New holistic approach for easy and cost efficient geno-typing of small numbers of genes

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PENTABASE

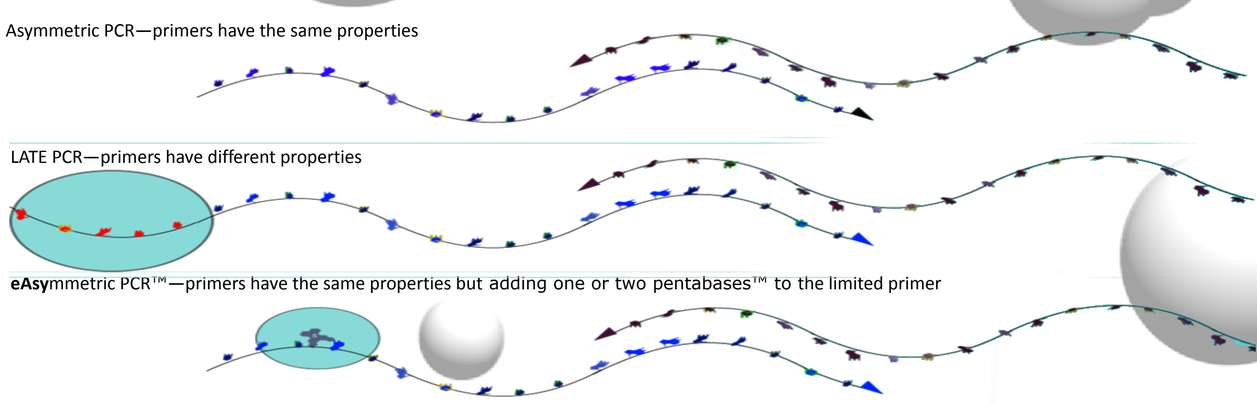
Abstract

We present here a new holistic way of endpoint genotyping, using primers and probes based on modified DNA. Our approach is a closed tube assay, minimizing the risk of cross contamination, it runs on standard equipment, can easily be multiplexed determining the three possible genotypes (homozygous wildtype, heterozygous or homozygous mutant) of up to at least four genes in each well, is very robust for input of sample material and is using standard procedures and reagents present in most laboratories.

The method we describe is an enhanced asymmetric PCR, which we call eAsymmetric PCR™ followed by an endpoint melt study using modified DNA probes called EasyBeacons™. The PCR reactions can be carried out on standard, inexpensive PCR machines and read-out of the genotypes requires a ten minutes melt study post PCR on a real-time PCR instrument or temperature controlled fluorescence reader to evaluate the genotypes of up to four different genes per well.

Logic of eAsymmetric PCR™

eAsymmetric PCR™ is an enhanced asymmetric PCR using two primers added in unequal amounts, one primer being limited and one in excess amounts. In **eAsy**mmetric PCR™ the two primers are designed to have the same properties, but adding one or two pentabases™, as shown in the figure below, during synthesis to the limited primer, to increase its affinity. The reason for that is that the affinity of a primer is dependent of the sequence and the concentration. So if two primers are designed equally the one present in lower amount will have a lower affinity for its complementary target, than the one present in higher amount. **eAsy**mmetric PCR™ is thus an easy approach to obtain an efficient asymmetric PCR, with similar results, known from the more complex Linear After Exponetial PCR (LATE PCR) approach. The **eAsy**mmetric PCR™ is a two phased amplification. Phase I is an exponential amplification until the limiting primer is used up yielding a limited number of double-stranded amplicons. Phase II is a linear amplification with the excess primer, generating a single-stranded DNA amplicon. The single-stranded amplicon can be detected real-time or in an endpoint-detection using probes that are either sequence specific or mismatch tolerant.



