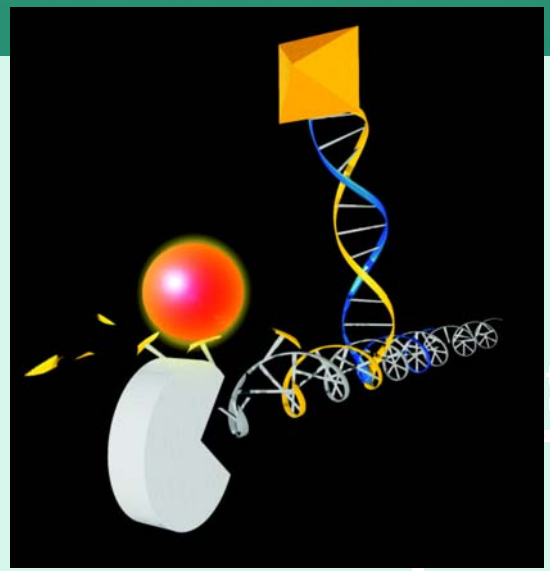

TRIPLEHYB®: A novel detection format for real-time fluorescence PCR

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2nd International qPCR Symposium

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TRIPLEHYB®: A novel detection format for real-time fluorescence PCR

1. Limitations of available real-time PCR detection formats and demands on a new platform technology
2. TripleHyb® Probe Format – Principle
3. Clinical Application: Quantification of Hepatitis B Virus copy number in PEI HBV reference plasma

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Limitations of available real-time PCR detection formats

From technical point:

- Susceptibility to inhibitors usually very high, robustness needs improvements
- Dogma: only one signal pulse per amplicon and cycle which is still limiting any further increase of RT assay sensitivity
- Known probe architecture usually open for only one measuring format
- Single probe formats with limited applicability to differ between single nucleotide polymorphism (SNP)
- Each assay type usually requires extra software for primer/probe design
- Mismatches between assay formats and real-time instruments

From financial point:

- Patents and licences very expensive (manufacturer of tests!)

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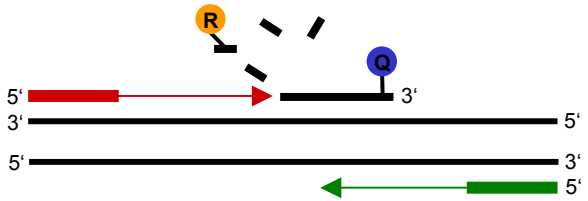
Demands on a new real-time PCR detection platform technology

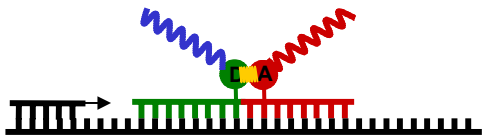
- Improved flexibility and robustness of new assay compared to available formats
- Preferably utilization of available, validated primers & probe sequences and standards
- Simple assay development using available primer & probe design software
- Full compatibility of results compared with established assays (e.g. TaqMan)
- New format must support multiplex PCR/RT-PCR
- Full compatibility with the most commonly used real-time instrument platforms
- No need for external licences

Three in one: TripleHyb® Probe Format

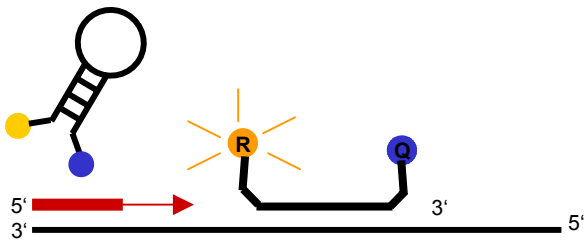
5`-Nuclease Assay (TaqMan™)



