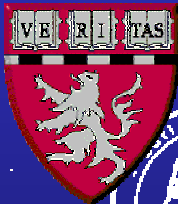


TripleHyb real time PCR for detection of single nucleotide polymorphisms in the VEGF promoter region



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aj ROBOSCREEN



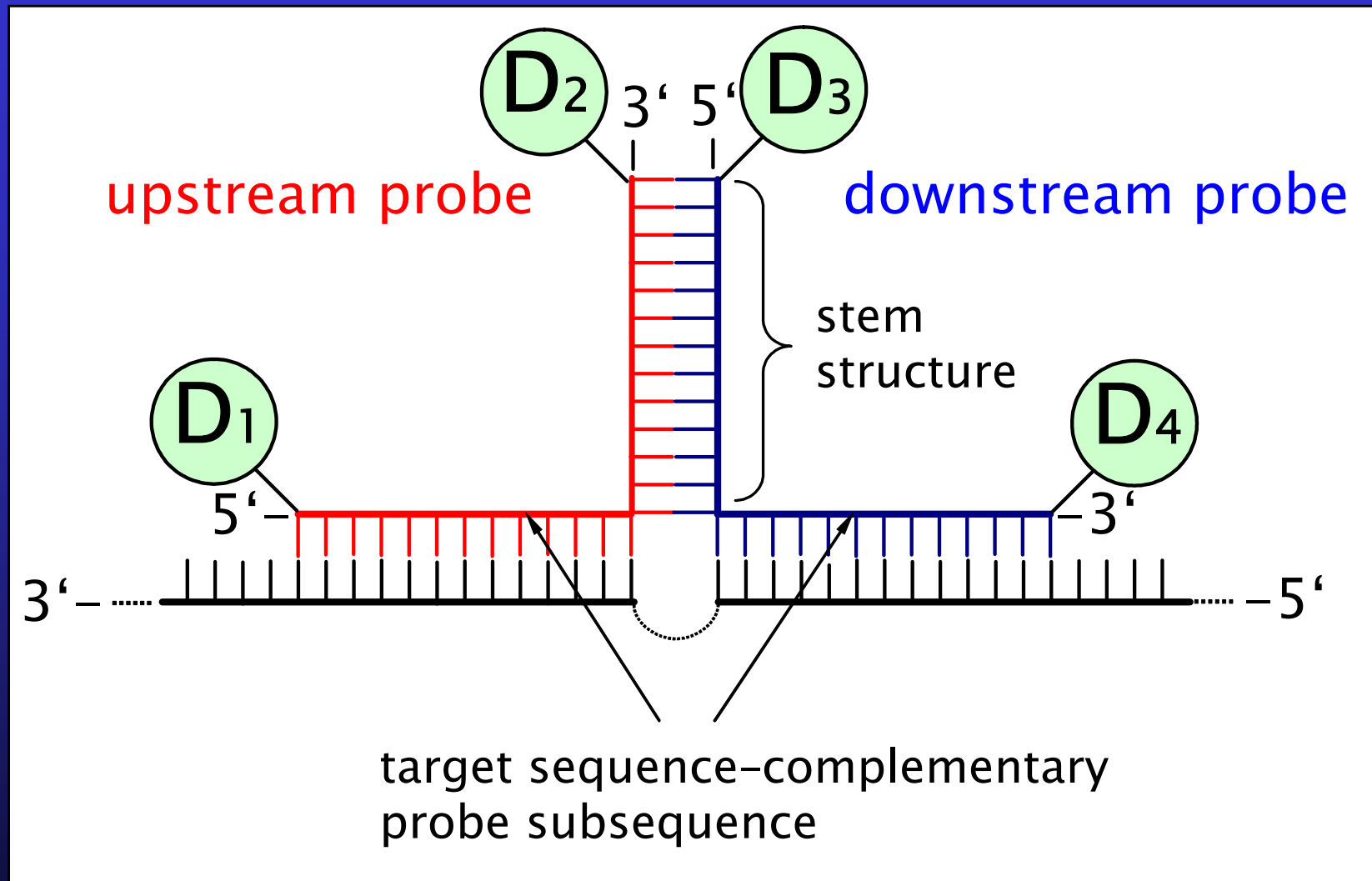
Granted by technology support with financial sources of the European Regional Development Fund and the State of Saxony

Application of a new detection format

- establishment of a novel detection format for real-time PCR supporting quantitative applications
- suitability for genetic polymorphism detection
- improved or same sensitivity, specificity, flexibility, and robustness compared to available formats
- full compatibility with the most commonly used real-time instrument platforms
- no need for external licenses

↳ development of the **TRIPLEHYB[®]** probe format

TRIPLEHYB[®] probe format: basic principle



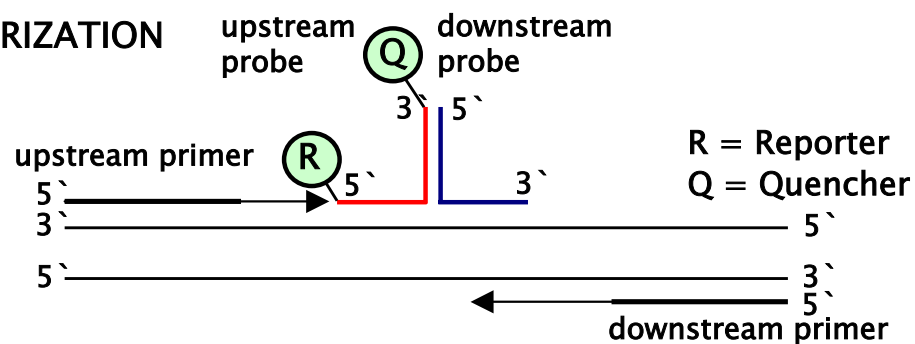
- D₁ – D₄: positions for the attachment of different dyes
 - ↳ D₁ → fluorescent dye & D₂ → quencher

TRIPLEHYB[®] probe format: probe design

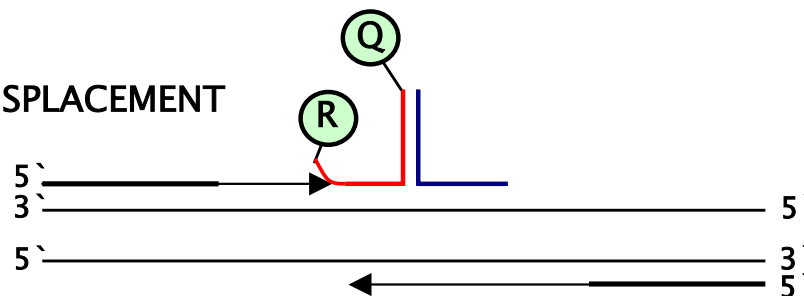
- design of TaqMan primers and probes using a suited software (e.g. PrimerExpress, Applied Biosystems)
- retaining of original TaqMan primers
- splitting of TaqMan probe into two target-complementary subsequences (about 9–15 bp length)
- appending of a target-unrelated, inter-molecular stem-forming subsequence to both the 3'-end of the upstream and the 5'-end of the downstream probe
- optimal stem length: 9 bp
- need for both up & do probes for high, robust signals