EasyBeacons™

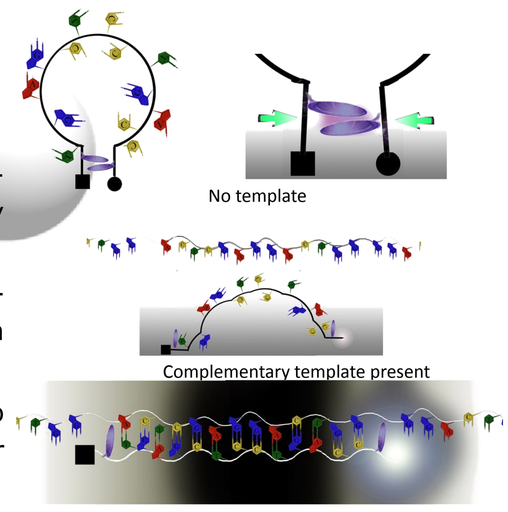
An EasyBeacon™ is composed of a fluorophore like FAM, HEX, ROX etc. and a quencher such as Tamra, Dabcyl, Black Hole Quencher etc. normally linked to the 5’ and 3’ end of a standard DNA oligo comprising two to eight pentabases™.

EasyBeacons™ combine some of the advantageous features associated with conventional probes ratio:

- High affinity
- High specificity
- Ease of design
- High signal-to-noise ratio due to low background fluorescence (inherent secondary structure of unbound probes)

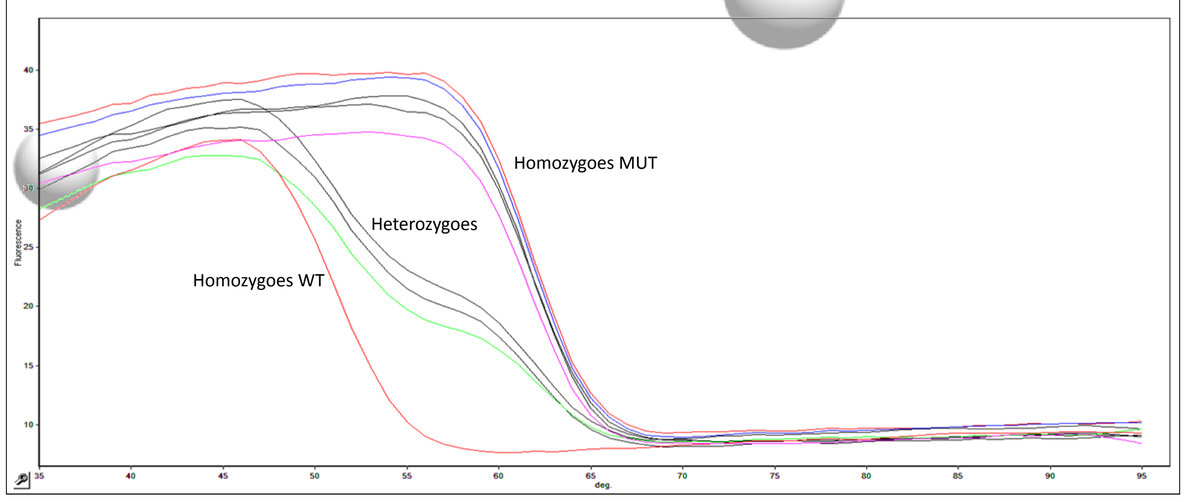
In addition, as EasyBeacon™ are nuclease resistand and not degraded during the PCR, they can be used in endpoint melt analysis.

A typical EasyBeacon™ comprises between 7 to 20 DNA nucleotides, 1 fluorophore, 1 quencher and 2 to 8 pentabases™.