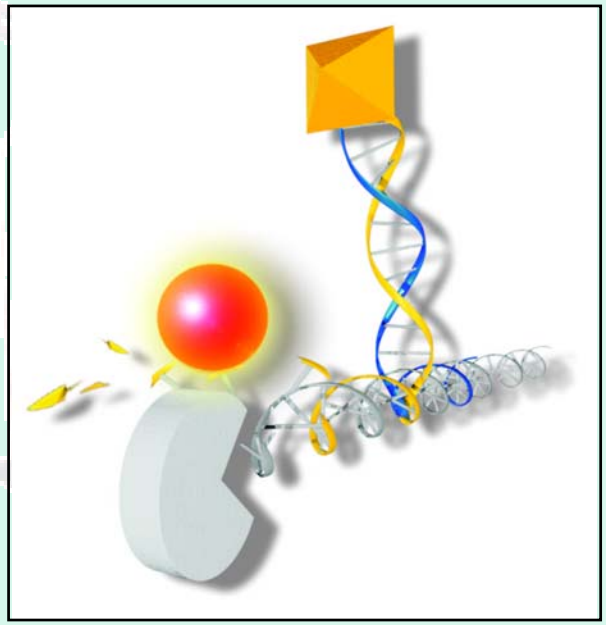
Hybridization Probes



Molecular Beacons

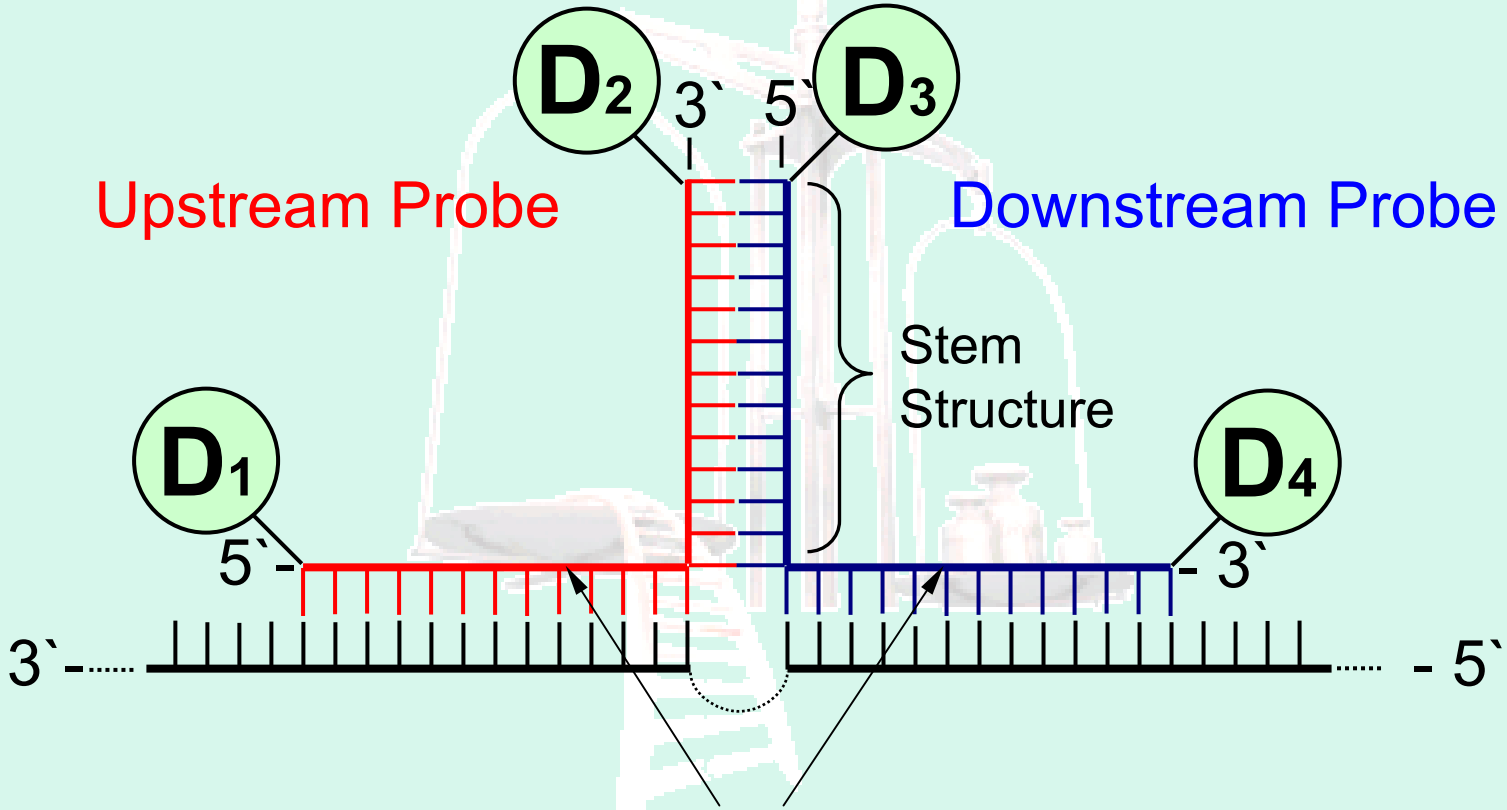


TripleHyb®



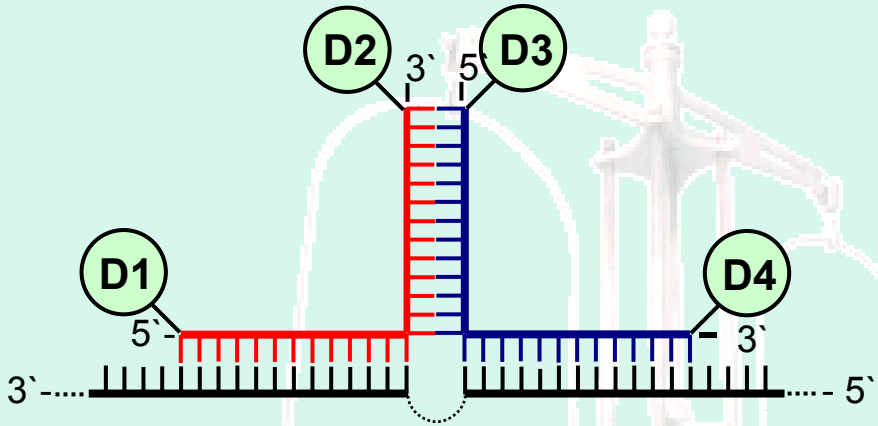
TripleHyb® Probe Format - Principle

Triple detection complex between paired probes and single-stranded target DNA



Target sequence–complementary probe subsequence

Possible labeling combinations



Combination	D1	D2	D3	D4
1	R	Q	∅	∅
2	∅	∅	R	Q
3	R	Q	R	Q
4	∅	D	A	∅
5	R	∅	Q	∅
6	R	Q	∅	Q
7	∅	Q	R	Q

D = Dye

R = Reporter

Q = Quencher

D = Donor

A = Acceptor

Prototype: probes and experimental design

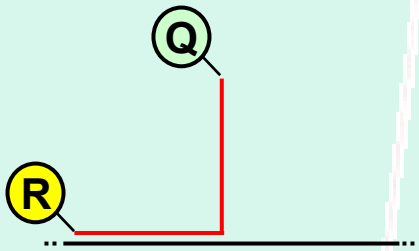
Based on a validated Hepatitis B Virus (HBV) TaqMan™ protocol:

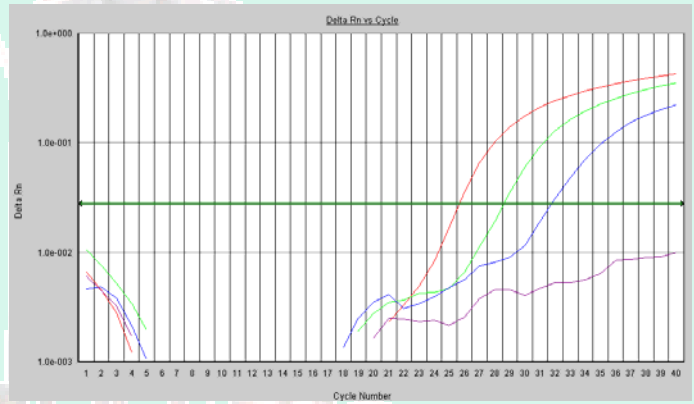
- Original TaqMan primers were kept
- TaqMan probe (20 b) splitted into two target-complementary subsequences
- To both the 3`-end of the upstream and the 5`-end of the downstream probe a target-unrelated, inter-molecular stem-forming subsequence was appended
- A set of corresponding labeled and unlabeled probe pairs was ordered

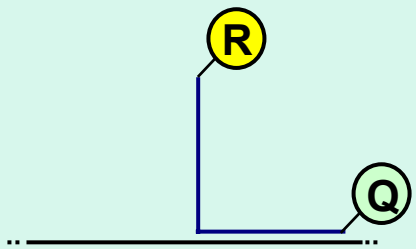
Experimental design (1)

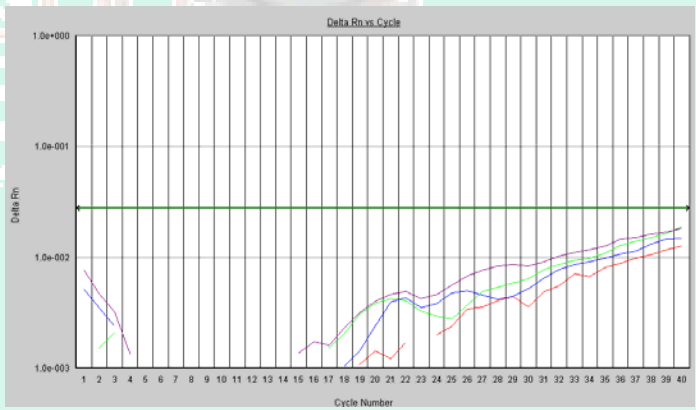
Target: „Intelligent tubes“ coated with copies, respectively

Pre-validated protocol: 3-step PCR (40 cycles, ABI PRISM 7000 SDS); 95°C, 00:30 min; 45°C, 00:15 min; 57°C, 01:15 min

