

# Chapter 2

## Fluorescent-Based Detection, Quantitation, and Expression of Viral Gene by qRT-PCR

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### Abstract

Using fluorescent reporter molecules, viral gene(s) can be quantified for diagnostics as well as for gene expression studies by quantitative PCR. The process is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. 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. Here we discuss the detailed explanation of various fluorescent molecules and strategies to determine the viral load. These experiments are equally efficient in determining viral gene expression studies.

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### 2.1 Introduction

Over last several years, the development of novel chemistries and instrumentation platforms enabling detection of PCR products on real-time basis has lead to wide spread adoption of quantitative PCR (Q-PCR/qPCR) as the method of choice for diagnostic and analyzing changes in gene expression [1]. It is called “real-time PCR,” because it allows us to monitor the increase in the amount of DNA as it is amplified. Real-time polymerase chain reaction is also known as kinetic polymerase chain reaction, because it is the most sensitive technique to amplify and simultaneously quantify a targeted DNA molecule compared to the commonly used Northern and Southern blotting techniques. 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. Q-PCR can be used to quantify RNA levels from much smaller samples. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell.

Real-time PCR or Q-PCR is a variation of the standard PCR technique used to quantify DNA or messenger RNA (mRNA) in a sample. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes needed for the PCR cycles. Real-time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e., SYBR Green) or sequence-specific probes. 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. Using sequence-specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. We use the term relative since this technique tends to be used to compare relative copy numbers between tissues, organisms, or different genes relative to a specific housekeeping gene (or reference gene). Housekeeping genes are constitutively expressed at a relatively constant level in cells, and they are always present in all known conditions. To obtain the value of the target gene under investigation and the value of the housekeeping gene in the same sample, a standard curve can be used. In this, the absolute concentration of the target gene is divided by the absolute concentration of the housekeeping gene. The resulting target/reference ratio that expresses the amount of target gene is then normalized to the level of the reference gene within each unknown sample [2]. The quantification arises by measuring the amount of amplified product at each stage during the PCR cycle. DNA/RNA from genes with higher copy numbers will appear after fewer melting, annealing, extension PCR cycles.

We present here how to quantitate 2b gene (RNAi suppressor) of *Cucumber mosaic virus* (CMV) in different plant samples, using fluorescent molecules [3]. CMV is the type member of the *Cucumovirus* genus, in the family *Bromoviridae*. Cucumber mosaic, first described in 1916, was one of the earliest plant diseases attributed to a virus. CMV genome consists of positive sense, single-stranded RNA. CMV encodes five proteins, distributed on three genomic RNAs, i.e., RNA1 which is the only monocistronic RNA, encoding the 1a protein that is required for viral replication and contains methyl-transferase and helicase motifs. RNA2 encodes the 2a protein, the viral polymerase, and the 2b protein, the RNAi suppressor. RNA3 encodes the movement protein (MP), and the coat protein (CP) expressed from the subgenomic RNA4 and the satellite RNAs (satRNAs). The subgenomic RNA4 and the satellite RNAs of CMV are small linear RNA that does not carry any apparent coding capacity (Fig. 2.1).

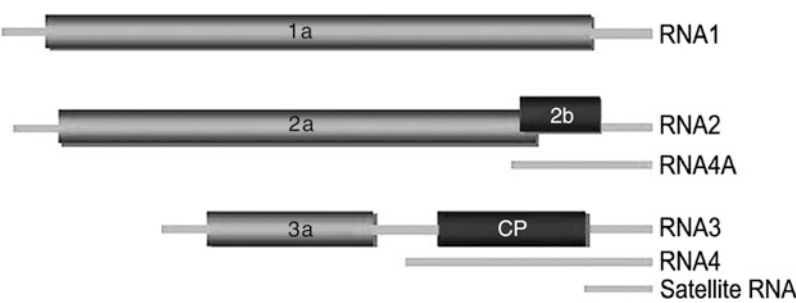


Fig. 2.1 CMV genome consists of single-stranded positive sense RNA.

