Comparison of different real-time PCR chemistries and their suitability for detection and quantification of genetically modified organisms

"Different real-time PCR chemistries: suitability for detection and quantification"

The real-time polymerase chain reaction (real-time PCR or qPCR) is currently the method of choice for quantifying nucleic acids. It is also widely used in the detection and quantification of genetically modified components of food and animal feed. The most frequently used real-time PCR fluorogenic systems are TaqMan and SYBR Green. While SYBR Green is an intercalating sequence unspecific DNA dye, the TaqMan chemistry relies on hydrolysis probes rendering this method sequence specific. Though SYBR Green displays lower efficiency than other systems it is the most widely used real-time chemistry owing to the fact that it does not require probes, which tend to be expensive and difficult to design. On the other hand, reliability of qPCR results increases significantly with the use of probes making them especially appropriate for quantification purposes.

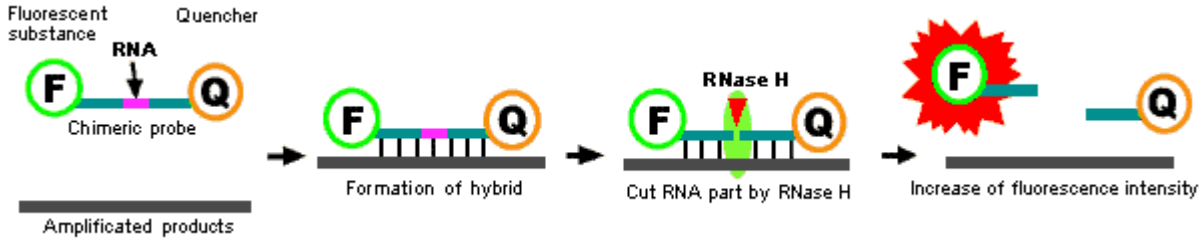


Figure 1: Cycling Probe Technology

In recent years alternative chemistries have also become available. Researchers from CSIC in Barcelona, the University of Girona, the National Institute of Norway and the National Institute of Biology from Slovenia evaluated several alternative chemistries: Lux, Plexor and Cycling Probe Technology, Molecular Beacons, MGB probes, Amplifluor and LNA technology. Using these chemistries, real-time PCR assays were designed and carried out. Their sensitivity, efficiency of amplification, limit of detection and quantification, repeatability, linearity, accuracy, specificity, robustness and overall applicability were subsequently compared. Practicality and cost-efficiency of each procedure were also taken into consideration.

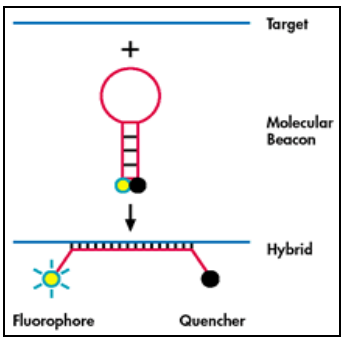


Figure 2: Molecular Beacon technology

In addition to the different chemistries, real-time PCR cycling modes was assessed. The conventional cycling mode was compared to the fast cycling mode, offered by certain new PCR machines, which reduces the reaction time by half. Cycle mode analysis was carried out for TaqMan, Molecular Beacon and MGB probe chemistries. The results showed that the cycling mode used during a qPCR reaction affects linearity while different chemistries influence efficiency of the reaction. Since prolonged heating reduces and enzyme's activity, an advantage of the fast cycling mode is that the reagents (especially the polymerase) are exposed to high temperatures for shorter periods of time.