Proposed mechanism of TRIPLHYB[®] real-time fluorescence PCR format

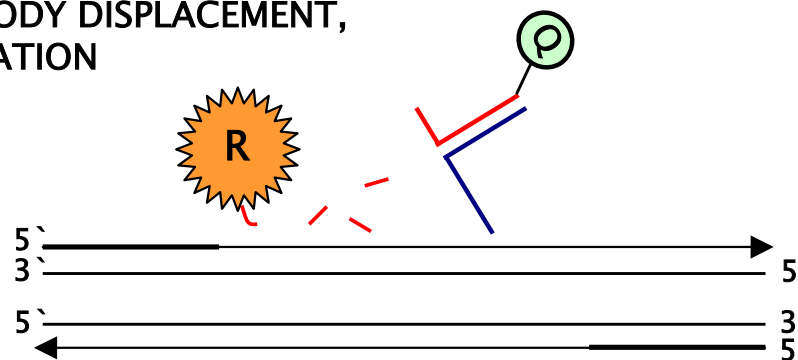
1. POLYMERIZATION



2. STRAND DISPLACEMENT



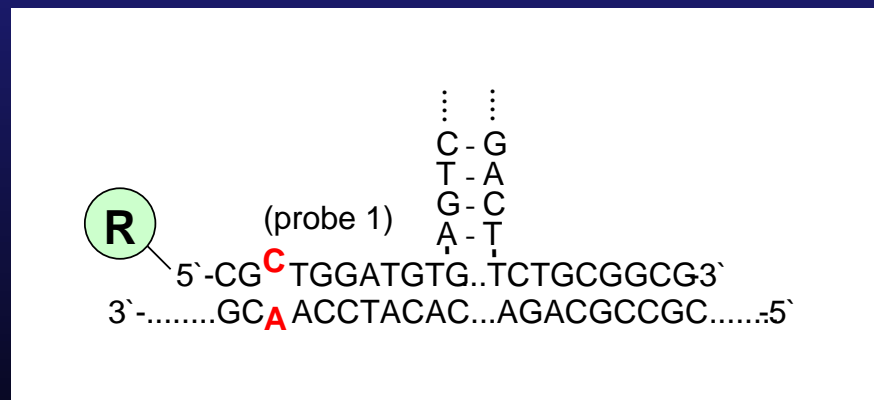
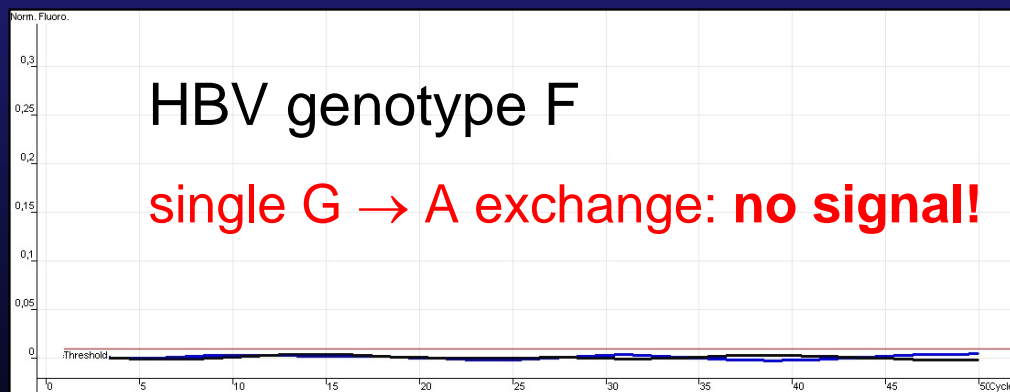
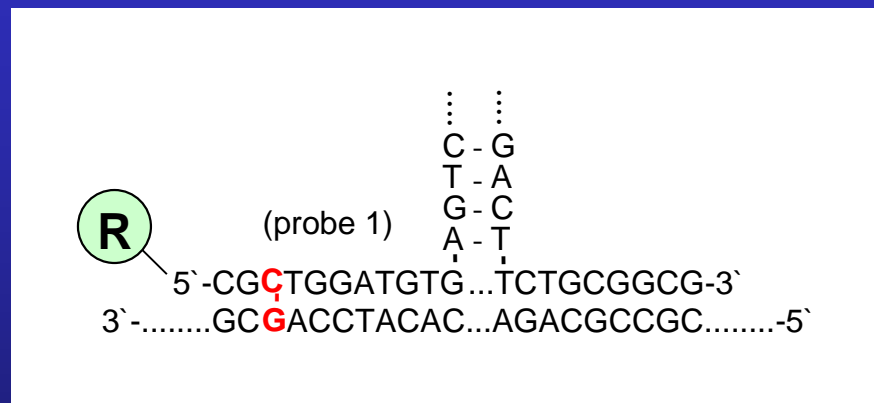
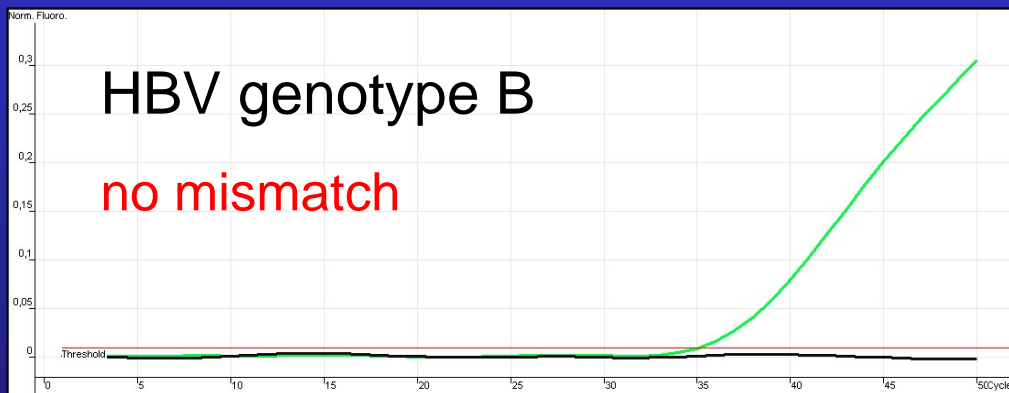
3. CLEAVAGE, TRIPLEX BODY DISPLACEMENT, COMPLETE POLYMERIZATION



TRIPLEHYB[®]: detection of point mutations

TripleHyb[®] particularly suited for detection of polymorphisms

↳ accidental finding during Hepatitis B virus genotyping!