CMV infects over 1,000 species of hosts, including members of 85 plant families, making it the broadest host range virus known. Tomatoes infected with the *Cucumber mosaic virus* develop a slight yellowing and mottling of the older leaves. The expanding leaves typically become twisted, curl downward, and develop a “shoestring” appearance as a result of a restriction of the leaf surface to a narrow band around the midrib of the leaf. Diseased plants are stunted and produce poor fruit yield.

2.2 Materials

2.2.1. Instrument and Set Up

1. Real-time PCR thermal cycler; Light Cycler<sup>®</sup> 480 II (Roche).
2. Clear LightCycler<sup>®</sup> 480 multiwell plates.
3. LightCycler<sup>®</sup> 480 Sealing Foil.
4. 2× Light CyclerR 480 SYBR Green 1 master mix/TaqMan probe.
5. Gene-specific primers.

2.2.2. QRT-PCR Reaction Requirements (Using TaqMan Probe)

Total RNA (as CMV is a RNA virus) from the infected plant leaf samples

1. RNA (10–100 ng)	30.0 µl
2. 10× TaqMan buffer	5.0 µl
3. MgCl <sub>2</sub> (25 mM)	5.0 µl
4. dNTPs (10 mM)	2.0 µl
5. Primer F (10 µM)	2.5 µl
6. Primer R (10 µM)	2.5 µl
7. TaqMan probe (10 µM)	1.0 µl
8. Taq Polymerase (5 U)	0.5 µl
9. M-MuLV Reverse Transcriptase (20 U)	0.5 µl
10. RNase inhibitor (20 U)	1.0 µl

**2.2.3. Cycling****Parameters**

1. Reverse transcription (using M-MuLV) at 48 °C for 30 min.
2. *Taq* activation 95 °C for 10 min.

*PCR profile:*

1. Denaturation at 95 °C for 15 s.
2. Annealing/extension at 60 °C for 1 min (repeated 40 times).

**2.2.4. Components****2.2.4.1. Primers and Probe**

Whenever possible, primers and probes should be selected in a region with a G/C content of 30–80 %. Regions with G/C content in excess may not denature well during thermal cycling, leading to a less efficient reaction. In addition, G/C-rich sequences are susceptible to nonspecific interactions that may reduce reaction efficiency and produce nonspecific signal in SYBR Green assays. For this same reason, primer and probe sequences containing repeats of four or more G bases should be avoided. A/T-rich sequences require longer primer and probe sequences in order to obtain the optimum melting temperatures. This is rarely a problem for quantitative assays; however, probes approaching 40 base pairs can exhibit less efficient quenching and produce lower synthesis yields. Primer should be highly purified ideally; HPLC purified primers should be used and their concentration should be in the range of 0.3–1  $\mu$ M, ideally 0.5  $\mu$ M. The last five bases on the 3' end of the primers should contain no more than two C and/or G bases, which is another factor that reduces the possibility of nonspecific product formation. Under certain circumstances, however, such as a G/C-rich template sequence, this recommendation may have to be relaxed to keep the amplicon under 150 base pairs in length. It should be followed as often as possible, and even when it is not possible, primer 3' ends extremely rich in G and/or C bases should be avoided. The  $T_m$  of primers is adjusted in the range of 58–60 °C as both the annealing and extension step are achieved in a single step of real-time PCR.

**2.2.4.2. Probe Selection  
Criteria**

1. Select the probe first and design the primers as close as possible to the probe without overlapping it.
2. Keep the G/C content in the 30–80 % range.
3. Avoid runs of an identical nucleotide, especially for guanine, where repeats of four or more should be avoided, and there should be no G on the 5' end.
4.  $T_m$  of the probe should be in the range of 60–70 °C.
5. Select the probe with more C compared to G bases.