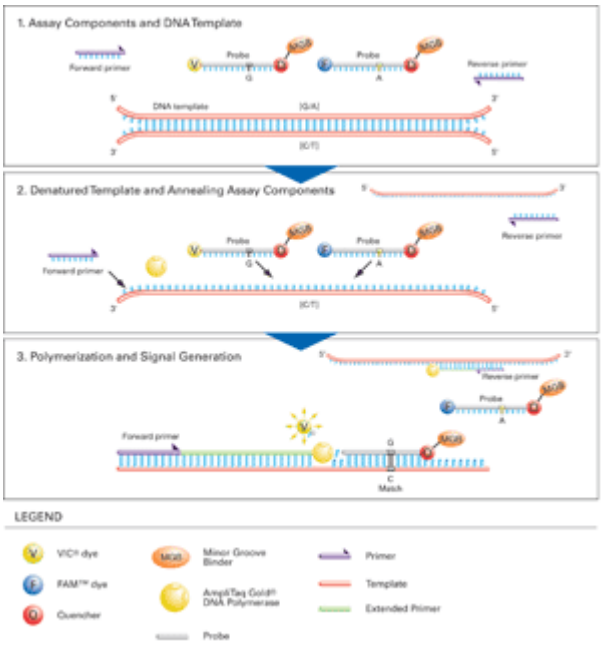


Figure 3: Minor Groove Binding probes

Although it was shown that no one fluorogenic system stands above the rest, there are certain characteristics that suggest that the LNA technology is a good choice as an alternative chemistry when designing assays for quantitative analysis, as the LNA probes are much shorter than the TaqMan counterparts and thus facilitate higher specificity. Plexor, on the other hand, might be the better choice when the prevailing experimental concerns are low cost, sensitivity and simplicity. Molecular Beacons (MB) showed the lowest efficiency and were most sensitive to changes in experimental setup, yet they were among the chemistries selected as being most suitable for quantification purposes (the others being the MGB and TaqMan probes). As expected, the MB system's performance improved under fast cycling conditions.

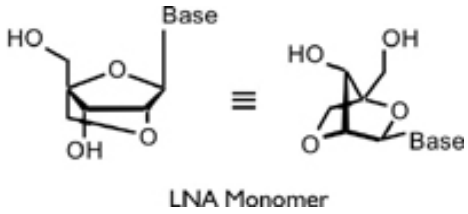


Figure 4: Locked Nucleic Acid

It is generally agreed that for the detection of a GMO junction sequence all real-time chemistries discussed previously can be designed to be equally specific, sensitive and robust. Essentially, the choice of the most appropriate detection chemistry depends on the structural properties of the target DNA, characteristics of the reaction, its performance, cost and its labor intensity.

External links:

http://www.biomedcentral.com/1472-6750/8/26

http://www.ncbi.nlm.nih.gov/pubmed/17177484

http://www.ncbi.nlm.nih.gov/pubmed/17488028

GLOSSERY ENTRIES

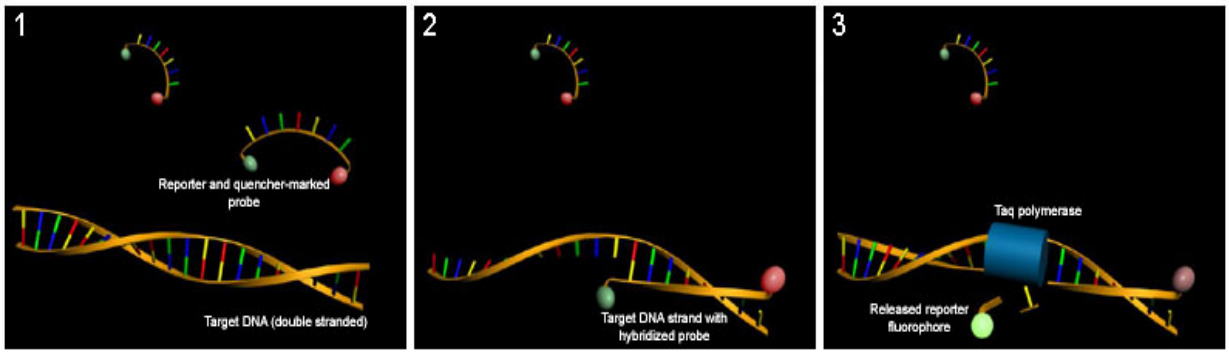
- real-time PCR (qPCR)

In Molecular Biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (qPCR) or kinetic polymerase chain reaction, is a laboratory technique based on polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-strand DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA.