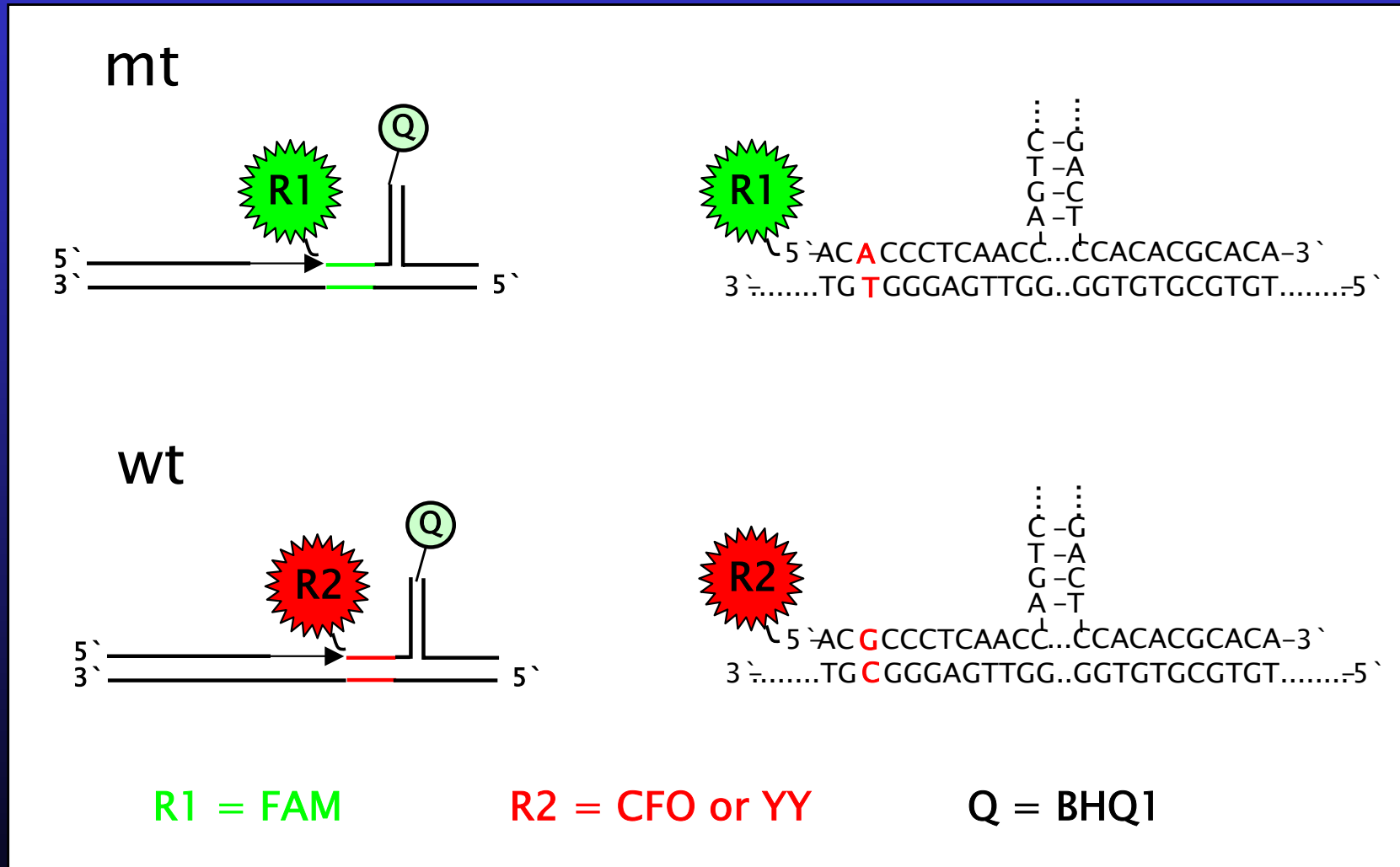


TRIPLEHYB[®]: evaluation in SNP detection

- should be generally useful for SNP detection due to short hybridization length in up-probe
- known: base exchange in position 3 abolishes up-probe binding to template → no signal
- unknown: at which positions it works too?
- model system: SNP C→T at position 460 of the VEGF promoter
- known association to different diseases including predisposition to tumorigenesis

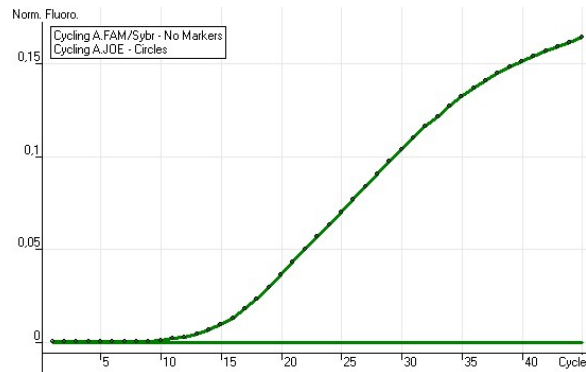
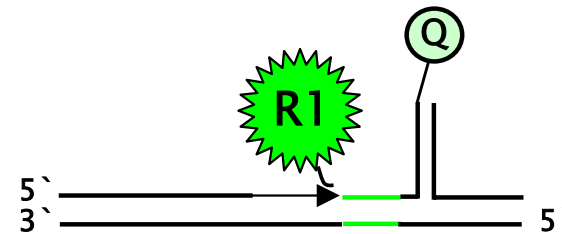
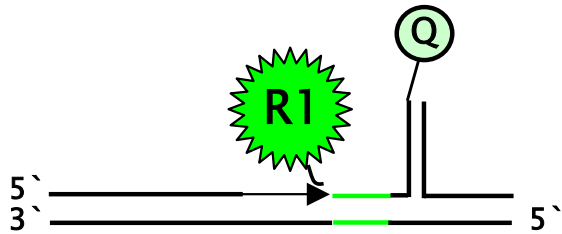
C460T polymorphism of the VEGF promoter

probes containing the SNP at position 3 of the upstream probe



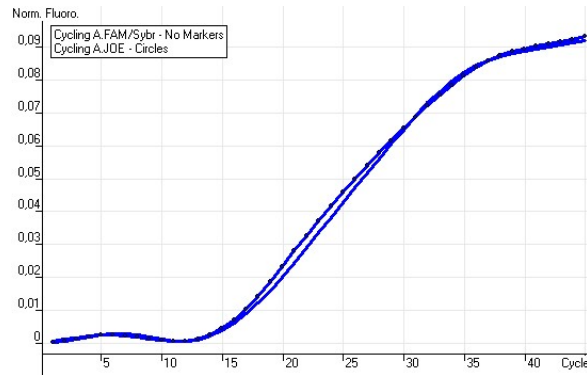
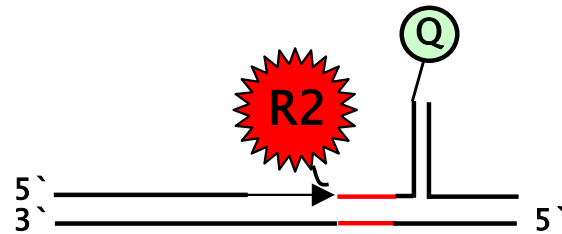
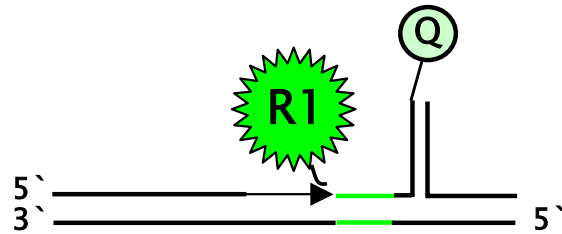
Genotyping of VEGF-C460T at position 3

