

# A New Real-time PCR Detection Method

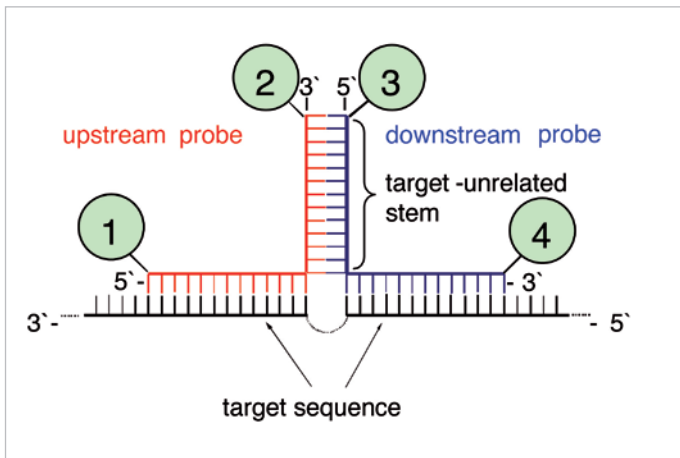


Fig. 1: Schematic triplex of the Triple Hyb assay. 1–4: possible positions for labelling with fluorophore (1,3) and quencher (2,4) or donor (2) and acceptor (3)

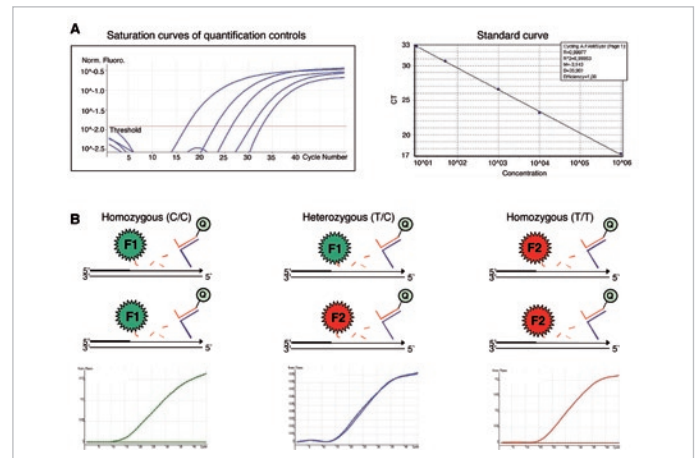


Fig. 2: Quantification (A) and genotyping (B) by Triple Hyb assay. *VEGF* gene polymorphism C-460T analysis is shown as an example (B). F1/2: two different fluorophores; Q: quencher

To date, a variety of probe-based real-time PCR technologies exists due to the mode of probe such as FRET-based [1], TaqMan [2], and so-called non-FRET probes like molecular beacons [3]. Though more or less satisfied in practical use, most technologies are restricted or not utilizable for genotyping. Taking into account the advantage of real-time PCR, AJ Roboscreen developed the TripleHyb assay that is applicable to both quantification and polymorphism analysis of nucleic acids.

## Principle

A triple detection complex is formed by complementary binding of the 5'-end of an upstream probe and the 3'-end of a downstream probe to the target, whereas target-unrelated stem sequences of the probes hybridise together (fig. 1). When the taq polymerase reaches the double-stranded triplex during PCR, its 5'→3' exonucleolytic activity detaches the fluorophore from the probe by hydrolysing the first nucleotides. In that result fluorescence is generated and the remaining core complex displaced (fig. 2B). Several probe positions were tested for labelling to enhance detection sensitivity. In a favoured version only the upstream probe is labelled with a fluorophore at the 5'-end and a fluorescence-quenching compound at the 3'-terminus.

## Evaluation and Application

Initially, the TripleHyb assay was tested applying a prototype of Hepatitis B Virus

(HBV) kit. Reaction parameters were found to be comparable to the TaqMan assay except for need for three PCR steps. The detection limit was determined to 50 IU/ml and the quantitative range was at least 6 logs using the Instant Virus DNA Purification Kit (AJ Innuscreen, Berlin). So the new assay provides a highly sensitive and accurate method for real-time quantification (fig. 2A). Its second application was more or less found by accident. Analysing the quantitation efficiency concerning all HBV genotypes (A-H), no signal was detectable for genotype F. Sequence alignment revealed a point mutation in the respective target corresponding to the 5'-end of the upstream probe. Accordingly, a mismatch at prominent position appears to destabilize the triplex resulting in no hydrolysis and absence of fluorescence. Therefore, the new assay is also suitable for allele-discrimination in one tube applying the TripleHyb assay are:

- applicable to quantification and genotyping of nucleic acids
- possible analysis of more than one mutation within a single reaction
- no need for melting curve analysis
- no requirement of 3'-OH-end protection of the probe
- advanced analysis of highly variable sequences due to short target-specific area of the upstream probe
- applicable to different real-time PCR machines

## Future Trends

To take advantage of the TripleHyb assay in molecular diagnostics, the development and evaluation of new *in vitro* diagnostic products has been aspired. The highly sensitive and accurate quantification of viral load is well suited for predicting and monitoring of therapeutic success. The potential of allele-discrimination is usable for analysis of functional polymorphisms that are discussed as biomarker for specified diseases. Consequently, CE-marked test kits based on the TripleHyb technology will be available on the market soon. The future trend of the new technology consists in the capability to rapid and correct combination of phenotype, genotype, and clinical process specially concerning disease progression and individualized medicine.

## References

- [1] Chen and Kwok: Genet Anal 14, 157–163 (1999)
- [2] Nurmi *et al.*: Nucleic Acids Res 28, E28 (2000)
- [3] Tyagi and Kramer: Nat Biotechnol 14, 303–308 (1996)

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