Using fluorescent reporter probes is the most accurate and most reliable of the methods. It uses a sequence-specific DNA-based probe to quantify only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and allows quantification even in the presence of some non-specific DNA amplification. This potentially allows for multiplexing - assaying for several genes in the same reaction by using specific probes with different-colored labels, provided that all genes are amplified with similar efficiency.

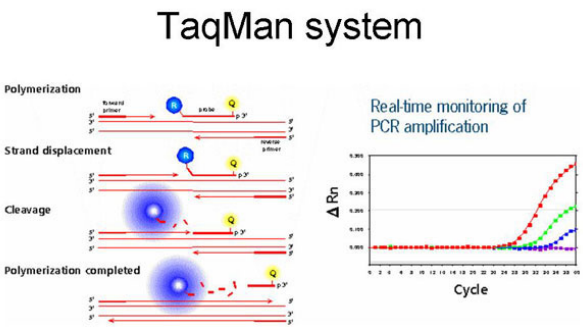
It is commonly carried out with an RNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of the taq polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter.



(source: http://en.wikipedia.org/wiki/Real_time_PCR)

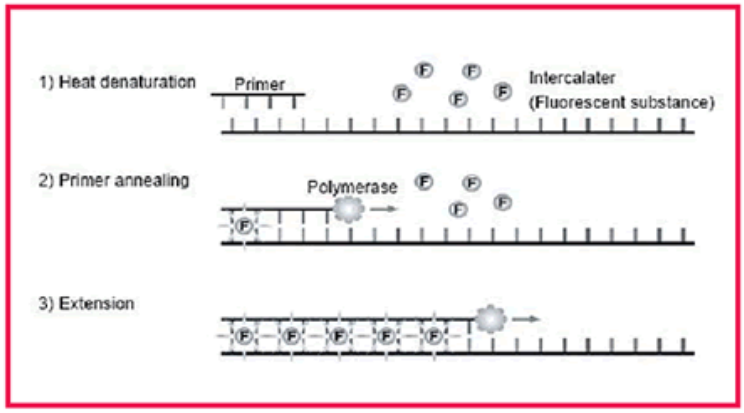
- TaqMan probes

In molecular biology, quantitative real time PCR methods often use a dual-labeled fluorogenic probe (called a TaqMan probe) as a rapid fluorophore-based real-time PCR method. The TaqMan Real-time PCR measures accumulation of a product via the fluorophore during the exponential stages of the PCR, rather than at the end point as in conventional PCR. The exponential increase of the product is used to determine the threshold cycle, C_T, i.e. the number of PCR cycles at which a significant exponential increase in fluorescence is detected, and which is directly correlated with the number of copies of DNA template present in the reaction.
In TaqMan real-time PCR a probe is added to the reaction, i.e., a single-stranded oligonucleotide complementary to a segment of 20-60 nucleotides within the DNA template and located between the two primers. A fluorescent reporter or fluorophore (e.g. *FAM* or TET) and quencher (e.g.TAMRA) are covalently attached to the 5' and 3' ends of the probe , respectively. The close proximity between the fluorophore and quencher attached to the probe inhibits fluorescence from the fluorophore. During PCR, as DNA synthesis commences, the 5' to 3' exonuclease activity of the Taq polymerase degrades that proportion of the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.