homozygous mutant



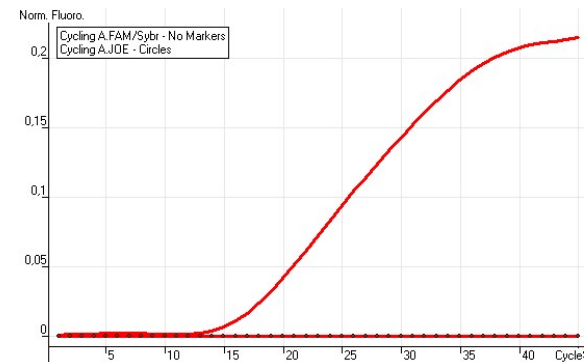
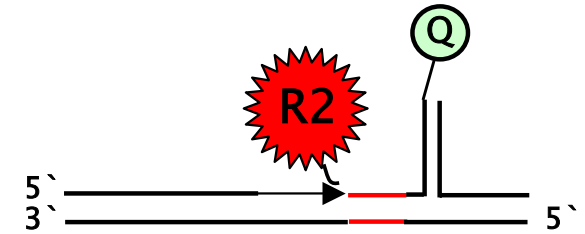
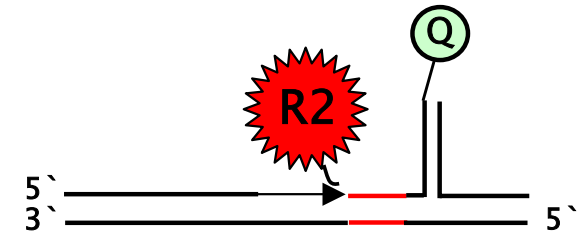
R1 = FAM

heterozygous



R2 = CFO or YY

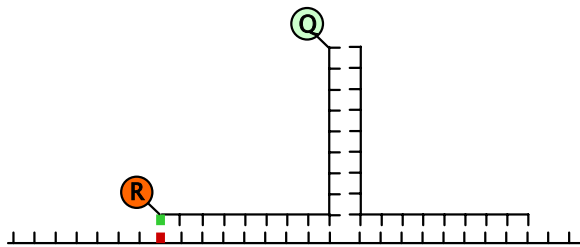
homozygous wild-type



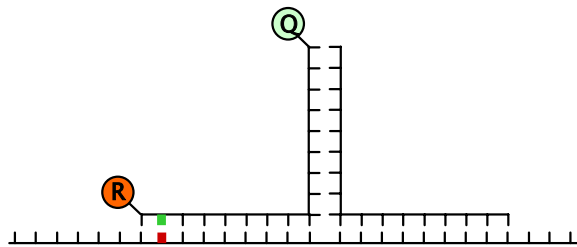
Q = BHQ1

SNP C460T at position 1–5 of the up–probe

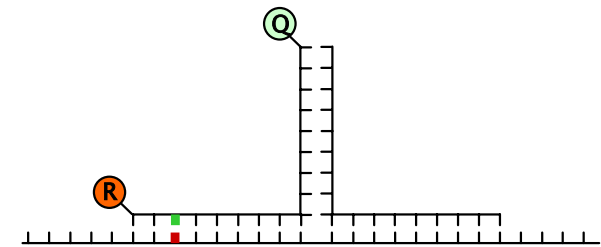
system 1



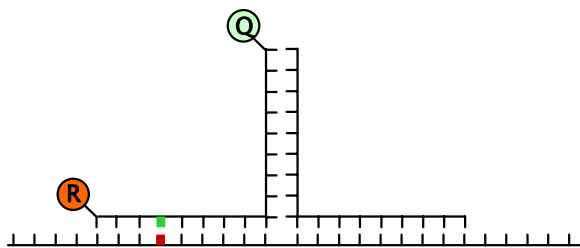
system 2



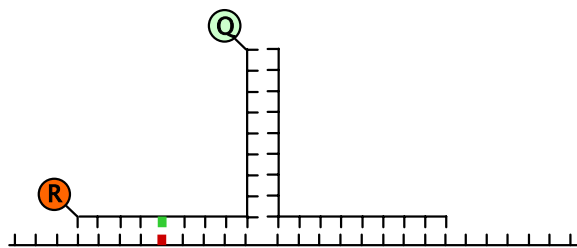
system 3



system 4



system 5



set of 3 probes for each of the 5 systems:

- up–probe for wt
- up–probe for mut
- do–probe

↪ systematic analysis of SNPs at positions 1 to 5 of the upstream probe

Optimization of assay performance

- systematic optimization of assay conditions and evaluation of system flexibility
 - ⇒ use of standard plasmids containing wt or mut
 - ⇒ probe ratios and concentrations
 - ⇒ primer concentrations
 - ⇒ MgCl₂ concentrations
- final aims:
 - ⇒ find suitable SNP-positions in up-probes
 - ⇒ validation using clinical samples
 - ⇒ relevance of VEGF C460T for prostate cancer?