One EasyBeacon™ detects 3 genotypes

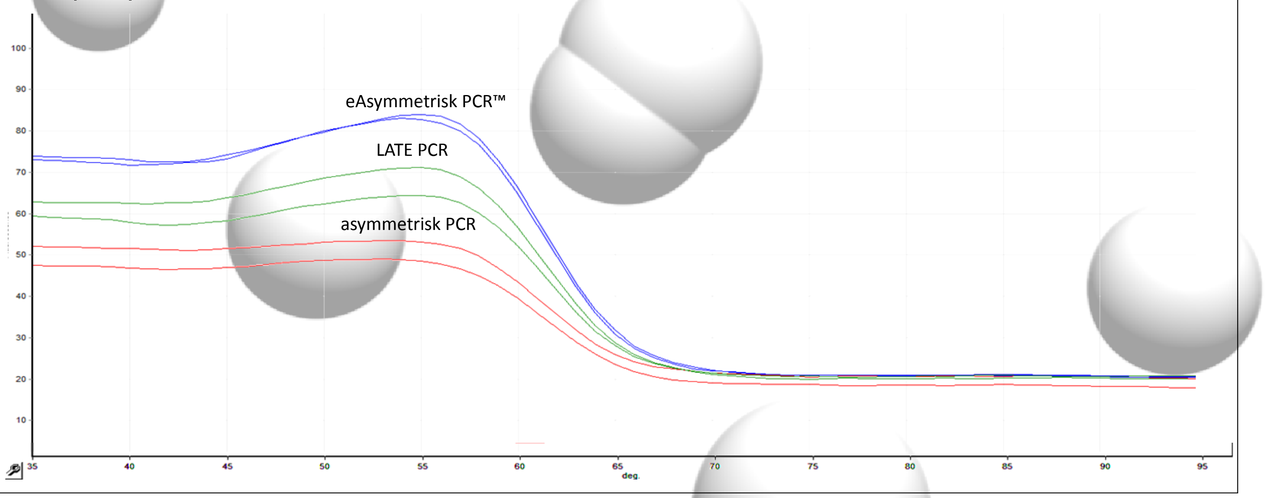
When hybridized to a complementary target, the fluorophore and the quencher of EasyBeacon™ are separated in spatial distance allowing the fluorophore fluoresce. The Pentabases ensures that, if there are no or fewer complementary targets than EasyBeacons™ in the reaction, the two ends of the probe will be kept in close proximity and thereby be turned off. This feature is used in endpoint melt analysis, where the temperature is increased from 35^0C, where the EasyBeacons™ bind to their target and have maximum fluorescence to 95^0C where there will be no binding between target and EasyBeacon™, and hence the EasyBeacon™ will be quenched efficiently. In the example shown below an EasyBeacon™ distinguishes between the three possible genotypes: Homozygous wildtype (T_m= 51^0C), heterozygous (dual melt T_m= 51^0C and 62^0C) and homozygous mutant (T_m= 62^0C) of NMB RS7180849.



eAsymmetric PCR™ as good as LATE PCR—but easier

eAsymmetric PCR™:	LATE PCR:	asymmetric PCR:
Excess primer 750-1500 nM	Excess primer 750-1500 nM	Excess primer 750-2000 nM
Limiting primer 50-200 nM	Limiting primer 50-200 nM	Limiting primer 25-200 nM

Though some optimization could improve results a little, as standard we use 800 nM for excess primer and 100 nM for limiting primer in **eAsy**mmetric PCR. Using this ratio in **eAsy**mmetric PCR™, LATE PCR and standard PCR respectively gives the results shown below. It is clear that **eAsy**mmetric PCR performs much better than asymmetric PCR and in this case also better than LATE PCR. In our studies we have found that **eAsy**mmetric PCR™ and LATE PCR in general performs equally well.



