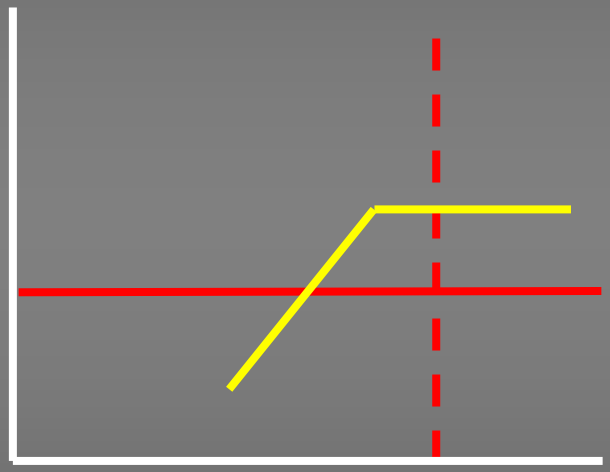




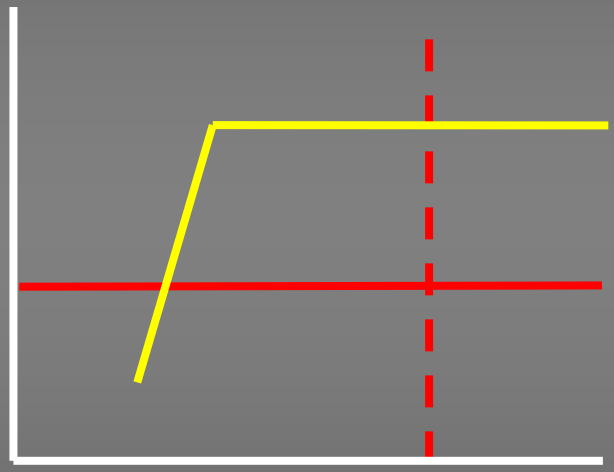
The JAK2 V617F mut primerset differs 0.8 Ct between new and old set