Selecting primers and probes with the recommended  $T_m$  is one of the factors that allow the use of universal thermal cycling parameters. Having the probe  $T_m$  8–10 °C higher than that of the

primers ensures that the probe is fully hybridized during primer extension. The required parameters for well-designed primers and probe have been well documented. These parameters include  $T_m$  for the probe that is 10 °C higher than the primers, primer  $T_m$  between 58 and 60 °C, amplicon size between 50 and 150 bases, and absence of 5' Gs.

#### 2.2.4.3. Template

A critical aspect of performing real-time PCR is to begin with a template that is of high purity. The DNA should be about 5–30 ng in concentration ideally; 25 ng DNA template is used in 20  $\mu$ l reaction mix. Size of amplicon should be <500 bp. Small amplicons are favored because they promote high-efficiency assays. In addition, high-efficiency assays enable relative quantification to be performed using the comparative method or threshold cycle ( $C_t$ ). This method increases sample throughput by eliminating the need for standard curves when looking at expression levels of a target relative to a reference control.

#### 2.2.5. General Recommendations for Real-Time RT-PCR

The optimal concentrations of the real-time PCR reagents are as follows:

1. Magnesium chloride concentration should be between 4 and 7 mM.
2. Concentrations of dNTPs in TaqMan reaction should be 200  $\mu$ M of each dNTPs.

Typically 1.25 U of *Taq* DNA polymerase is used in a 50- $\mu$ l reaction mixture. This is the minimum requirement; if necessary, optimization can be done by increasing this amount by 0.25 U increments.

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## 2.3 Methods

#### 2.3.1. Standard Curve Method

In this method, a standard curve is first plotted from DNA/RNA sample of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for samples of unknown DNA/RNA concentration. Nucleic acids like DNA, RNA, in vivo generated ssDNA or any cDNA sample can be used to construct standard curve. For the standard curve first the standard sample is quantified accurately, spectrophotometrically and is then converted to copy number based on molecular weight of the sample used. In this method, a standard curve is first plotted from DNA/RNA sample of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for samples of unknown DNA/RNA concentration. Nucleic acids like DNA, RNA, in vivo generated ssDNA or any cDNA sample can be used to construct standard

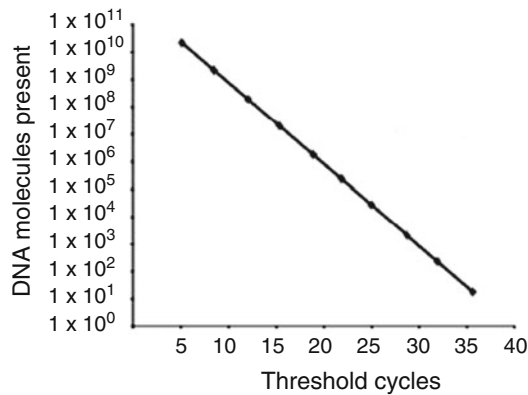


Fig. 2.2 Standard curve for absolute quantitation.

curve. For the standard curve first the standard sample is quantified accurately, spectrophotometrically and is then converted to copy number based on molecular weight of the sample used (Fig. 2.2).

Though RNA standards can be used, their stability can be a source of variability in the final analyses also; using RNA standards involves the construction of cDNA plasmids that have to be in vivo transcribed into the RNA standards. To check the variation introduced due to the variable RNA inputs, normalization can be done using a housekeeping gene.

### 2.3.2. Comparative Threshold ( $C_t$ ) Method

This involves comparing the  $C_t$  values of sample of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The  $C_t$  value of both the sample of interest and calibrator are normalized to an appropriate endogenous housekeeping gene.

The comparative  $C_t$  method is also known as  $2^{-[\Delta][\Delta]} C_t$  method, where:

$$[\Delta][\Delta] C_t = [\Delta] C_{t, \text{sample}} - [\Delta] C_{t, \text{reference}}$$

Here,  $[\Delta] C_{t, \text{sample}}$  is the  $C_t$  value for any sample normalized to the endogenous housekeeping gene and  $[\Delta] C_{t, \text{reference}}$  is  $C_t$  value for the calibrator also normalized to the endogenous housekeeping gene.