TRIPLEHYB[®]: ratio of probes

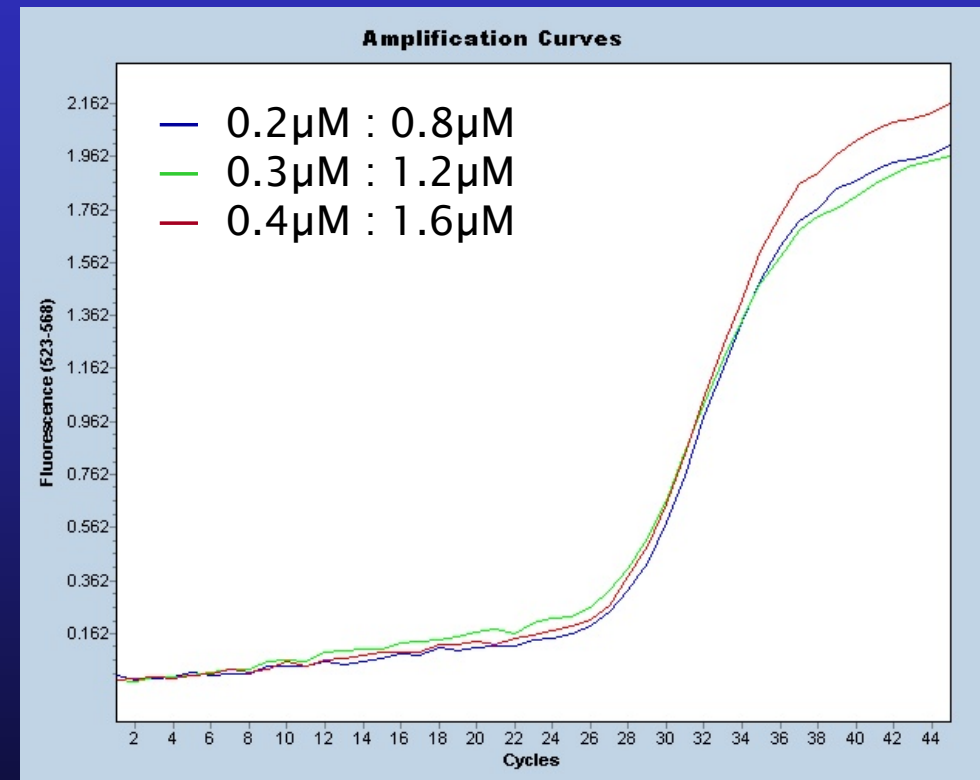
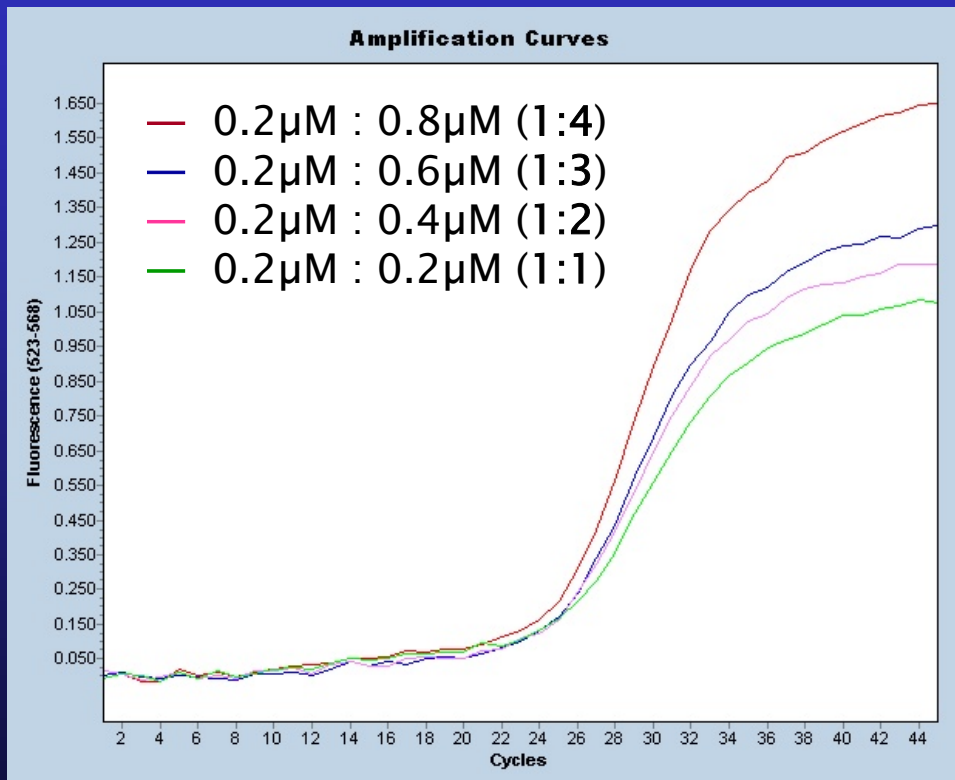
probe matrix: molar excess of downstream probe required

upstream : downstream

1:1 / 1:2 / 1:3 / 1:4

1:4 at different concentrations:

similar performance

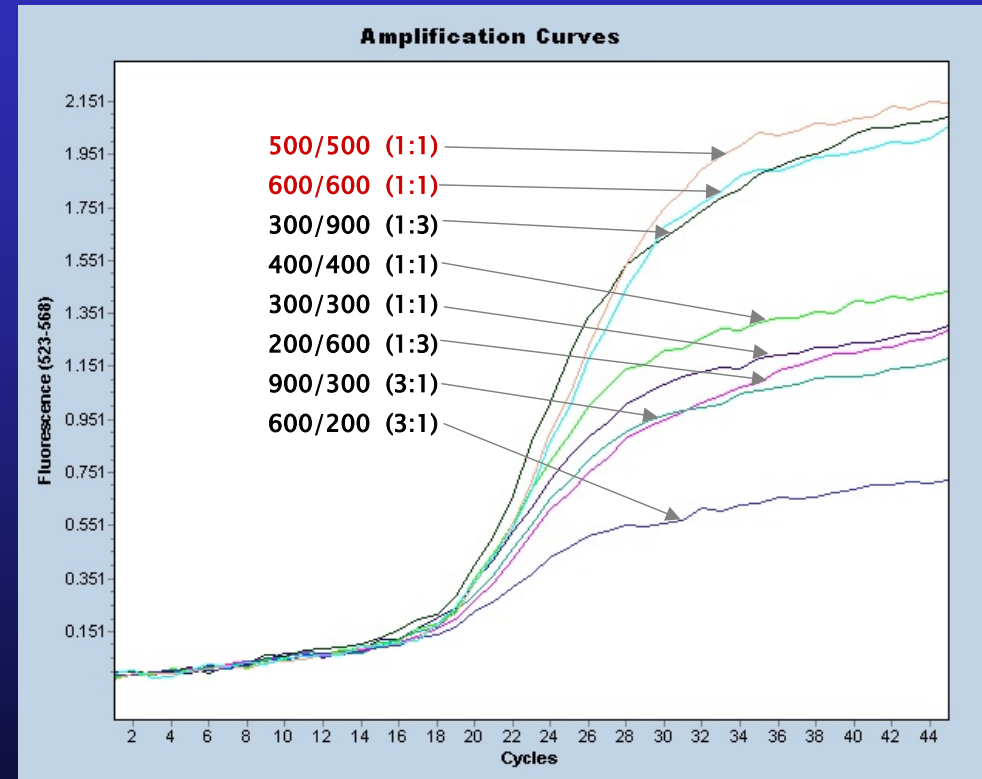
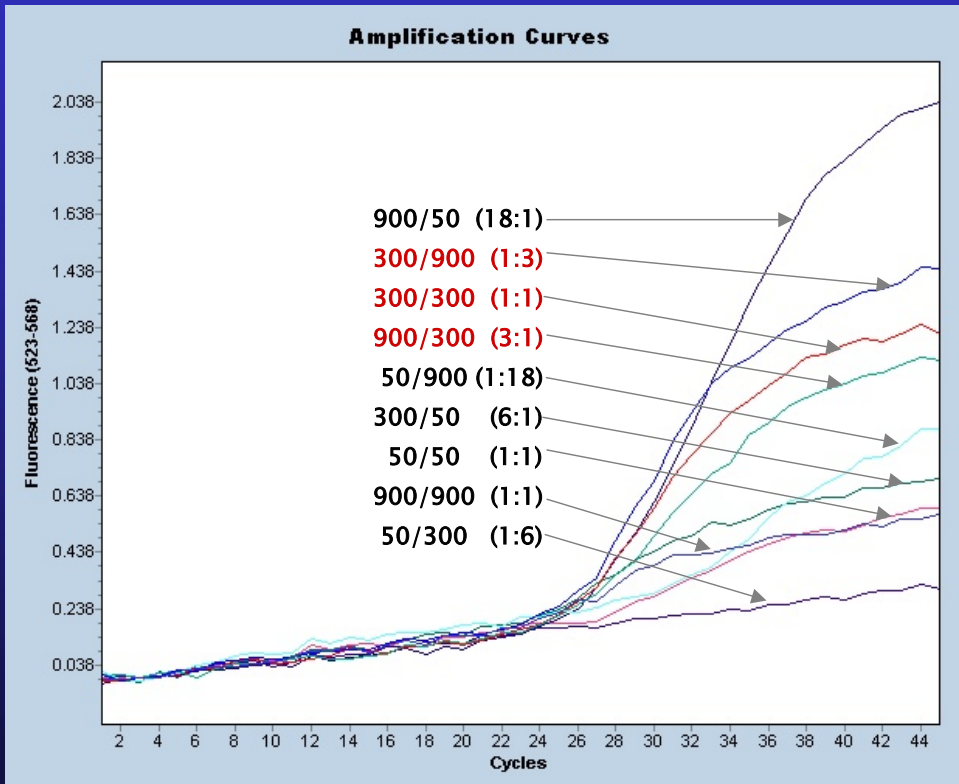