(source: http://en.wikipedia.org/wiki/TaqMan)

- SYBR Green



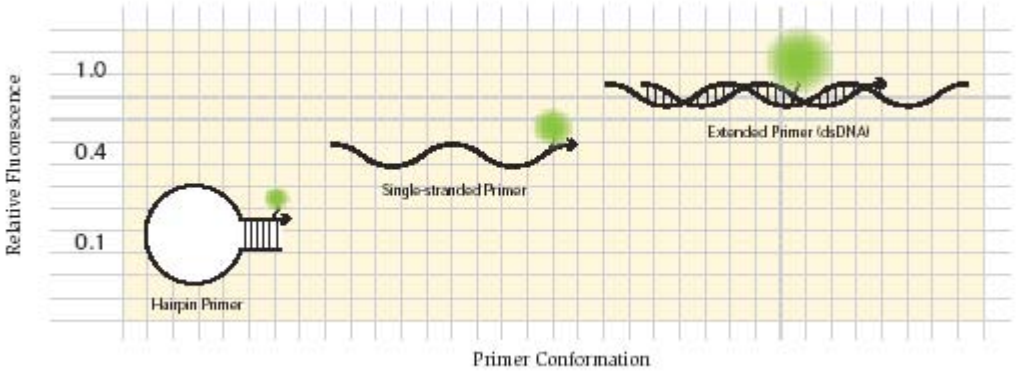
SYBR® Green I Method

SYBR Green is the most commonly used real-time PCR chemistry that is not based on a probe. It is an intercalating dye whose fluorescence increases significantly upon intercalation with double stranded DNA. Thus fluorescence emission is independent of the DNA sequence. A dissociation curve is needed to confirm the product by analyzing the mealting temperature (Tm).

(source: La Paz J.L. et. al., Comparison of Real-Time PCR Detection Chemistries and Cycling Modes Using Mon810 Event-Specific Assays as Model, *Journal of Agricultural and Food Chemistry*, **2007**, 55, 4312-4318)

- Lux technology

The LUX (Light Upon eXtension) effect presents a novel fluorescent detection mechanism for real-time analysis. LUX Primers are oligonucleotides labeled with a single fluorophore, custom-synthesized according to the DNA/RNA of interest. Typically 20-30 bases in length, they are designed with the fluorophore close to the 3´ end in a hairpin structure. This configuration, an advancement from the dual-labeled probe format, intrinsically renders fluorescence quenching capability so that a separate quenching moiety is not needed. When the primer is incorporated into the double-stranded PCR product, the fluorophore is
dequenched, resulting in a significant increase in fluorescent signal. This signal increase is the basis for the LUX detection platform.



(source: http://tools.invitrogen.com/content/sfs/brochures/711_01953LUXBrochure.pdf)

- Plexor technology

Plexor technology is a real-time PCR chemistry that differs from other such fluorogenic systems in its strong fluorescence signal at the beginning of the reaction which decreases proportionally to the increase of PCR products formed during the reaction. One of the primers contains a synthetic base, isocytosine, linked to the fluorophore at the 5'-end. During the amplification phase the iso-dGTP from the reaction solution preferentially binds to the opposite DNA strand and because it is linked to the quencher, the signal decreases after the binding.
For details see animation:
http://www.promega.com/paguide/animation/selector.htm?coreName=plexor01