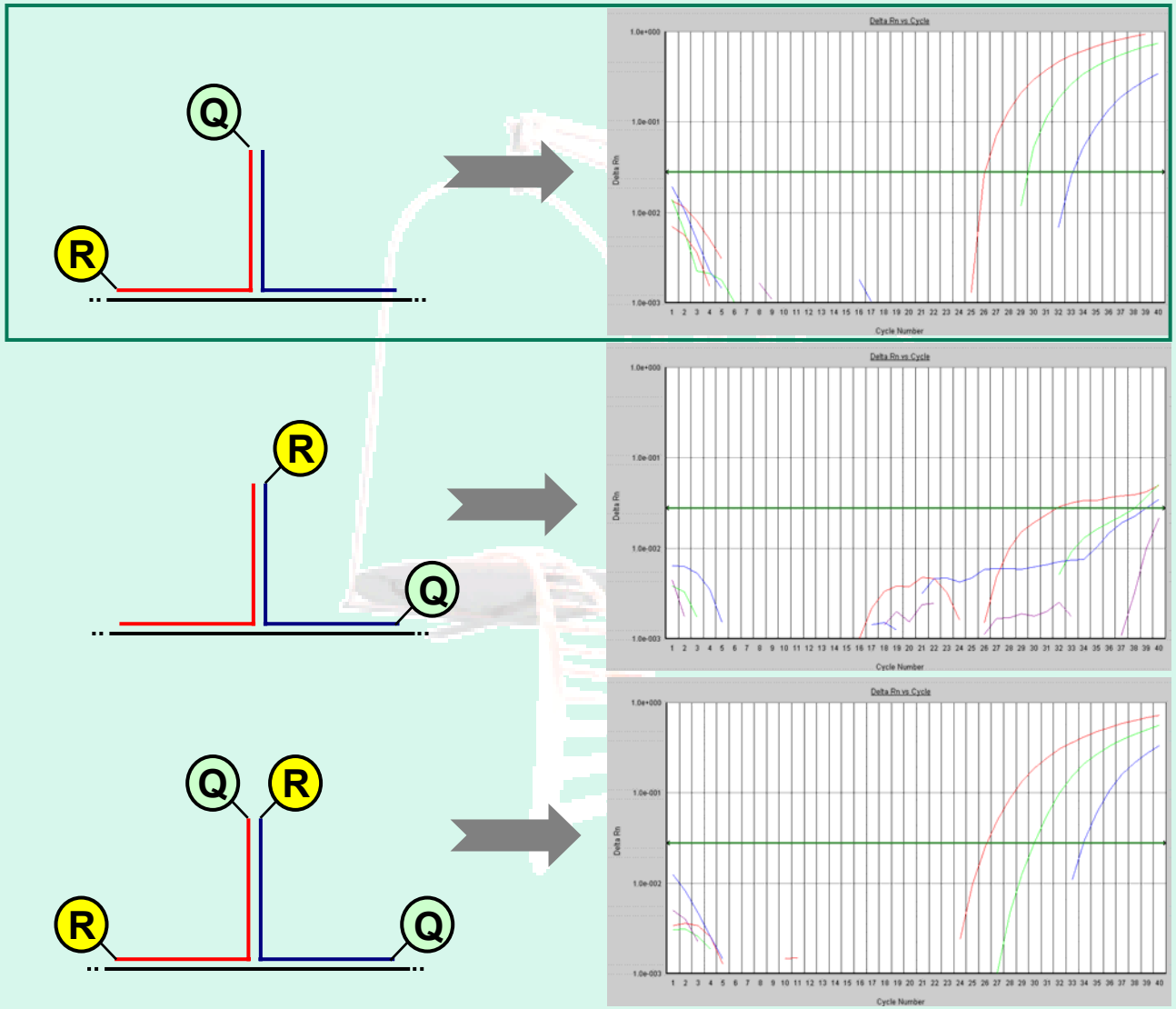








Experimental design (2)



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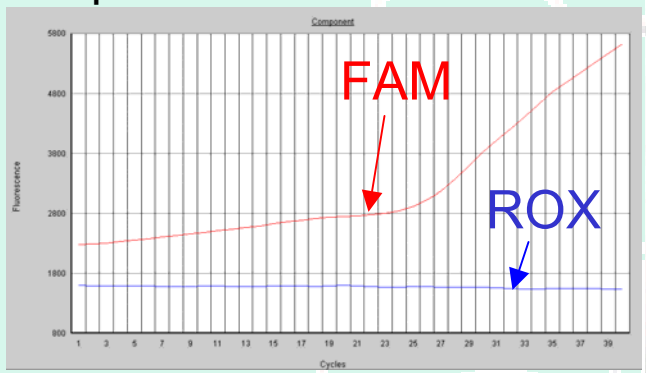
Higher signal intensity and amplification efficiency using paired upstream/downstream probes compared with unpaired upstream probe