qPCR Inhibition

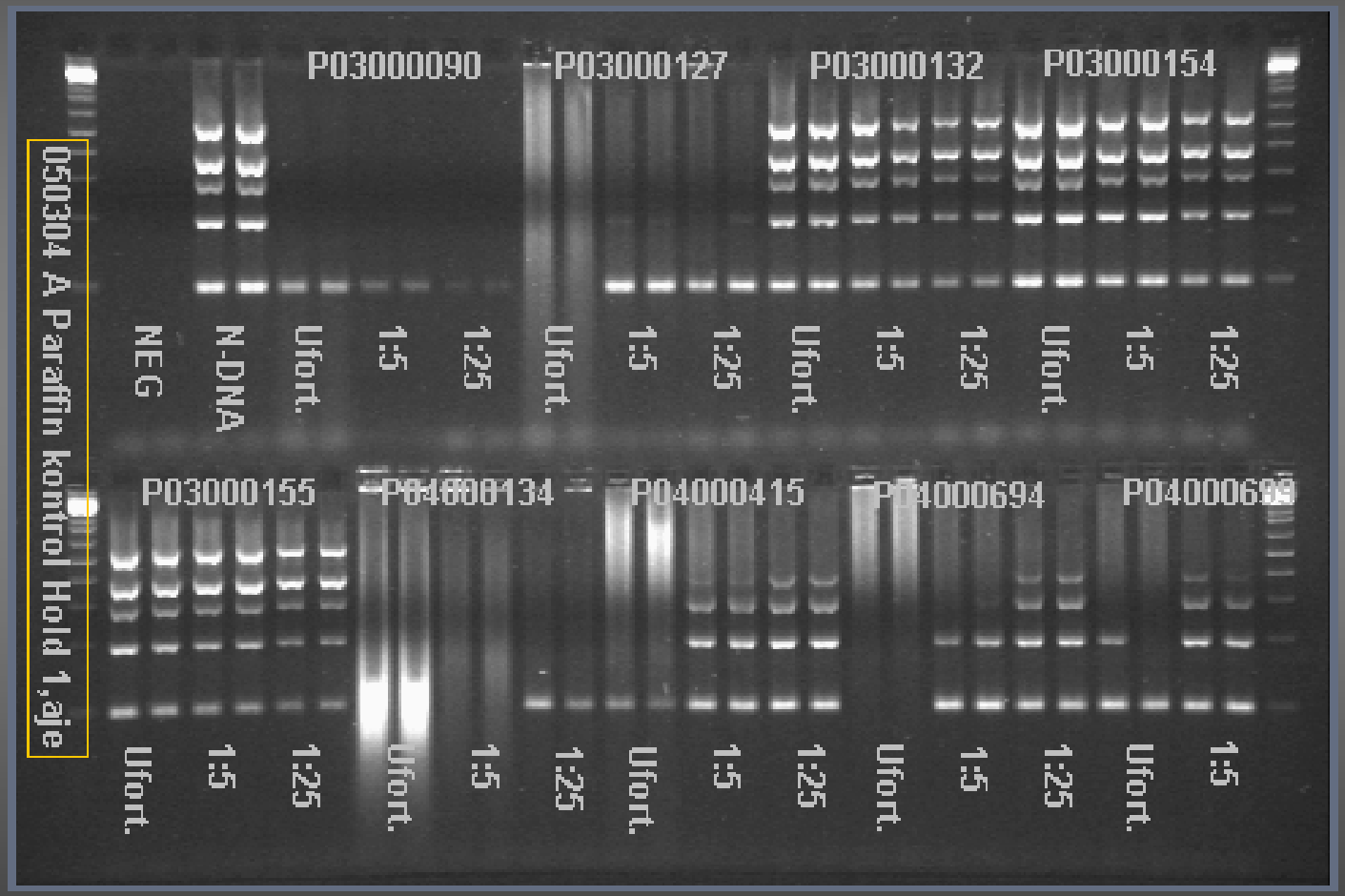
Non-diluted DNA



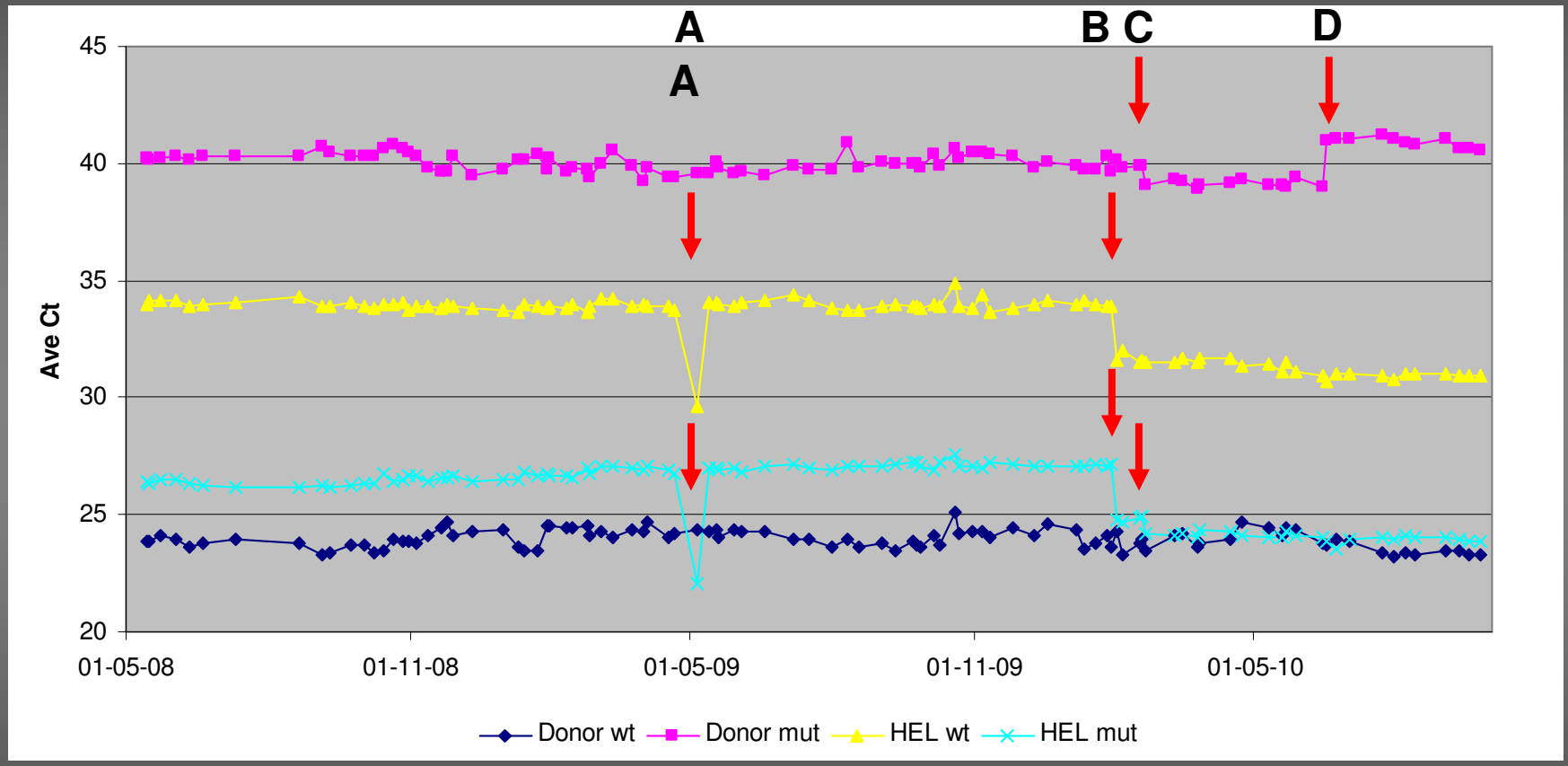
Diluted DNA



FFPE DNA QC – Integrity and Inhibition

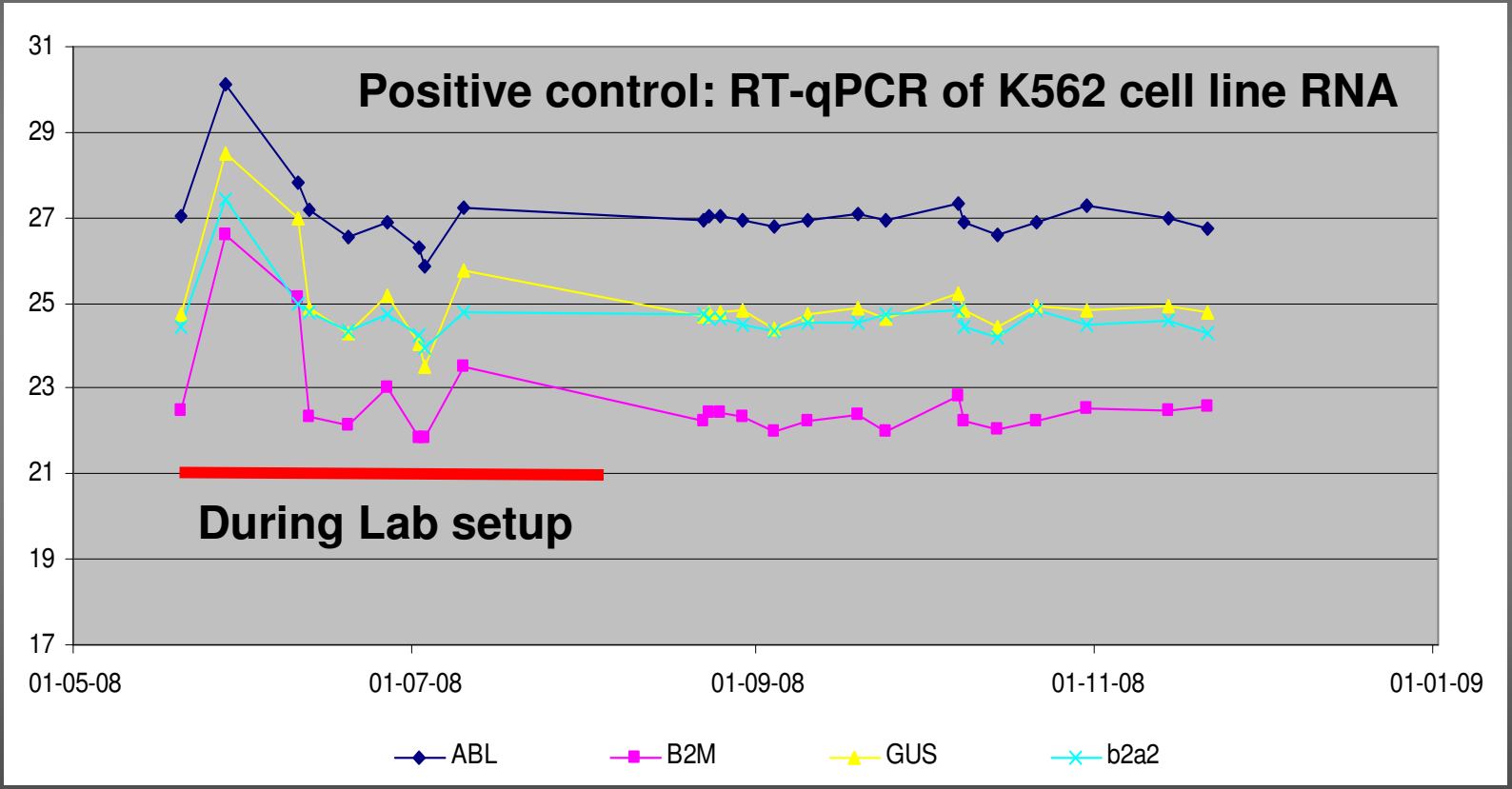


JAK2 QC - internal (day to day)