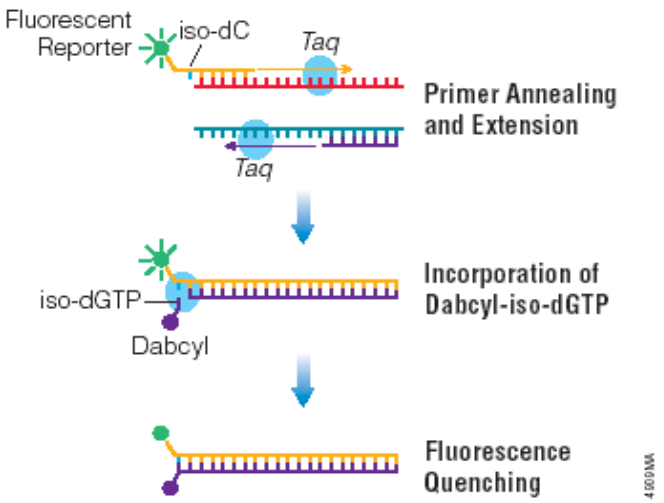
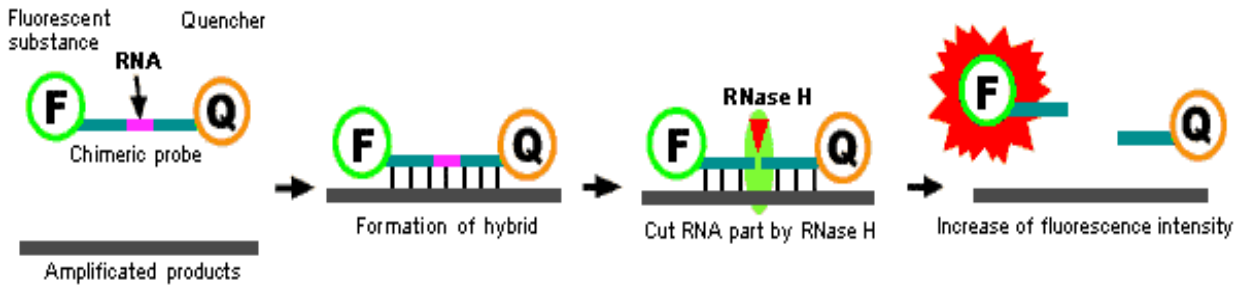


Figure 1. Quenching of the fluorescent signal by dabcyl during product accumulation.

- Cycling Probe technology

Cycling Probe Technology (CPT) is a highly sensitive detection method which utilizes a combination of a chimera probe, composed of RNA and DNA, and RNase H. The specific sequence of the target gene to be amplified can be detected efficiently during or after amplification by this method. One end of the probe is labeled with a fluorescent substance and the other end is labeled with a quencher, which quenches the fluorescence emitted from the fluorescent substance. As long as this probe remains intact, no strong fluoescence can be emitted because of the quenching function. When this probe forms a hybrid with the complementary sequence of amplified product, RNase H specifically cuts the RNA region of this probe, resulting in emission of strong fluorescence. By measuring the intensity of emitted fluorescence, the amount of amplified product can be monitored.



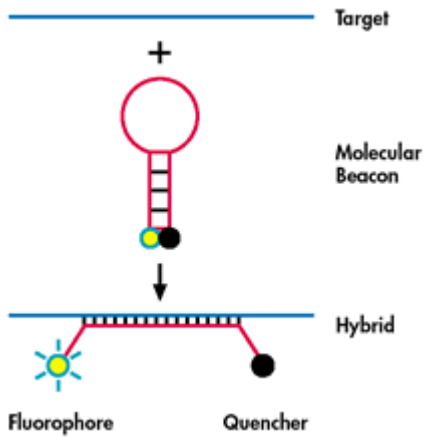
Principle of Cycling Probe Technology

(source: http://catalog.takara-bio.co.jp/en/product/basic_info.asp?unitid=U100005882)

- Molecular Beacons

Molecular beacons are single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure. The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore is covalently linked to the end of one arm and a quencher is covalently linked to the end of the other arm. Molecular beacons do not fluoresce when they are free in solution. However, when they hybridize to a nucleic acid strand containing a target sequence they undergo a conformational change that enables them to fluoresce brightly.

In the absence of targets, the probe is dark, because the stem places the fluorophore so close to the nonfluorescent quencher that they transiently share electrons, eliminating the ability of the fluorophore to fluoresce. When the probe encounters a target molecule, it forms a probe-target hybrid that is longer and more stable than the stem hybrid. The rigidity and length of the probe-target hybrid precludes the simultaneous existence of the stem hybrid. Consequently, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem hybrid to dissociate and the fluorophore and the quencher to move away from each other, restoring fluorescence.