For the  $[\Delta][\Delta] C_t$  calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be estimated by looking at how  $[\Delta] C_t$  varies with template dilution. If the plot of cDNA dilution versus  $\Delta C_t$  is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method is preferred (Fig. 2.3).

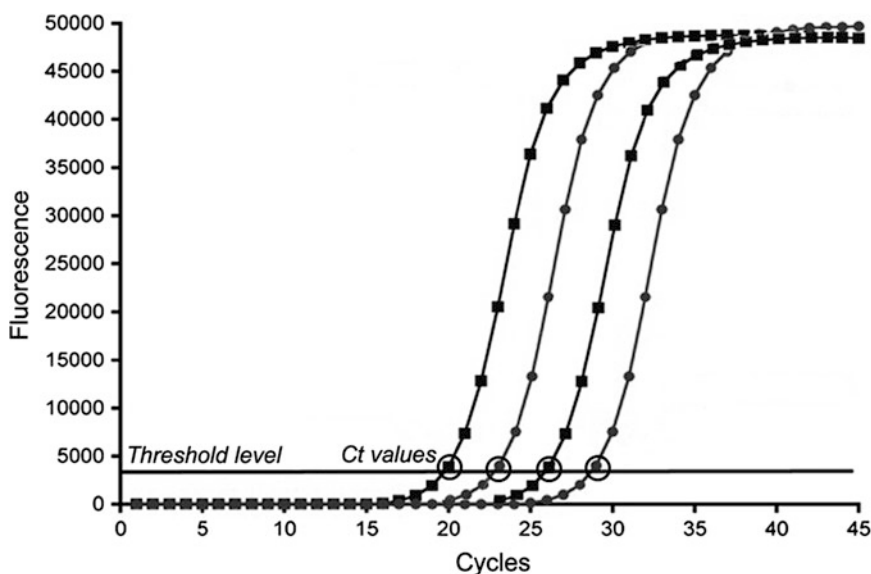


Fig. 2.3 Graph representing a typical curve with critical threshold value.

### 2.3.3. Reaction Set and Thermal Cycling

1. Prepare the reaction mix (according to the components given in Sect. 2.2.2) and load it on the multi-well plate.
2. Set up the cycling parameter (shown in Sect. 2.2.3 for CMV-2b gene).
3. Run the program and analyze the results.

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## 2.4 Notes

The quantification is done by measuring the amount of amplified product at each stage during the PCR cycle. DNA/RNA from genes with higher copy numbers will appear after fewer PCR cycles. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes required for the PCR cycles. Real-time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e., SYBR Green) or sequence-specific probes (TaqMan probes). 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.

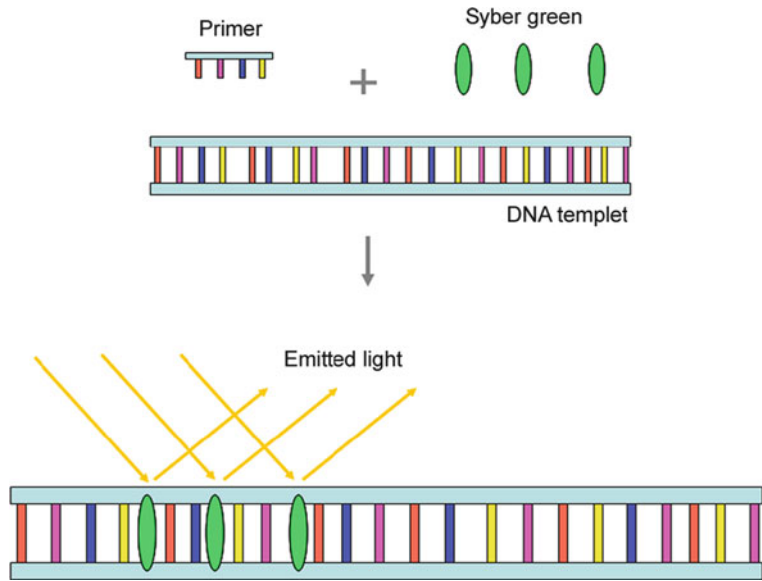


Fig. 2.4 SYBER Green; A fluorescent dye binds with double-stranded DNA.

