REAL-TIME PCR AND ITS APPLICATIONS IN HUMAN PAPILLOMAVIRUS QUANTITATION AND PHYSICAL STATUS IDENTIFICATION

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ABSTRACT

The basic principles and advantages of real-time PCR are reviewed in this paper as well as the recent literature regarding to detection and research of human papillomavirus (HPV) in the routine and research laboratory. Real-time PCR assays are not only fast and simple to quantitatively detect and type HPV DNA, but also can define the physical state of the virus, providing valuable information with regard to the progression of HPV infection. Thus, identification of integrated HPV and measurement of the viral load provides a new potential prognostic tool with which to estimate the risk of cervical cancer in individual patients.

Introduction

Human papillomaviruses (HPVs) comprise more than 120 putative virus types, of which 85 types have been fully sequenced. More than 40 types of human papillomavirus (HPV) infect the genital epithelium and several high-risk types including HPV types 16, 18, 31, 33, and 45 are found in almost all cases of high-grade cervical intraepithelial neoplasia and cervical cancer (12). Laboratory diagnosis of HPV infection is dependent upon molecular techniques such as DNA hybridization or nucleic acid amplification. Several polymerase chain reaction (PCR) methods have been developed to detect a broad spectrum of HPV types using various primer sets (5, 10).

Irrespective of the PCR amplification method used, several techniques are used for the detection of PCR products, such as agarose gel electrophoresis and ethidium bromide staining, fluorescence labeling and analysis using polyacrylamide gels, and radioactive labeling and Southern blotting or detection by phosphorimaging. All these techniques require intensive and laborious post-PCR manipulation, make use of hazardous chemicals, and carry a potential risk for laboratory contamination (7).

The introduction of the new procedure based on fluorescence – kinetic PCR enables quantification of the PCR product in "real-time." This sensitive and accurate technique measures PCR product accumulation during the exponential phase of the

reaction. The technique is much faster than the previous endpoint PCR as it designed to provide information as rapidly as the amplification process itself, thus requiring no post-PCR manipulations (1).

Prinlciple of Real-time PCR and detection chemistries available

Two important findings led to the discovery of real-time PCR: first, the finding that the *Taq* polymerase possesses 5'-exonuclease activity; second, the construction of dual-labeled oligonucleotide probes, which emit a fluorescence signal only on cleavage, based on the fluorescence resonance energy transfer (FRET) principle (**Fig. 1**). FRET is a process that shifts energy from an electronically excited molecule (the donor fluorophore) to a neighboring molecule (the acceptor or quencher), returning the donor molecule to its ground state without fluorescence emission.

In the TaqMan assay are combined both important findings mentioned above (**Fig. 2**). In this real-time PCR method the *Taq* polymerase enzyme cleaves an internal labeled nonextendable probe, the so-called "TaqMan" probe, during the extension phase of the PCR. The probe is dual-labeled, with a reporter dye, e.g., FAM (6-carboxyfluorescein), at one end of the probe and a quencher dye, e.g. TAMRA (6-carboxytetrameth-ylrhodamine), at the other extremity. As long as the probe is intact (in its free form), fluorescence energy transfer occurs through which the fluorescence emission of the reporter dye is absorbed by the quenching dye. On nuclease degradation of the probe during the PCR, the reporter and quencher dyes are separated, and the reporter dye emission is no longer transferred to the quenching dye (no more FRET), resulting in an increase of reporter fluorescence emission (e.g., for FAM at 518 nm). This process occurs in every cycle and does not interfere with the exponential accumulation of PCR product (13).

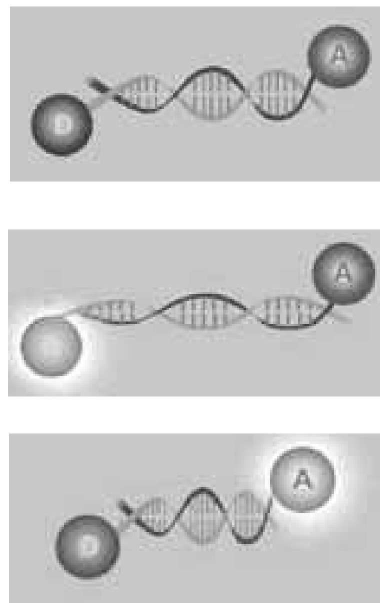

Fig. 1. Principle of FRET. FRET features a donor (D) and an acceptor (A) dye molecule attached at different points along a sample. When the donor is photo-excited at longer distances from the acceptor, fluorescence comes exclusively from the donor. As the space between them contracts, fluorescence is increasingly transferred to the acceptor.