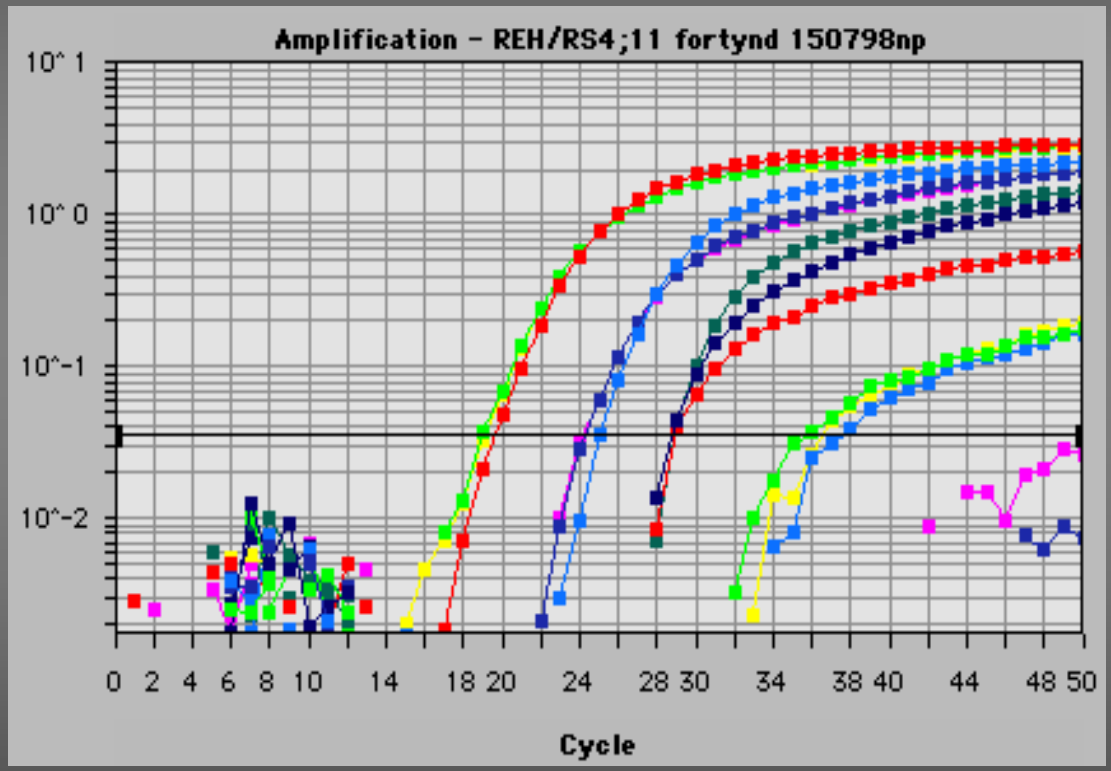
A: Wrong batch of HEL DNA (high concentrated DNA) used as positive control, B: New batch of HEL DNA of higher concentration, C: New batch of JAK2V617F primer set, D: Addition of blocking oligo to increase assay specificity.

CML RT-qPCR QC



Optimization of primers for t(12;21) (II)

Ten fold dilutions of t(12;21)+ REH cell line RNA into 1 µg RS4;11 RNA (negative for t(12;21))



Bad t(12;21) primer set

PCR

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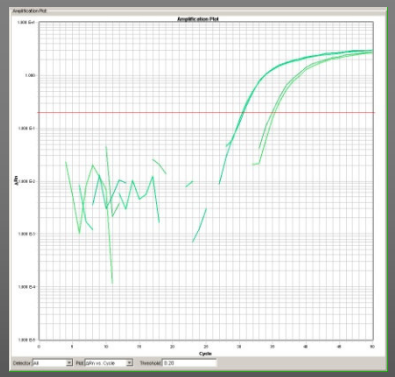
Plasma cfDNA Results