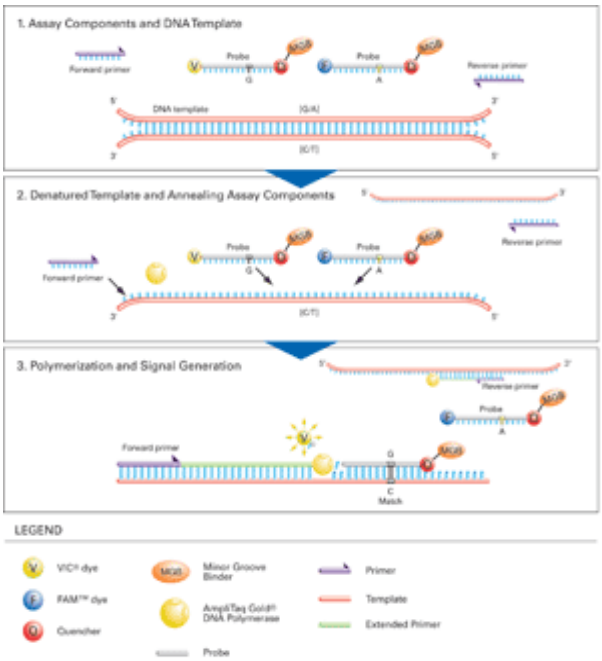


(source: http://www.molecular-beacons.org/Introduction.html)

- MGB probes

MGB or minor groove binding probes are similar to TaqMan probes by design and function. They contain a conjugated minor groove binder group that increases the melting temperature of the oligonucleotides. This causes improvements in hybridization specificity and makes it possible to design and use shorter

probes. The fluorescent signal they emit relys on FRET (i.e. fluorescence resonance energy transfer between the dye and the quencher).



(pic. Source: http://images.google.com/imgres?imgurl=http://www.appliedbiosystems.eu.com/innovations04/04_prods_mouse_fig1.gif&imgrefurl=http://marketing.appliedbiosystems.com/mk/submit/INNOVATIONS04JULY2007_RD%3F_JS%3DT%26rd%3Db&h=323&w=299&sz=15&hl=sl&start=6&um=1&tbnid=9wCbpubfoiopUM:&tbnh=118&tbnw=109&prev=/images%3Fq%3DMGB%2Bprobes%26um%3D1%26hl%3Dsl%26sa%3DN%26domains%3Den.wikipedia.org).

(source: Andersen C. B., et. al., Equal Performance of TaqMan, MGB, Molecular Beacon and SYBR Green-based Detection Assays in Detection and Quantification of Roundup Ready Soybean, *Journal of Agricultural and Food Chemistry*, **2006**, 54: 9658-9663.)

- Amplifluor technology
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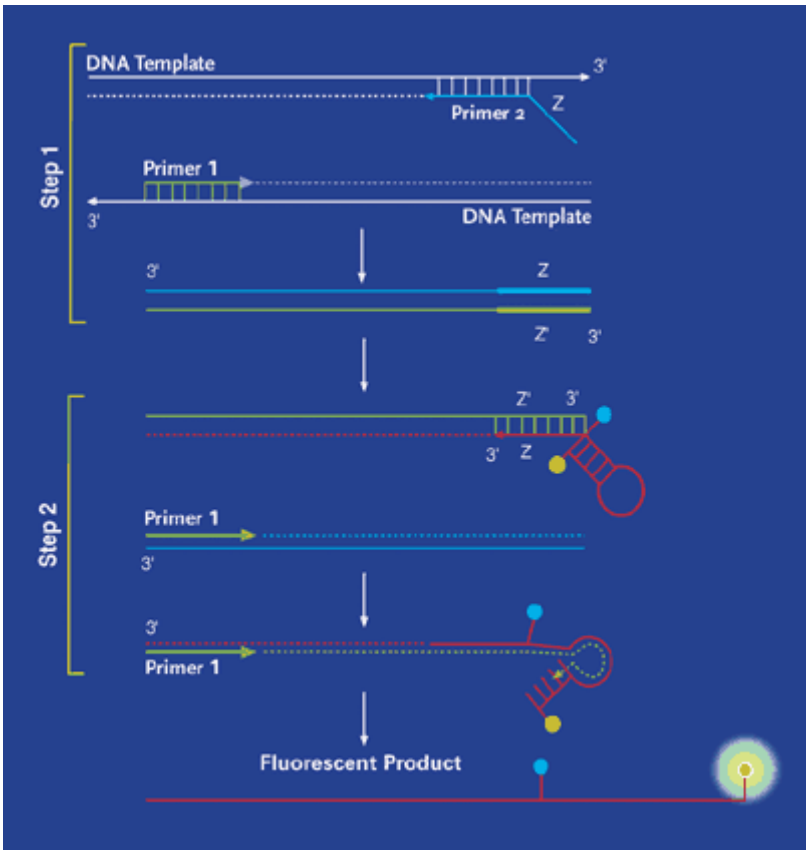