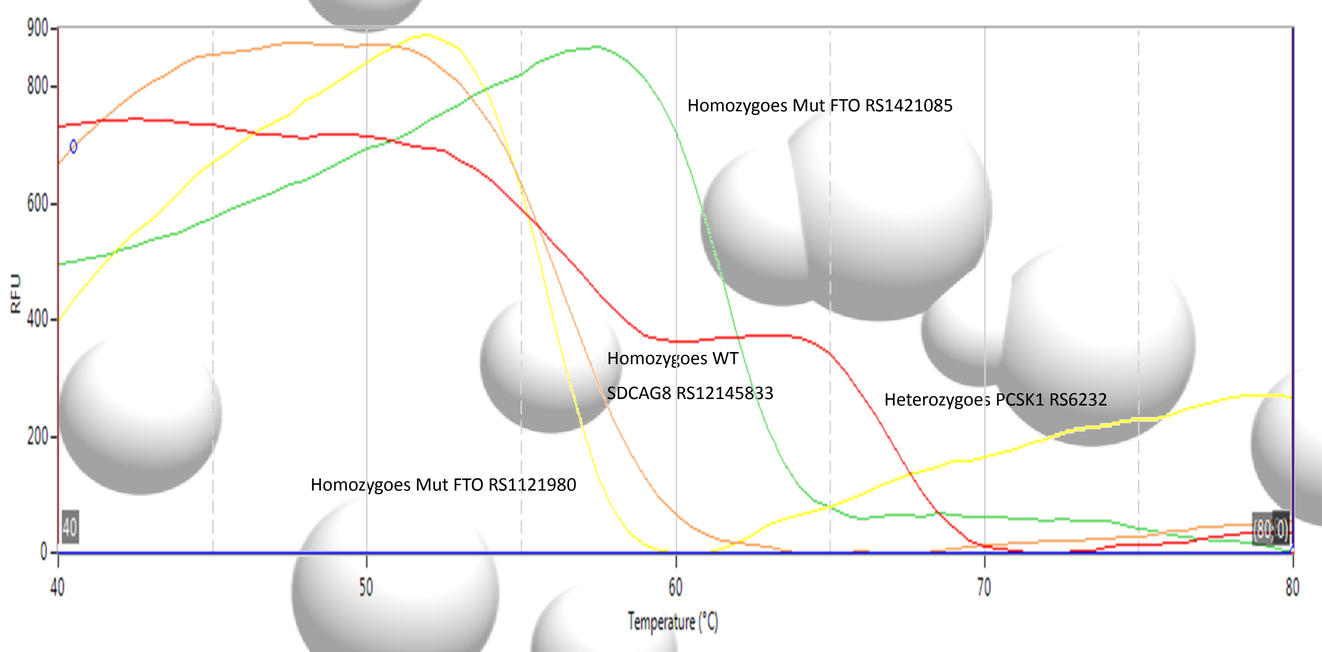
Four-plex reaction ⇒ 12 genotypes in one vial!

It is shown that combining **eAsy**mmetrisk PCR™ with EasyBeacon™ is an easy, closed tube procedure, which is able to:

- Combine at least four different SNP-detection tests in one well
- Detect up to at least 12 different genotypes in one well

Amplification can be carried out on standard inexpensive PCR machines. Read-out of the genotypes requires only a ten minutes melt study post PCR on a real-time PCR instrument or temperature controlled fluorescence reader.

In the figure to the right is an example of a four-plex SNP detection, giving clear signals and easy determination of the different genotypes. The four different probes in the assay is labelled with FAM (Green), Hex (Yellow), CalFlour red 610 (Orange) and Cy5 (Red). FAM detects the genotype of FTO RS1421085 and is homozygous mutant (T_m= 61^0C), Hex detects the genotype of FTO R1121980 and is homozygous mutant (T_m= 51^0C), CalFlour Red 610 detects the genotype of SDCCAG8 RS12145833 and is homozygous wildtype (T_m= 51^0C), Cy5 detects the genotype of PCSK1 RS6232 and is heterozygous (dual melt T_m= 52^0C and 68^0C).



FTO RS1421085	TT	TC	***CC***	PCSK1 RS6232	AA	***AG***	GG
FTO RS1121980	CC	CT	***TT***	SDCCAG8 RS12145833	***TT***	TG	GG