Plasma cfDNA analysis

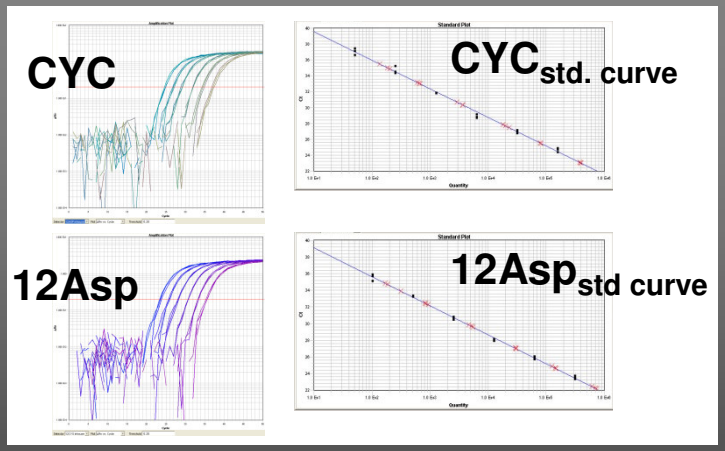
Blood sample

Plasma

DNA purification

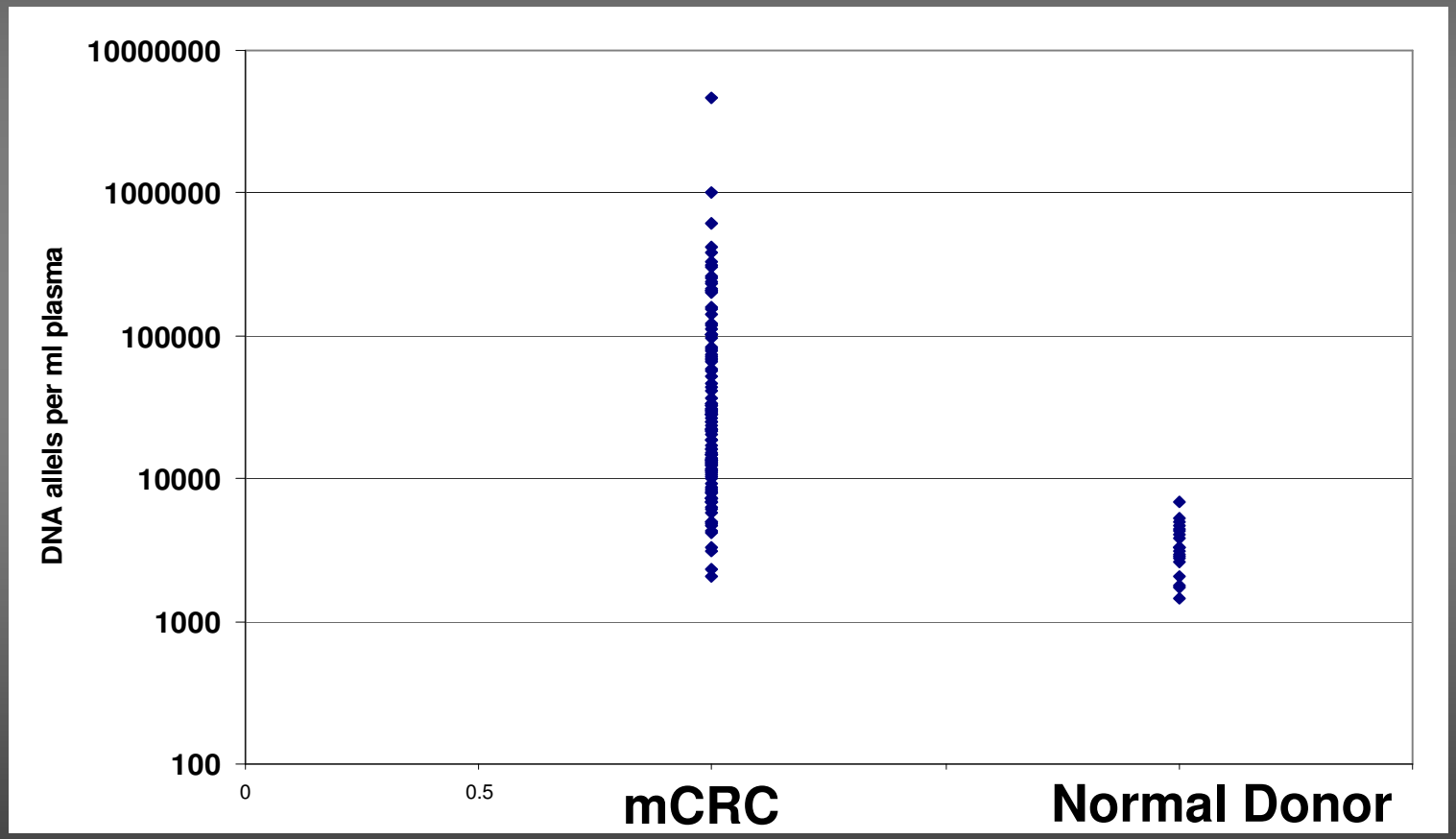


qPCR analysis
(cfDNA$_{cyc}$ and KRAS$_{mut}$)



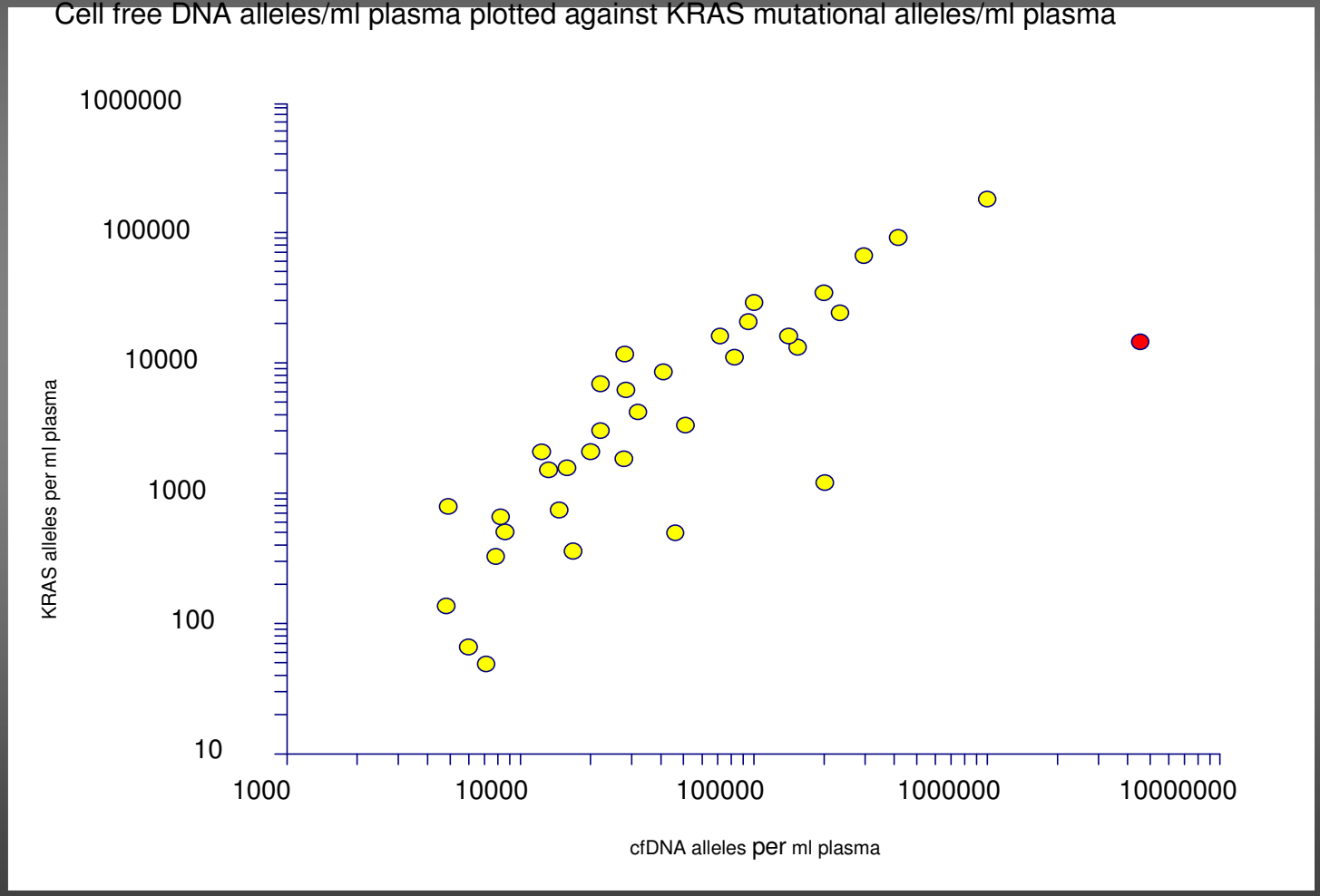
Calculation of allele copy number
based on standard curves

Plasma cfDNA level in mCRC Pt.