3-step PCR (45 cycles; LC480): 95°C/15s; 45°C/1s; 59°C/40s, system 3;
primers: 0.5 μ M each; probes: CFO-up + do, varying ratios and concentrations;
MgCl₂: 5mM; templates: 10⁴ molecules wt-plasmid

TRIPLEHYB[®]: primer matrix

primer matrix 1: 50 / 300 / 900nM
ratios 1:1 / 1:3 / 3:1 promising
medium concentration range

primer matrix 2: 200–900nM
equimolar best
medium concentration range



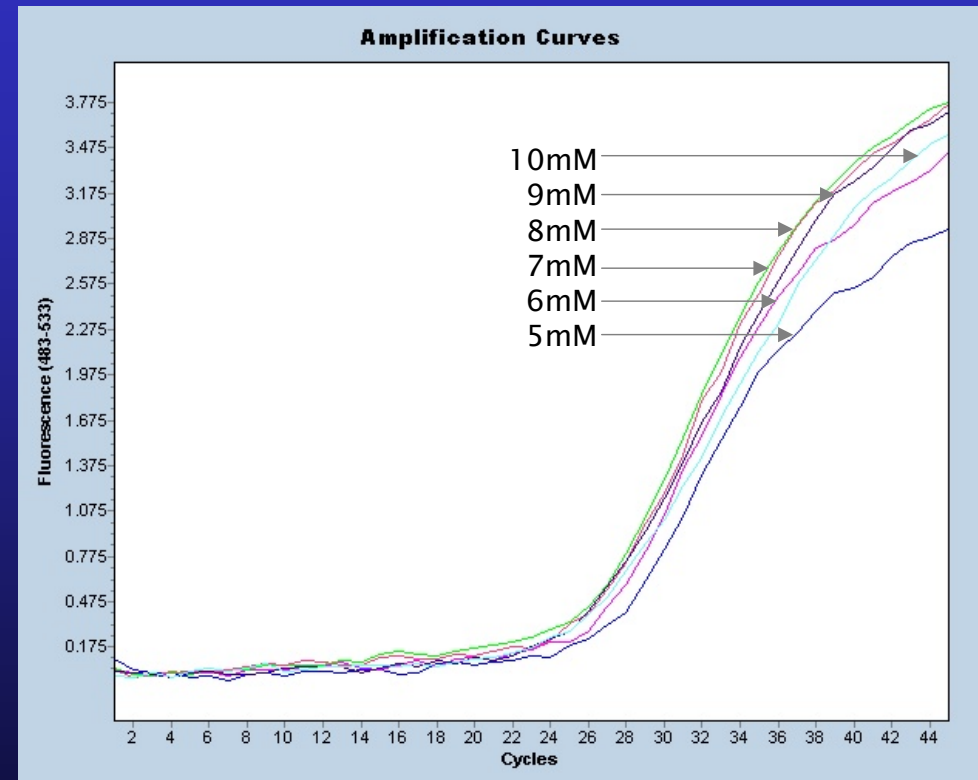
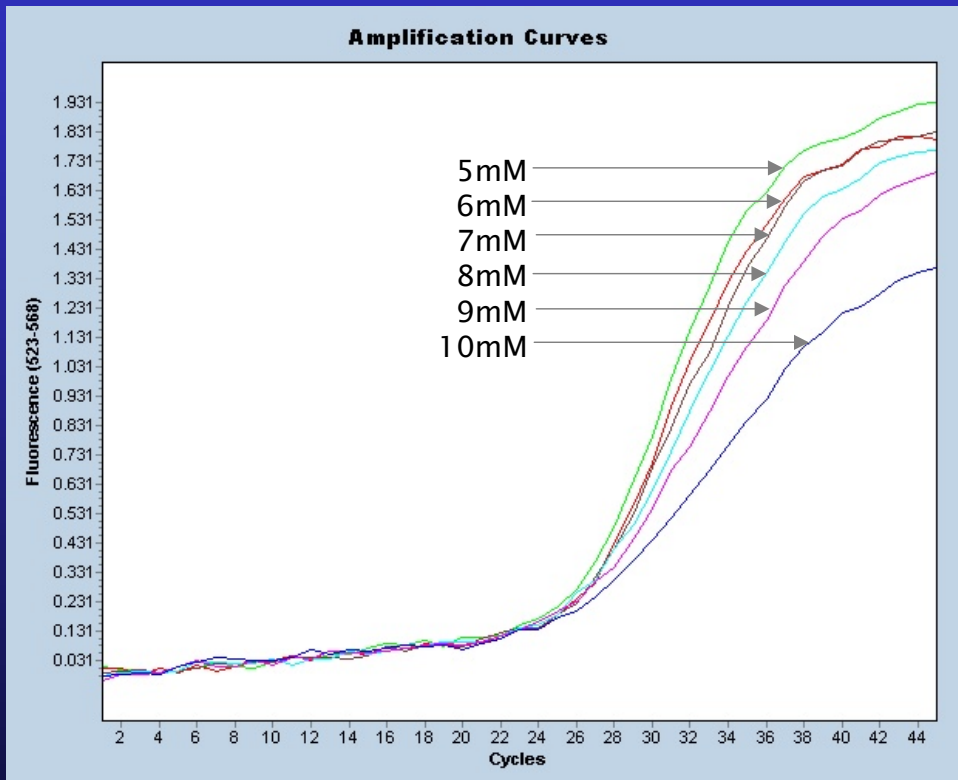
3-step PCR (45 cycles; LC480): 95°C/15s; 45°C/1s; 59°C/40s, system 3;
primers: varying; probes: 0.3µM CFO-up/0.4µM FAM-up + 1.2µM do;
MgCl₂: 7.5mM; templates: 10⁴ molecules wt-plasmid (detection of CFO)

TRIPLEHYB[®]: dependence on MgCl₂

relatively high MgCl₂ concentrations required

system 3: up-CFO + do

system 3: up-FAM + do



3-step PCR (45 cycles; LC480): 95°C/15s; 45°C/1s; 59°C/40s, system 3;
primers: 0.5µM each; probes: 0.3µM CFO-up + 1.2µM do;
MgCl₂: varying between 5–10mM; templates: 10⁴ molecules wt-plasmid

Evaluation of SNP-positions in up-probes

detection of **reliable reaction** curves with the right template?