#### 2.4.1. SYBER Green

SYBER Green provides the simplest and most economical format for detection and quantitation of PCR products in real-time reactions. SYBER Green binds double stranded DNA and upon excitation emits light. An increase in DNA product during PCR leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified (Fig. 2.4).

The advantages of SYBER Green are that it is inexpensive, easy to use, and sensitive. However, dsDNA dyes such as SYBR Green binds to all dsDNA PCR products, including nonspecific PCR products (such as “primer dimers”). This can potentially interfere with/or prevent accurate quantification of the intended target sequence. For single product PCR reactions with well-designed primers, SYBER Green can work extremely well, with spurious nonspecific background showing up in very late cycles.

1. The reaction is prepared as usual, with the addition of fluorescent dsDNA dye (instead of TaqMan probe as described in protocol).
2. The reaction is run in a thermocycler, and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). With reference to a standard dilution, the dsDNA concentration in the PCR can be determined.

Like other real-time PCR methods, the values obtained do not have absolute units associated with it (i.e., DNA/RNA copies/cell). A comparison of a measured DNA/RNA sample to a



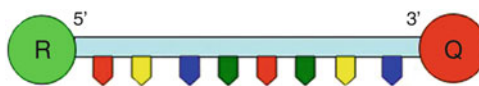


Fig. 2.5 The TaqMan probe. The *red circle* represents the quenching dye that disrupts the observable signal from the reporter dye (*green circle*) when it is within a short distance.

standard dilution will only give a fraction or ratio of the sample relative to the standard, allowing only relative comparisons between different tissues or experimental conditions. To ensure accuracy in the quantification, it is usually necessary to normalize expression of a target gene to stably expressed housekeeping genes, e.g., Ubiquitin. SYBR Green is the most widely used double-strand DNA-specific dye reported for real-time PCR. SYBR Green binds to the minor groove of the DNA double helix. In the solution, the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double-strand DNA. SYBR Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. Ethidium bromide can also be used in real-time PCR for detection of amplification but its carcinogenic nature renders its use restrictive.

#### 2.4.2. Hydrolysis Probe

The hydrolysis probe chemistry relies on the 5′–3′ exonuclease activity of *Taq* polymerase, which degrades a hybridized non-extendible DNA probe [4] during the extension step of the PCR, e.g., TaqMan probes. TaqMan probe is designed to hybridize in a region within the amplicon and is dual labeled with a reporter dye and a quenching dye. The reporter dye is attached to the 5′ end of the probe and the quencher at the 3′ end. The close proximity of the reporter to quencher prevents detection of its fluorescence (Fig. 2.5).

During the annealing stage of the PCR, both probe and primers anneal to the DNA target. Polymerization of a new DNA strand is initiated by TaqMan polymerase from the primers, and once the polymerase reaches the probe, its 5′–3′ exonuclease activity degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in fluorescence, and this fluorescence is detected and measured in the real-time PCR thermocycler. The more times the denaturing and annealing takes place, the more opportunities there are for the TaqMan probe to bind and, in turn, the more emitted light is detected, and its geometric increase corresponding to exponential increase of the product is used to determine the threshold cycle ( $C_t$ ) in each reaction.

Fluorescent reporter probes are more accurate and reliable of the method. It uses a sequence-specific RNA or 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 nonspecific 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. Well-designed TaqMan probes require very little optimization.