Amplifluor technology is based on energy transfer from an excited fluorophore to a complexed acceptor moiety that results in quenching of the fluorescence. This quenching is accomplished by linking the fluorophore and the acceptor to an oligonucleotide primer. The optimised design of the UniPrimer results in a large increase in fluorescent emission only upon incorporation into the amplification products produced during each PCR cycle. Unincorporated primers have low fluorescence, eliminating the need to purify the PCR reaction products prior to quantitation.

Each UniPrime consists of a different 3' primer sequence and a 5' hairpin region that is labelled with a unique energy transfer pair. The primer sequences enable the UniPrimer to work with target specific unlabelled primers to the researcher's target specific sequence. The target specific primers are synthesised with a 5' tail sequence identical to the 3' region of a particular Uniprimer, allowing it to hybridise to the PCR product.

During PCR, the incorporated UniPrimer serves as a template for DNA polymerisation. This process involves the displacement and replication of the hairpin sequence catalysed by DNA polymerase. The fluorophore is located at the 5' base of the primer and the quencher is linked to the nucleotide that is complementary to the 5' base. Efficient fluorescence quenching occurs when the primer is in this hairpin conformation. Upon incorporation into the doublestranded product, the hairpin structure unfolds and disrupts the energy-transfer between the fluorophore and the quencher. The fluorescent signal produced during each PCR cycle directly correlates to the amount of amplified DNA generated.

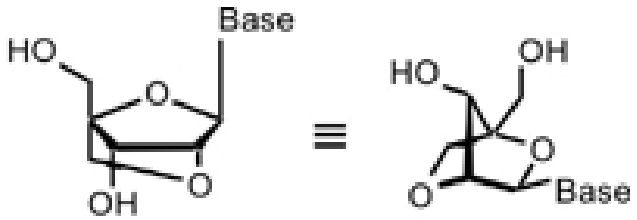
(source: http://www.flowgen.net/page019.asp)

- LNA technology

LNA chemistry is a real-time quantitative PCR chemistry which increases thermal duplex stability and improves specificity of probe hybridization to its target sequence. As such, background fluorescence from spurious binding is reduced and the signal-to-noise ratio is increased.

The LNA (locked nucleic acid) monomer chemical structure enhances the stability of the hybridization of the probe to its target. As a result, the duplex melting temperature (T_m) may increase by up to 8°C per LNA monomer substitution in medium salt conditions-compared to a DNA fluorescent probe for the same target sequence-depending on the target nucleic acid. This increase in hybridization creates a significant broadening in the scope of assay conditions and allows for more successful single-tube multiplexing.

Further, it is possible to optimize the T_m level and the hybridization specificity through specific placement of the LNA base(s) in the probe design.



LNA Monomer

(source: http://www.sigmaaldrich.com/Brands/Sigma_Genosys/DNA_Probes/Product_Lines/LNA_Probes.html)