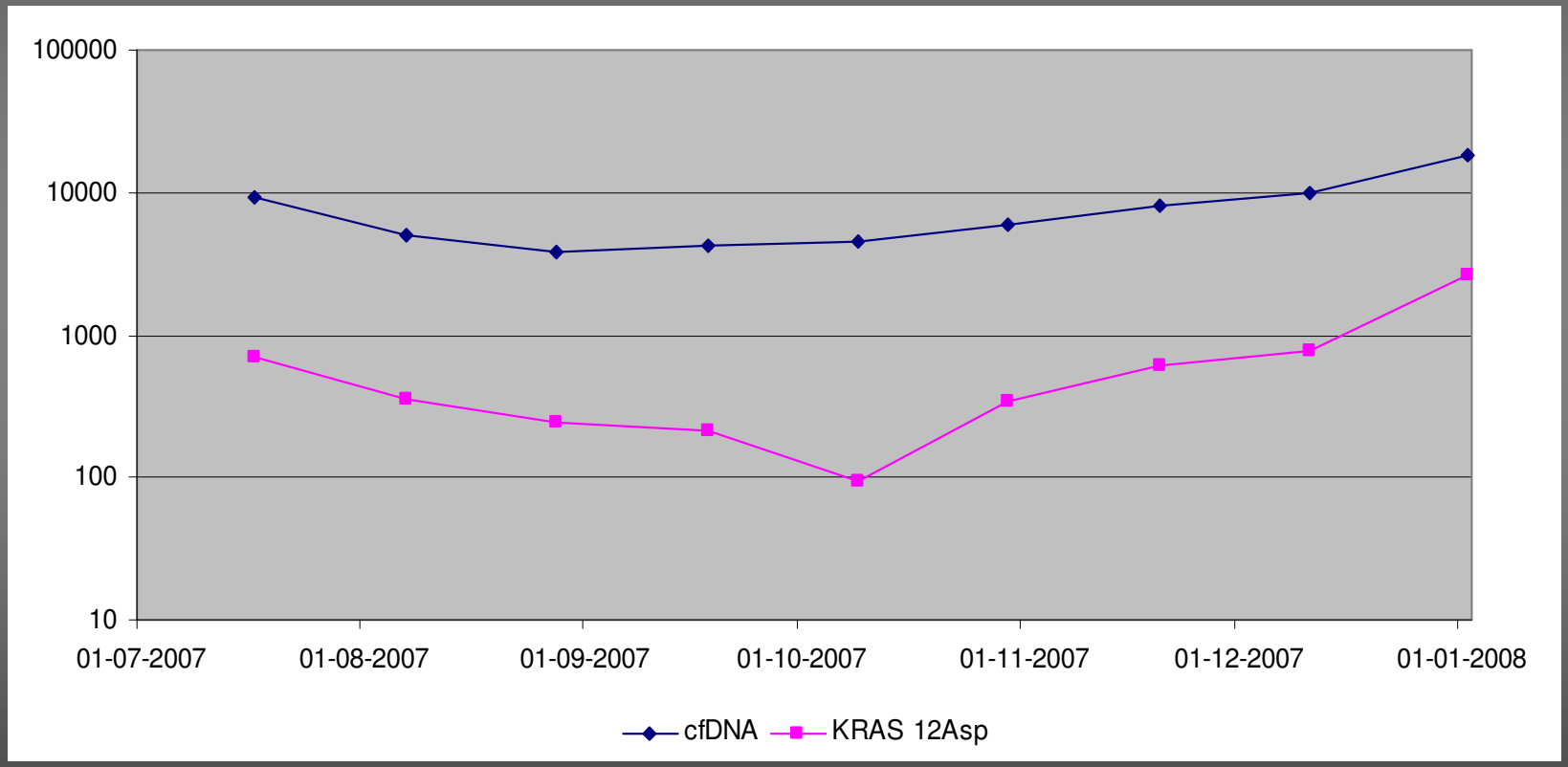
CRC Pt. n=117, ND n=19

Mutated *KRAS* alleles versus cfDNA alleles

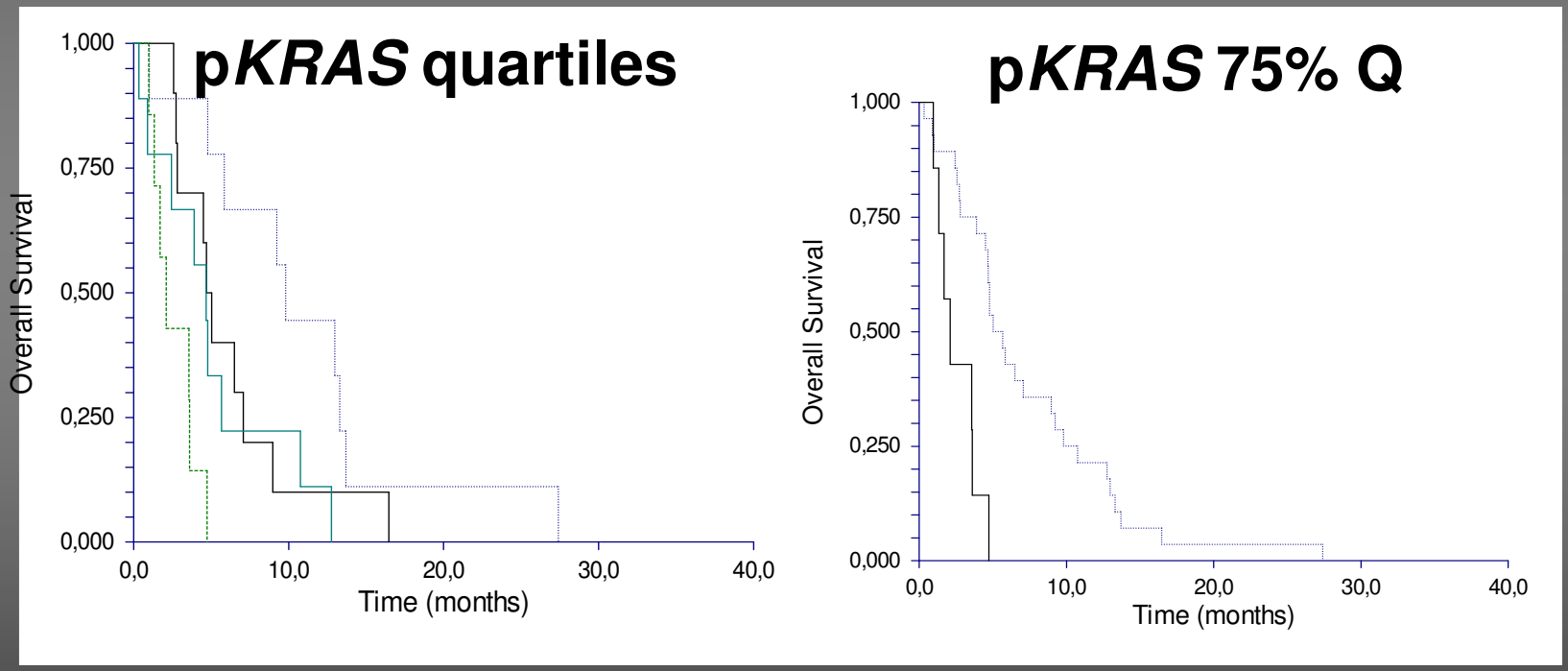


Spindler et al **Clin Cancer Res 2012**

cfDNA and p*KRAS* in 3. line mCRC 12Asp+ Pt. treated with Erbitux

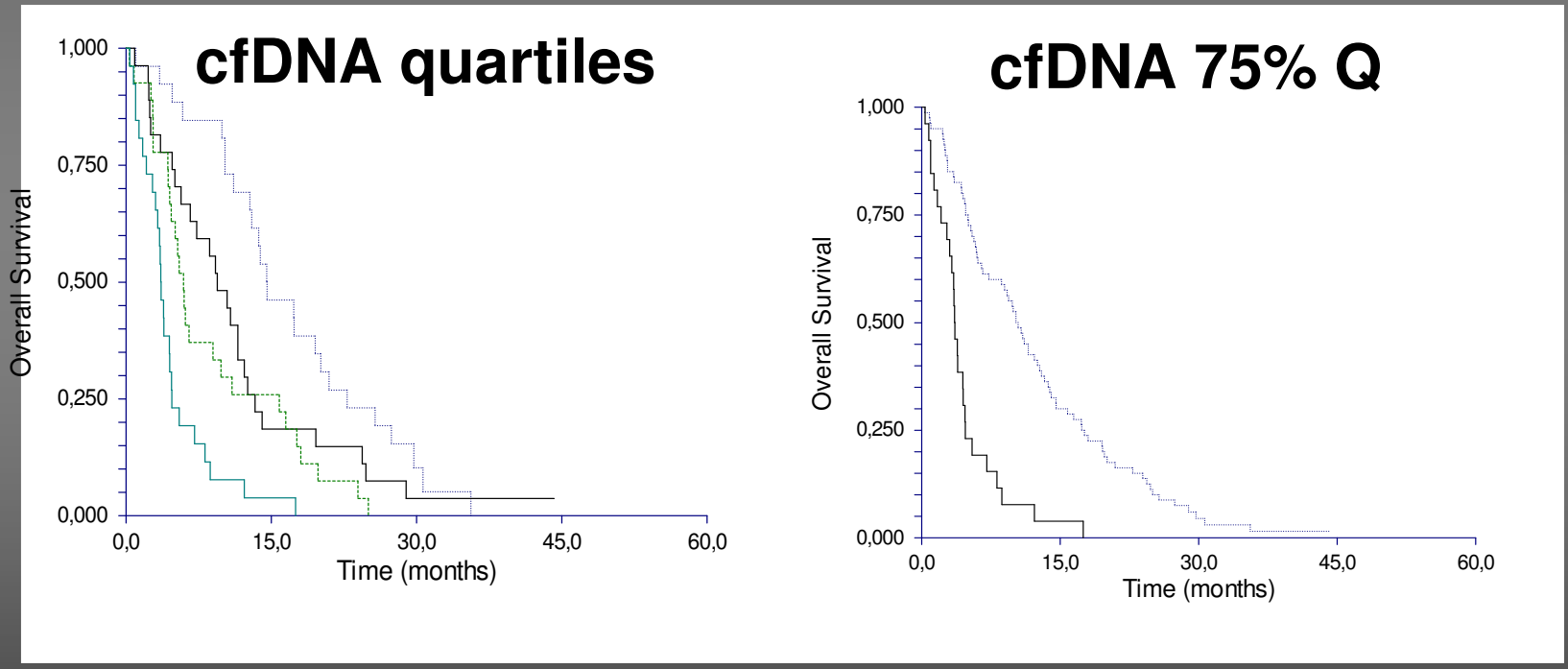


Overall Survival



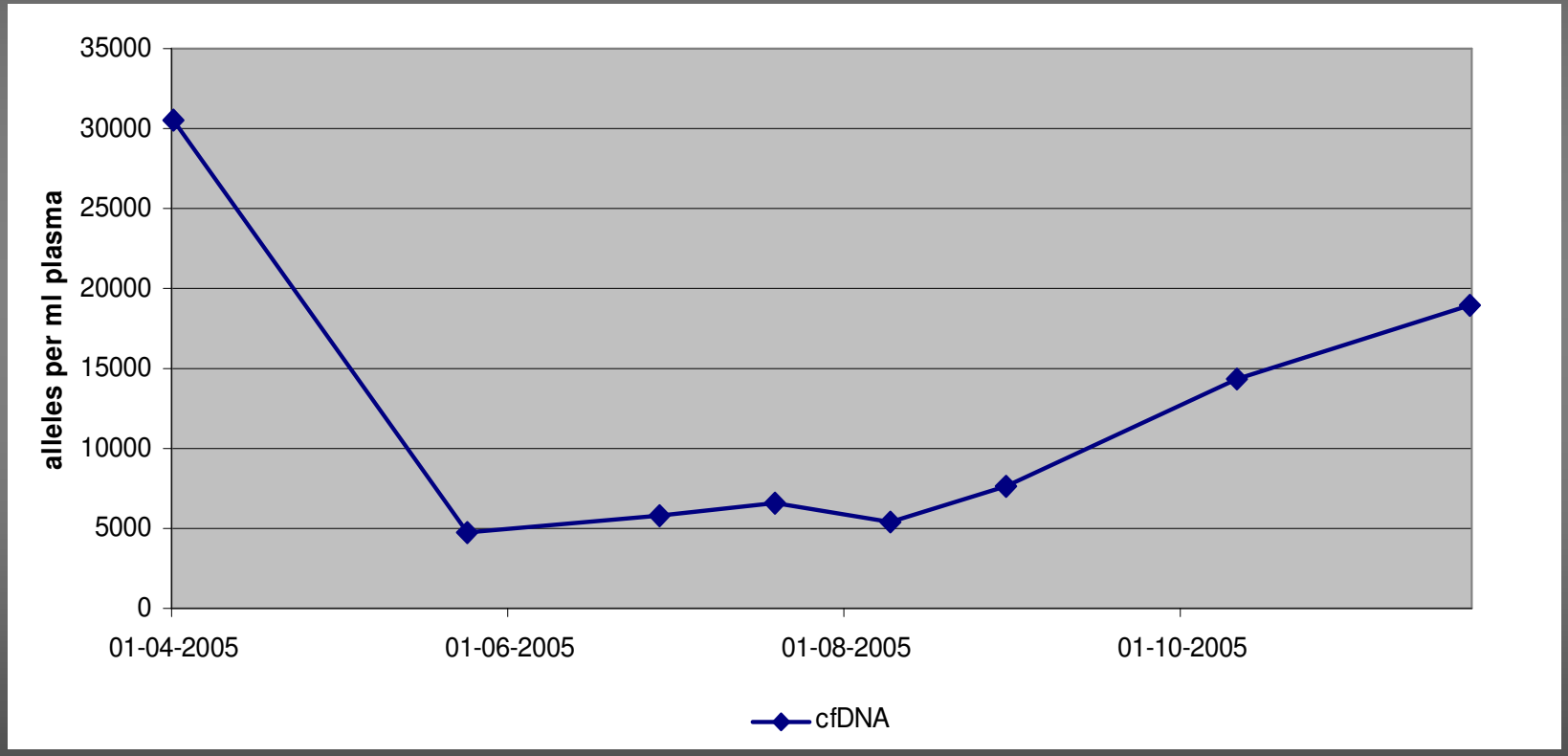
Spindler et al **Clin Cancer Res 2012**

Overall Survival



Spindler et al **Clin Cancer Res 2012**

Plasma cfDNA in 3. line mCRC *KRAS* wt Pt. treated with Erbitux