#### **2.4.3. Hybridization Probe**

The hybridization probes are used for DNA detection and quantitation, providing maximum specificity for product identification. Two specifically designed, sequence-specific oligonucleotide probes, labeled with different dyes, are used. The sequences of the probes are selected so that they can hybridize to the target sequences on the amplified DNA fragment in a head-to-tail orientation, thus bringing the two dyes into close proximity. The donor dye (fluorescein) is excited by the blue light source and emits green fluorescent light at a slightly longer wavelength. At close proximity, the energy emitted excites the acceptor dye attached to the second hybridization probe, which then emits fluorescent light at a different wavelength. The amount of fluorescence emitted is directly proportional to the amount of target DNA generated during the PCR reaction.

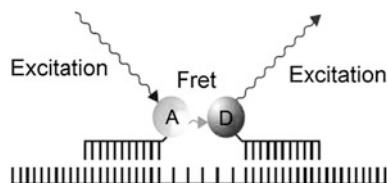
##### **2.4.3.1. FRET Probes**

Fluorescence resonance energy transfer (FRET) is transfer of energy from excited state, i.e., from the initially excited donor (D) to an acceptor (A). The hybridization probe system consists of two oligonucleotides labeled with different marker fluorescent dyes and these two probes designed to hybridize in close proximity on the target DNA. Interaction of the two dyes can only occur when both are bound to their target. The donor probe is labeled with fluorophore at the 3' end and the acceptor probe at 5' end. During PCR, the two different oligonucleotides hybridize to adjacent regions of the target DNA such that the fluorophores, which are coupled to the oligonucleotide, are in close proximity in the hybrid structure. The donor fluorophore is excited by an external light source and then passes part of its excitation energy to the adjacent acceptor fluorophore. The excited acceptor fluorophore emits light at a longer wavelength, which can then be detected and measured (Fig. 2.6). The light source cannot excite the acceptor dye.

Applications of FRET probes are in:

1. Quantitative PCR.
2. DNA copy number measurements.
3. Pathogen detection assays.
4. Single nucleotide polymorphism (SNP) genotyping.
5. Verification of microarray results.

Fig. 2.6 Fluorescence resonance energy transfer (FRET).



#### 2.4.3.2. Molecular Beacons

Molecular beacons are hairpin-shaped oligonucleotide in their unhybridized state containing a fluorophore on one end and a quenching dye on the opposite end. Under conditions when probe is not hybridized to its complementary target, the fluorescent and quenching dye remain proximal to one another, thus preventing fluorescence resonance energy transfer (FRET). Whereas, when the probe encounters a target molecule, it forms a probe–target hybrid that is longer and more stable than the stem hybrid; this causes the fluorophore and the quencher to move away from each other and causes emission of fluorescence. Molecular beacons are designed so that their probe sequence is just long enough for a perfectly complementary probe–target hybrid to be more stable than the stem hybrid. The length of the probe sequence (10–40 nt) is chosen in such a way that the probe target hybrid is stable in the conditions of the assay. The stem sequence (5–7 nt) is chosen to ensure that the two arms hybridize to each other but not to the loop sequence (Fig. 2.7).

The computer program is used to predict melting temperature of the stem and also to predict whether the intended stem-and-loop conformation will occur or not. Molecular beacons can be synthesized that possess differently colored fluorophores, enabling assays to be carried out that simultaneously detect different targets in the same reaction.

Molecular beacons are thus ideal probes for use in diagnostic assays designed for genetic screening, SNP detection, and pharmacogenetic applications. In summary, molecular beacons have three key properties that enable the design of new and powerful diagnostic assays:

1. They only fluoresce when bound to their targets.
2. They can be labeled with a fluorophore of any desired color.
3. They are so specific that they easily discriminate single-nucleotide polymorphisms.