Rotor-Gene 3000

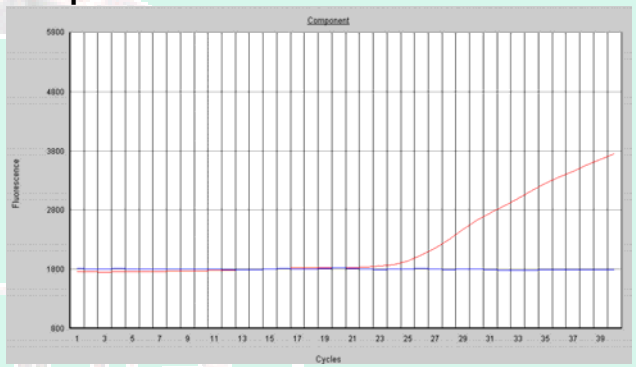
Creeping fluorescence increase eliminated by optimization of stem length

Spectral components view, ABI PRISM 7000 SDS

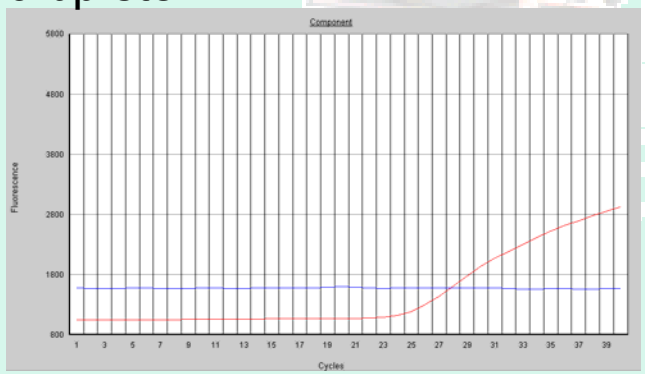
12 bp stem



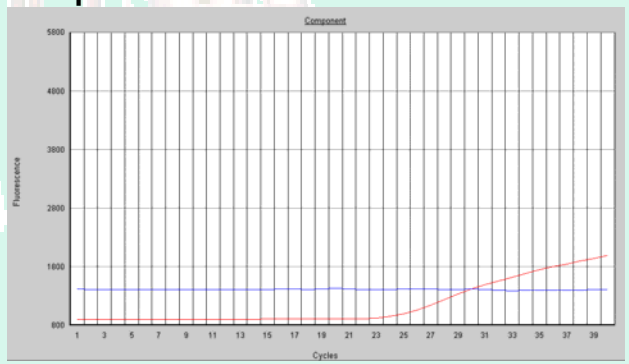
9 bp stem



6 bp stem

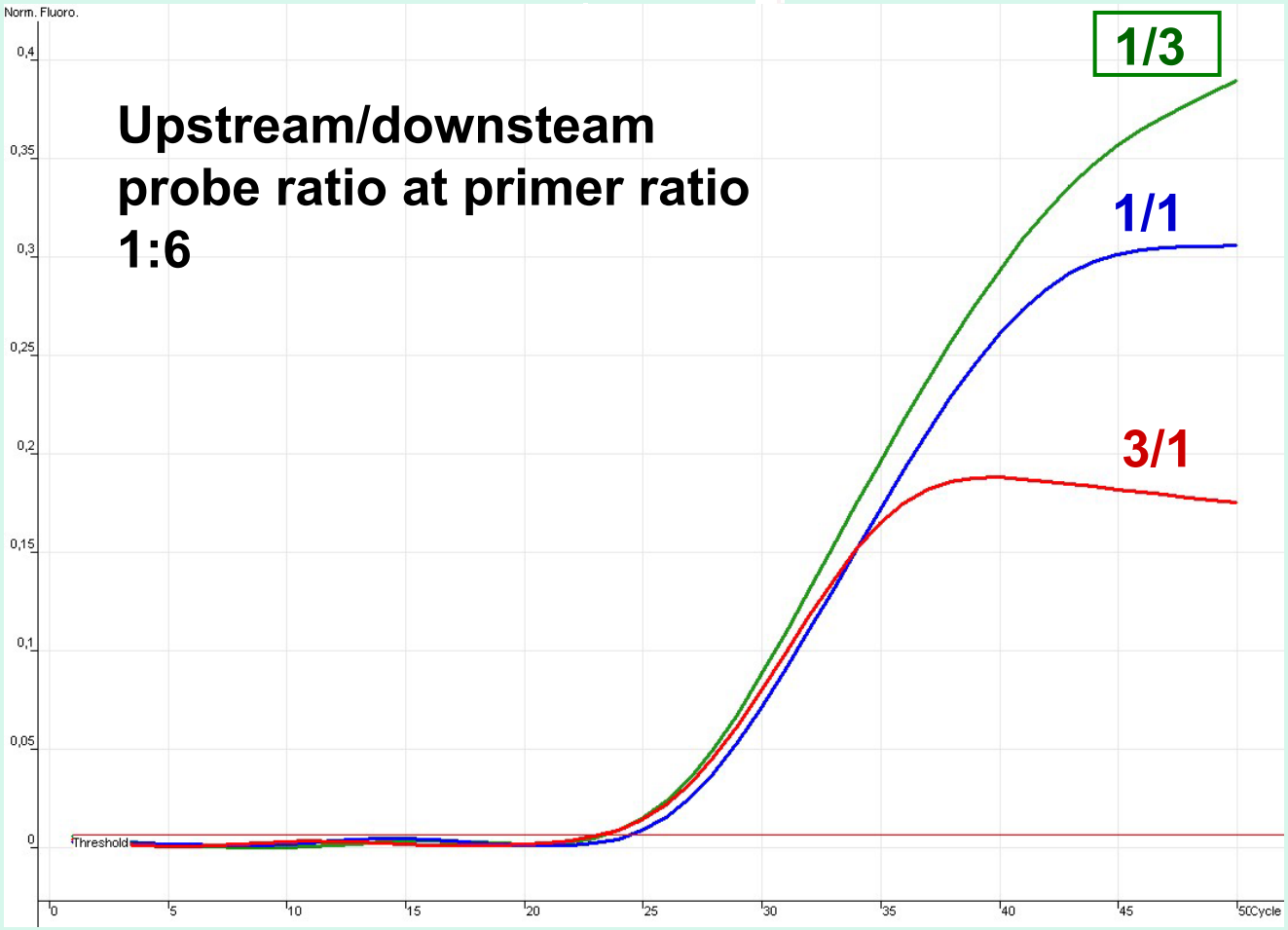


3 bp stem

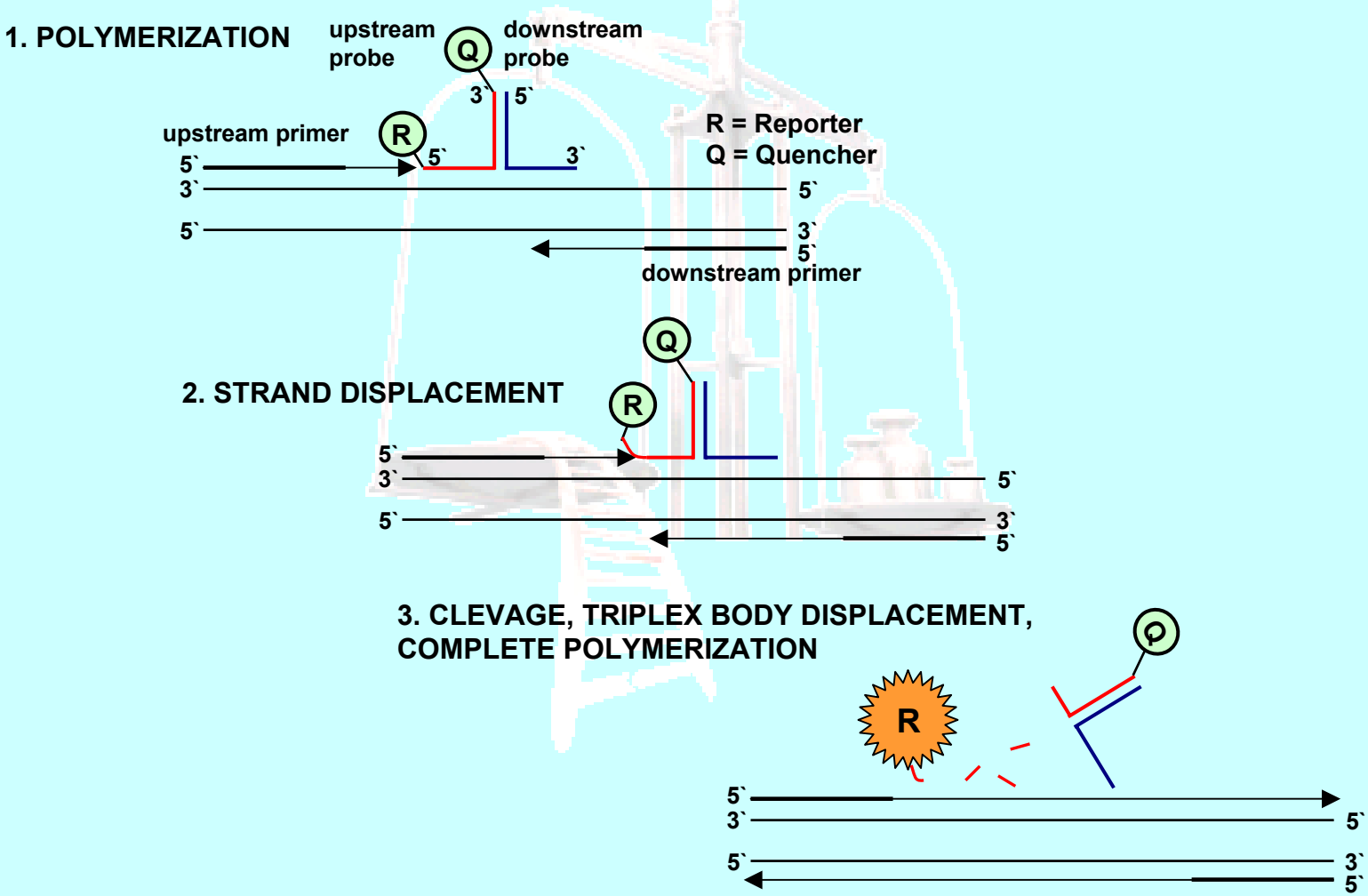


Optimization of probe ratio: probe matrix

Rotor-Gene 3000 (Corbett Research)



Supposed mechanism of TripleHyb® real-time fluorescence PCR format