↪ after further optimization for each system: **YES**

↪ but **not for system 1 & 2 with up-CFO (wt)**

system	1	2	3	4	5
up-FAM mut	+	++	+++	+++	+
up-CFO wt	-	-	+++	+++	+

Evaluation of SNP-positions in up-probes

detection of **reliable reaction curves** with the right template?

↪ after further optimization for each: **YES**

↪ but **not for system 1 & 2 with up-CFO (wt)**

system	1	2	3	4	5
up-FAM mut	+	++	+++	+++	+
up-CFO wt	-	-	+++	+++	+
up-FAM wt	n.t.	++	n.t.	n.t.	n.t.

n.t. = not tested

Evaluation of SNP-positions in up-probes

detection of **reliable reaction curves** with the right template?

↪ after further optimization for each: **YES**

↪ but **not for system 1 & 2 with up-CFO (wt)** → quenching by G?

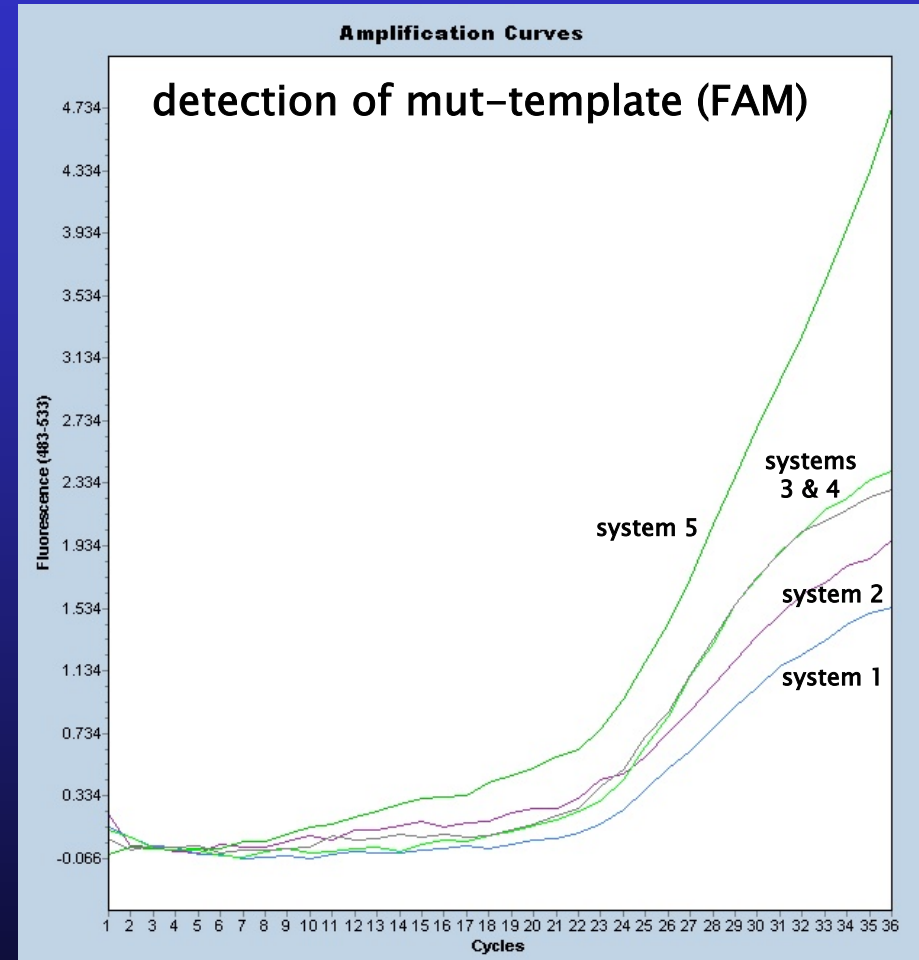
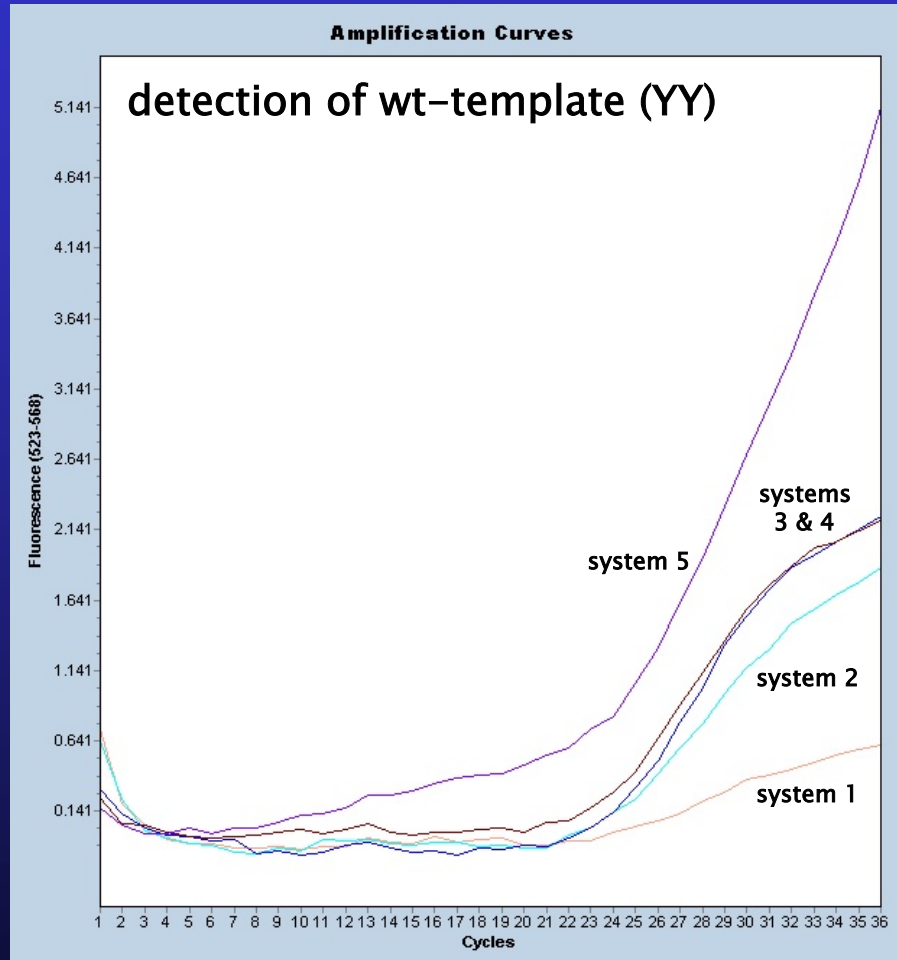
system	1	2	3	4	5
up-FAM mut	+	++	+++	+++	+
up-CFO wt	-	-	+++	+++	+
up-FAM wt	n.t.	++	n.t.	n.t.	n.t.
up-YY wt	+	++	+++	+++	+

n.t. = not tested

Systems 1–5: YY–up(wt)/FAM–up(mut) + do

best performance for systems 3 & 4

no or very weak detection of curves in the other channel



3–step PCR (37 cycles; LC480): 95°C/15s; 45°C/1s; 59°C/40s, systems 1–5;
primers: 0.5μM each; probes: 0.3μM YY–up/0.4μM FAM–up + 1.2μM do;
MgCl₂: 5mM; templates: 10⁵ molecules wt– or mut–plasmid