#### 2.4.3.3. Scorpions

In Scorpion probes, sequence-specific priming and PCR product detection are achieved using a single oligonucleotide. The Scorpion probe maintains a stem–loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the

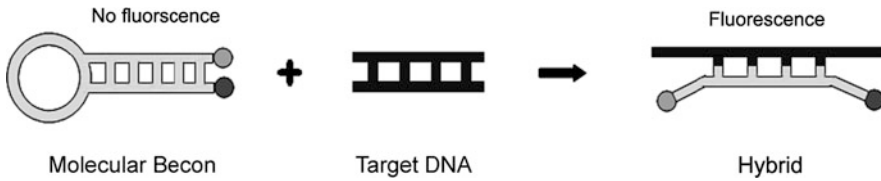


Fig. 2.7 Molecular beacon; a hairpin fluorescent probe.

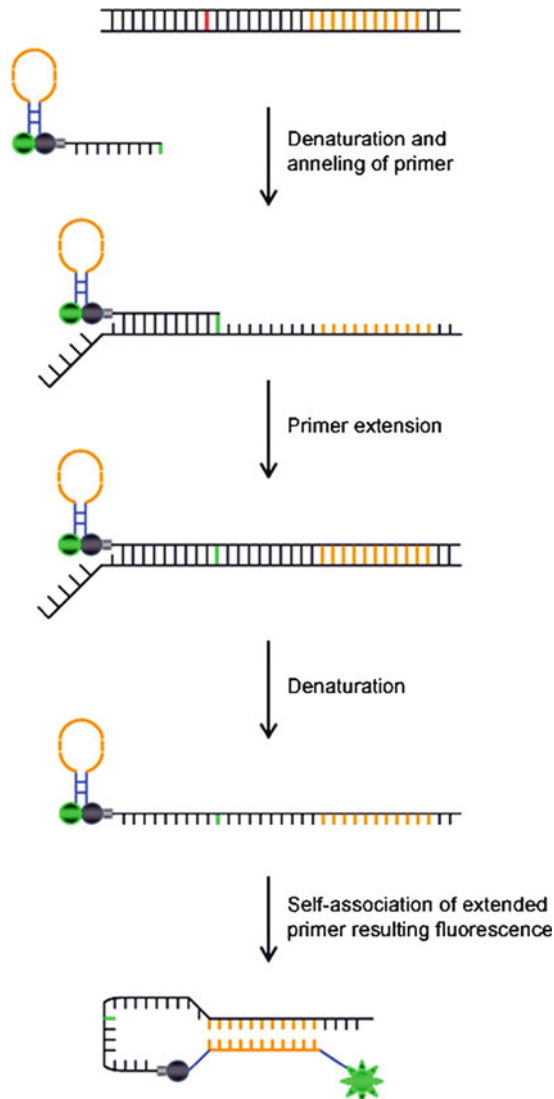


Fig. 2.8 Scorpion probe; a single oligonucleotide used in priming as well as in probing.

5' end of a specific primer via a non-amplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon thus opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is observed (Fig. 2.8).

It is possible to choose between the open and closed Scorpion format. Closed format means that the probe part of the Scorpion is designed to have two stems at each end that are complimentary to each other so that it will be in a beacon-like (link) secondary structure when it is not yet hybridized to the primer's extension product. This way a fluor and quencher that are attached to the 5' and 3' ends of the probe are in close proximity to each other. Hence, when the Scorpion is free in solution no fluorescence can be detectable. When the Scorpion unfolds as the probe binds to the extended primer, the fluor and quencher will be separated and fluorescence can be detected as to quantify the amount of PCR product. In the open format, the probe part of the Scorpion does not have a specific secondary structure in the unhybridized form and contains a fluor. A separate quencher oligonucleotide is designed simultaneously. This quencher will bind to the probe part of the Scorpion when the Scorpion is not bound to its intended target so as to prevent fluorescence. As the Scorpion binds to the target, the quencher and probe will be separated from each other when the probe hybridizes to the extension product of the primer and hence, fluorescence can be detected and used to quantify the amount of PCR product.

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