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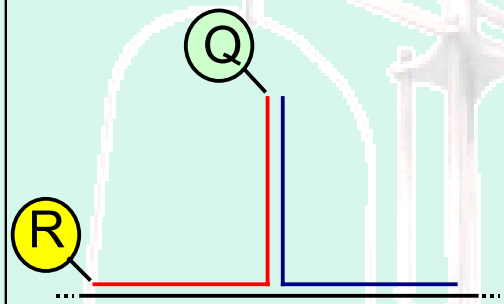
Clinical Application

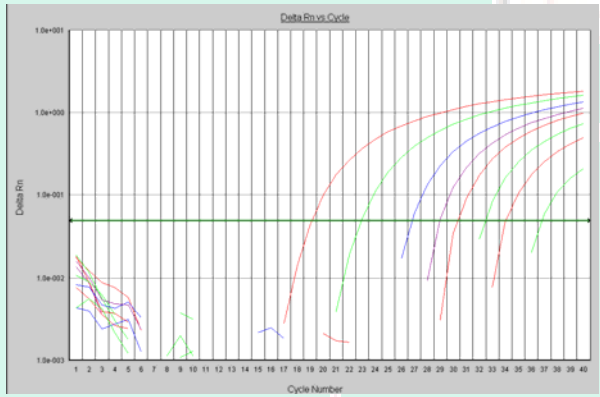
Quantification of Hepatitis B Virus copy number in PEI HBV reference plasma

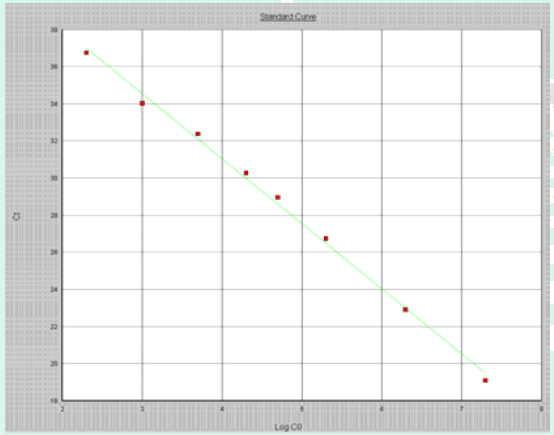
HBV copy number quantification using comparable TripleHyb® and TaqMan™ assays

	TRIPLEHYB	TaqMan
Format		
Primer	upstream/downstream primer (21/23 bases)	upstream/downstream primer (21/23 bases)
Probes	upstream/downstream probe (21/23 bases)	TaqMan probe (20 bases)
Amplicon	73 bp	73 bp
Standards	RoboGene HBV "Intelligent Tubes" (200 - 20x 10^6 IU/mL)	RoboGene HBV "Intelligent Tubes" (200 - 20x 10^6 IU/mL)
$MgCl_2$	7 mM	5 mM
Cycle program	95°C, 10:00 min (heat activation) 3-step PCR (40 cycles) 95°C, 00:30 min 45°C, 00:15 min 57°C, 01:15 min	95°C, 10:00 min (heat activation) 2-step PCR (40 cycles) 95°C, 00:30 min 59°C, 01:30 min

Comparable well amplification using Hepatitis B Virus (HBV) Control DNA coated strips on ABI PRISM 7000

RoboGene HBV (TRIPLEHYB)