Systems 3–5: CFO–up(wt)/FAM–up(mut) + do

best performance for systems 3 & 4

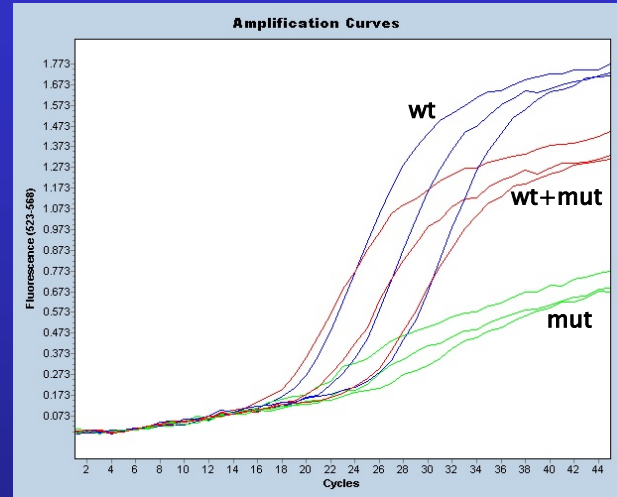
detection of CFO–signal:

- wild–type
($10^6/10^5/10^4$ mol.)
- heterozygous
($5 \times 10^5/10^4/10^3$ each)

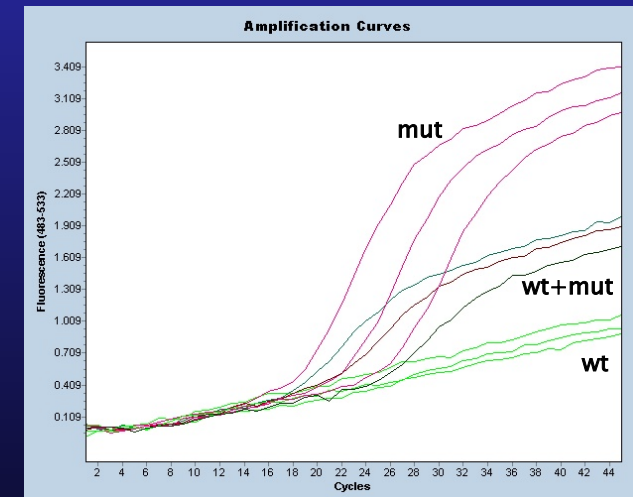
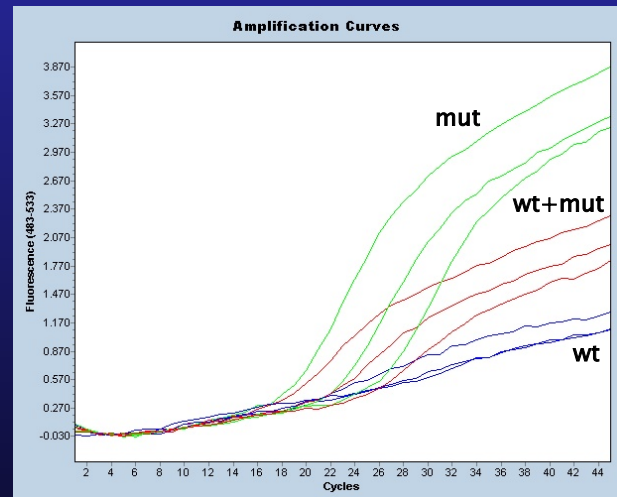
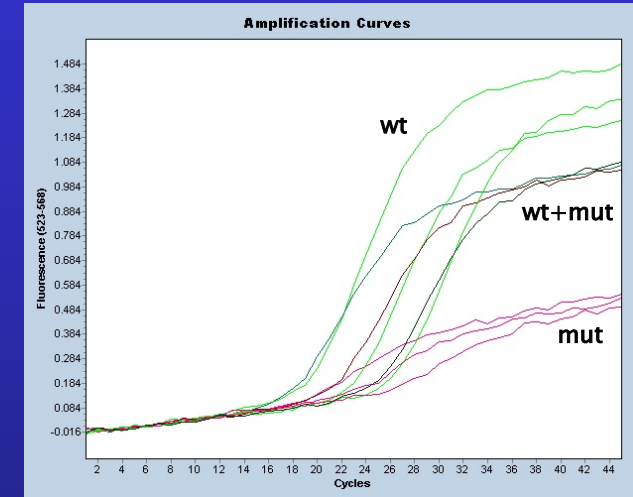
detection of FAM–signal:

- mutant
($10^6/10^5/10^4$ mol.)
- heterozygous
($5 \times 10^5/10^4/10^3$ each)

system 3



system 4



3–step PCR (37 cycles; LC480): 95°C/15s; 45°C/1s; 59°C/40s, systems 3–5;
primers: 0.5μM each; probes: 0.3μM CFO–up/0.4μM FAM–up + 1.2μM do;
MgCl₂: 5mM; templates: 10⁴ / 10⁵ / 10⁶ molecules wt– or mut–plasmid or 1:1 mixtures

Conclusion

- SNP at position 3 works best (HBV, VEGF), also at positions 4 & 5 using plasmid templates
- only preliminary data on clinical samples:
 - ↳ so far VEGF C460T detectable in tumor DNA by systems 3 & 4 (CFO-up)
 - ↳ further optimization needed (background elimination, other dyes, use of genomic DNA, evaluation of other positions and models)
- TRIPLEHYB® format as promising genotyping tool with more flexibility regarding SNP position

TRIPLEHYB[®] in summary

Advantages:

- universal format: broad applicability for both genotyping and quantitative target analysis
- open format: may be run on several real time platforms e.g. tested with LightCycler, ABI 7000 SDS, Rotor-Gene 3000
- safe format: intercalating dyes which are frequently used for genotyping may be replaced
- fast format: usable with fast cycling, SNP analysis does not require subsequent melting point analysis
- reliable format: since the detector probe hybridization area may be very short (9–12 bases), analysis particularly of targets with short conserved subsequences e.g. RNA viruses
- multiplexing capability

Disadvantages:

- assay set-up more complex particularly multiplex assays