Mutations status during treatment

< Erbitux >

Primary tumor	Metastasis	Plasma baseline	Plasma progression
12Val	?	wt	12Val
12Arg	13Asp	13Asp	13Asp
12Ala	wt	wt	wt
12Val	?	12Val +V600E	-
wt	wt	wt	V600E
wt	wt	wt	12Ala
wt	wt	wt	12Arg
wt	wt	wt	12Val

Time

Spindler et al in press

Conclusions of cfDNA in Plasma

- Plasma DNA (cfDNA) levels are elevated or highly elevated in the majority of mCRC Pt.
- *KRAS* mutation in CRC primary tumor may be detected in plasma from mCRC in 80%
- Quantitative levels of cfDNA and p*KRAS* were strongly correlated to clinical outcome of 3. line treatment of mCRC
- Mutational status may change during the course of treatment
- *KRAS* and *BRAF* analysis in plasma samples could be an alternative to tissue analysis
- Quantification of plasma cfDNA holds promise of clinical application

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PCR

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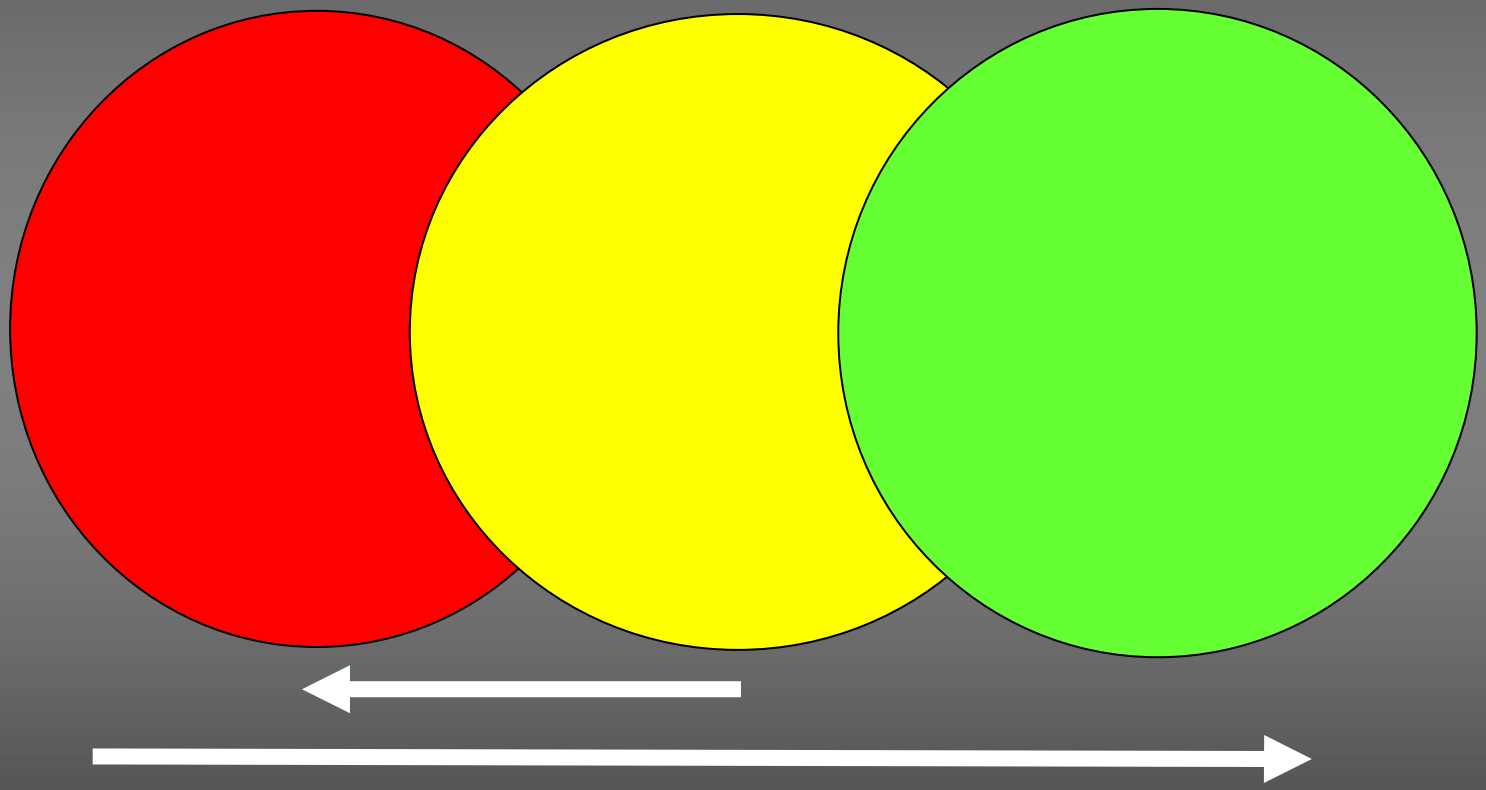
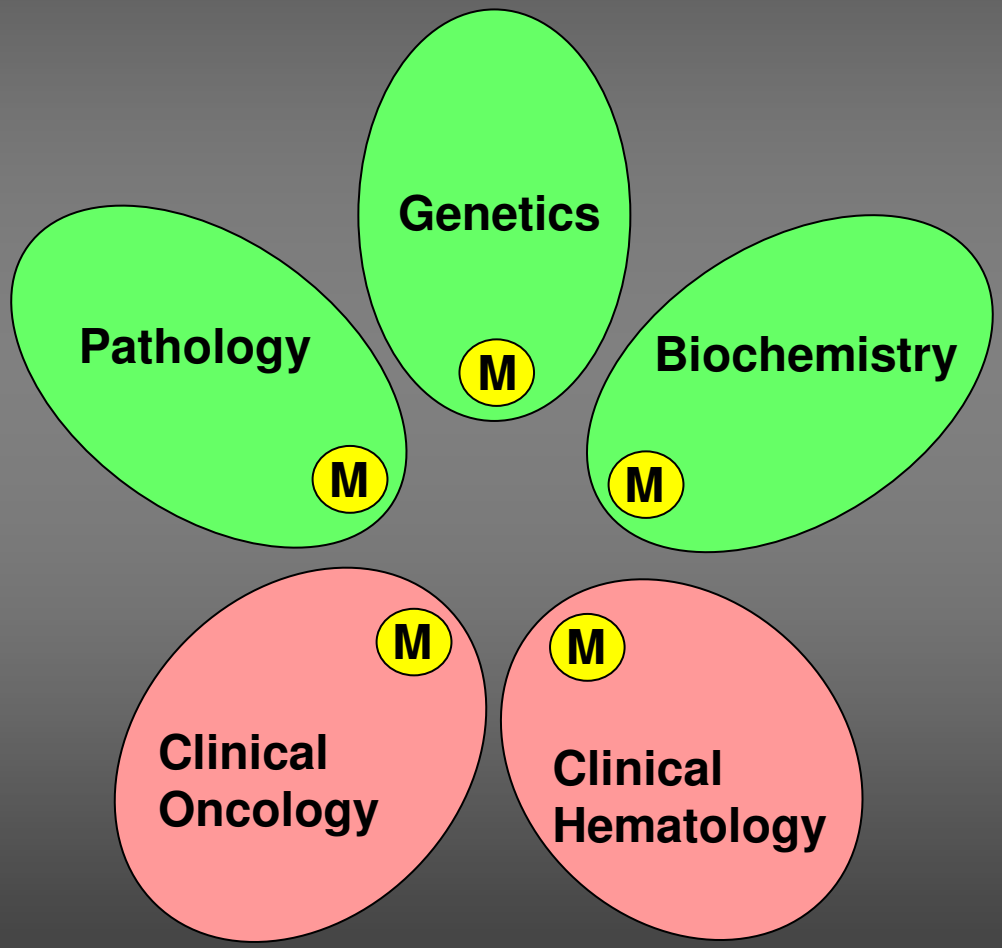
Potential Clinical Results