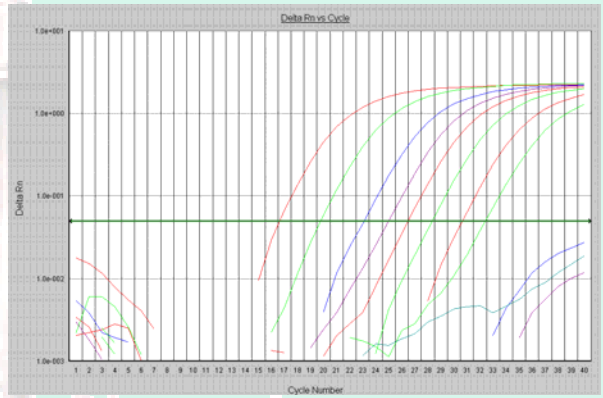


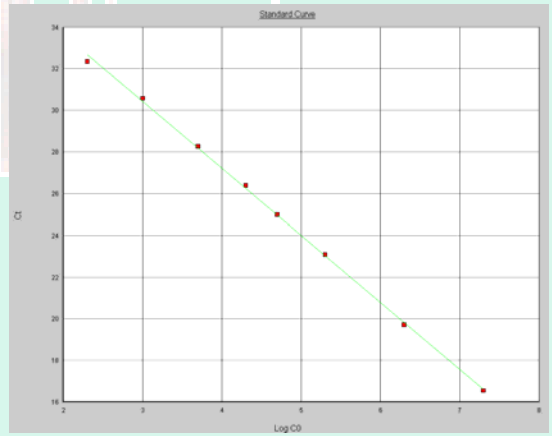


Slope = -3,498; Intercept = 45,028,

R^2 = 0,997

RoboGene HBV (TaqMan)





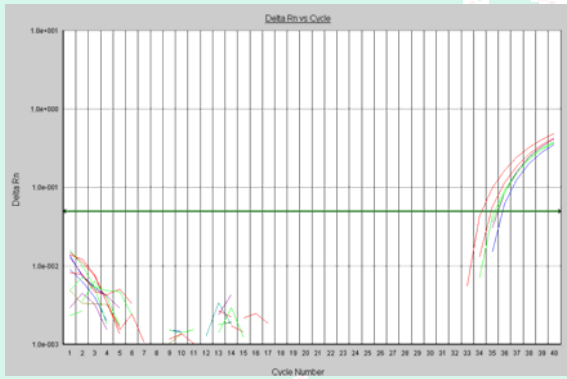
Slope = -3,209; Intercept = 40,046,

R^2 = 0,999

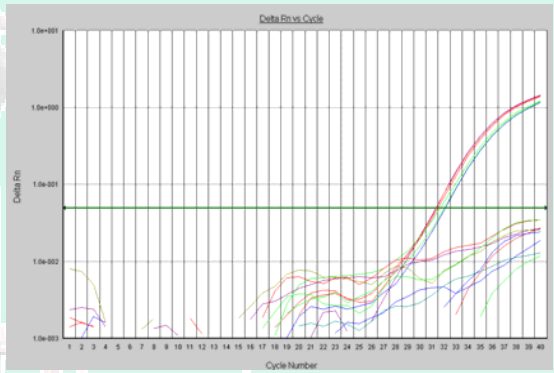
HBV Quantification in Clinical samples

Higher recovery rate of TRIPLEHYB vs. TaqMan™

RoboGene HBV (TRIPLEHYB)



RoboGene HBV (TaqMan)



Record No.	IU/ml TaqMan	IU/ml Triplex
1	523	1316
2	527	673
3	300	455
4	466	585
5	394	822
6	291	613
mean	**417**	**744**
SD	106	305

Clinical sample: PEI HBV reference plasma (50,000 IU/ml (Lot #1872/01, genotype D, subtype ayw2/3) diluted to 1000 IU/ml with plasma of a HBV negative blood donor

Similarities and advantages of TripleHyb® format compared to TaqMan

	TRIPLEHYB	TaqMan
Principle	5`-Nuclease cleavage during PCR amplification and FRET	5`-Nuclease cleavage during PCR amplification
Interaction between probe and target	Triple detection complex	Duplex detection complex
3`-OH blockade of probes	not required	always required
Maximum sites for dye incorporation	4	2
Mg^{2+} dependency	comparable	comparable
Optimization	primer and probe matrix	primer matrix
Sensitivity	very high	very high
Robustness	high	rather low

Summary

- TRIPLEHYB combines features of TaqMan, molecular beacons and hybridization probes format in one
- Only working real-time PCR assay which exploits a triple detection complex
- TRIPLEHYB probes with 4 possible sites for dye incorporation
- No 3'-OH blockade of fluorescently labeled probes required
- May be operated by exploiting both 5'-nuclease activity of taq polymerase and classical FRET analysis
- Compatibility with the most commonly used real-time instrument platforms (tested on ABI7000, Rotor-Gene 3000, LightCycler 1.0)
- Allows qualitative and quantitative multiplex analysis of at least two targets in one tube supposed no interference between stems
- Full compatibility with TaqMan assay in clinical application (HBV)
- Excellent sensitivity and robustness