Tasks for Molecular Biology Laboratory

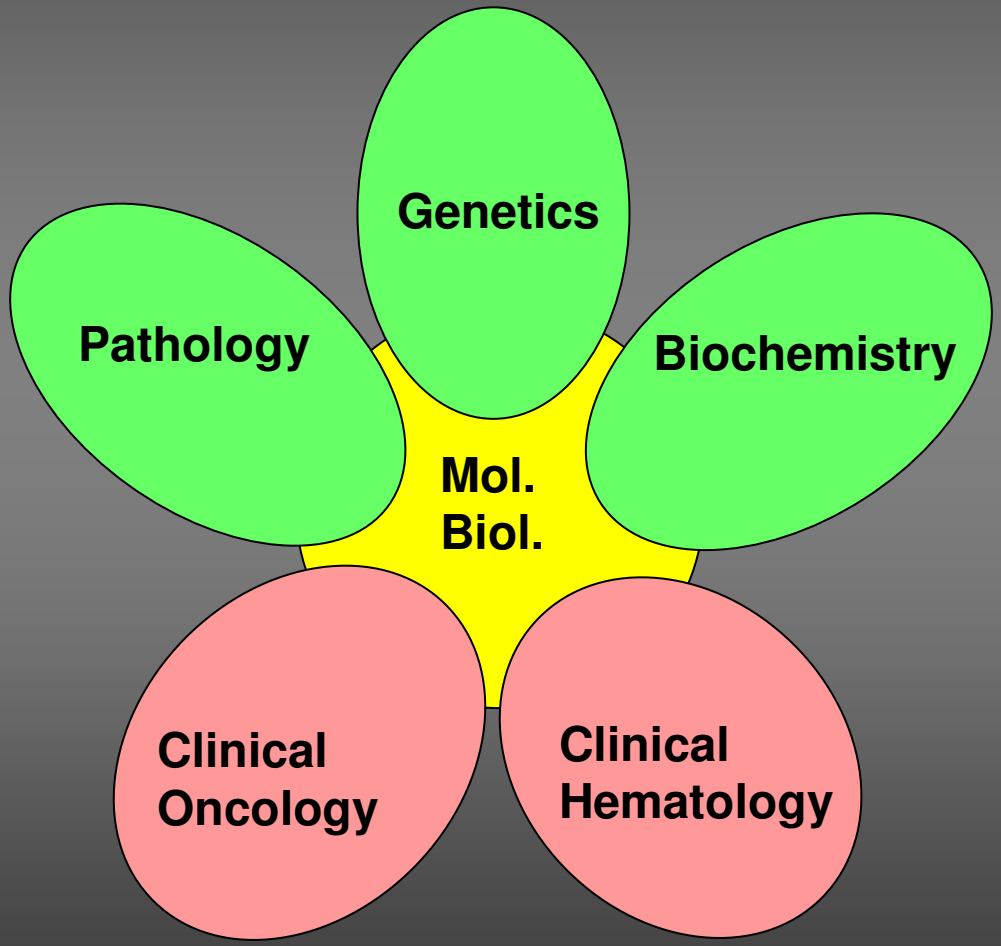


Present (typical) Organization



Each department have own molecular biology laboratory (M) with equipment and staff.

Future Organization



Molecular biology laboratory as one central unit.
Advantage:

- Synergy between personal;
- Broader range of equipment
- Backup equipment and staff
- Synergy in workflow
- Common analyses

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- Cheaper analyses
- Shorter analysis time
- Better research possibilities
- Synergy in development
- More stabile production

Close Communication between Laboratory and Clinical Practice is Essential