More recently, other sophisticated systems have been developed, such as molecular beacons, scorpions, and hybridization probes. These systems all rely on the FRET

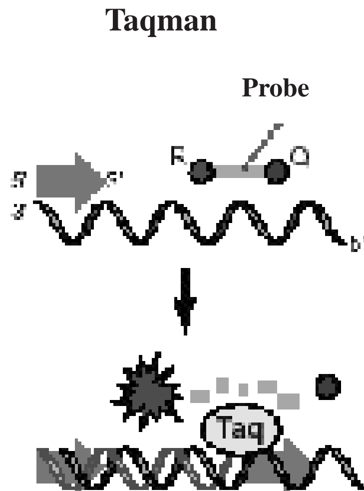

Fig. 2. TaqMan system. After denaturation, primers and probe anneal to the target. Fluorescence does not occur because of the proximity between fluorophore and quencher. During the extension phase, the probe is cleaved by the 5'-3' enzymatic activity of *Taq* polymerase. Thereby quencher and fluorophore are separated, allowing fluorescence emission from the reporter dye.

principle, although without the need for hydrolysis by the nuclease activity of the *Taq* polymerase. Molecular beacons are probes that form a stem-and-loop structure from a single-stranded DNA molecule (**Fig 3**). A fluorophore is linked to one end of the molecule and a quencher is linked to the other end. Fluorescence is quenched when the probe is in a hairpin-like structure (stem-and-loop structure) due to the proximity between quencher and fluorophore allowing FRET. When the probe sequence in the loop anneals to a complementary nucleic acid target sequence, a conformational change allows the formation of a linear structure whereby FRET no longer occurs, increasing the fluorescence emission. Molecular beacons are especially suitable for identifying point mutations. They can distinguish targets that differ by only a single nucleotide and significantly more specific than oligonucleotide probes of equivalent length (1, 7, 13).

Finally, the use of dsDNA-binding dyes, such as SYBR Green I®, was found to be very useful in detecting PCR product formation. Using this system, the need for an expensive, although specific probe can be avoided. SYBR Green I® is a DNA-binding dye that incorporates into dsDNA

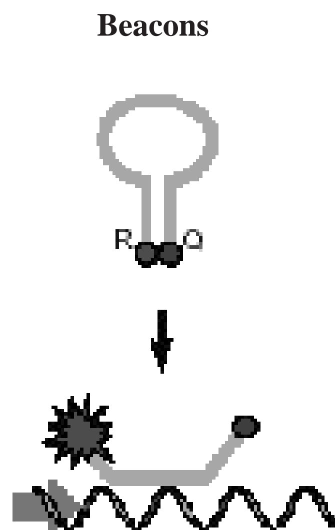

Fig. 3. Molecular beacons. The probe has a stem-and-loop hairpin shape, so that quencher and fluorophore are in very close proximity, avoiding fluorescence emission. As soon as probe hybridizes to the target, the probe is conformationally changed to a linear structure, separating quencher and fluorophore. This results in an increase in fluorescence emission.

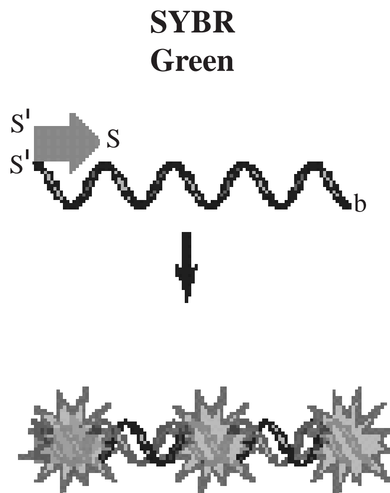

Fig. 4. DNA-binding dyes (SYBR Green I®). The dyes free in the solution do not emit fluorescence light. As soon as the SYBR Green binds to the dsDNA, target fluorescence occurs.

(**Fig 4**). It has an undetectable fluorescence when it is in its free form, but once bound to the dsDNA it starts to emit fluorescence. Its greatest advantage is that it can be used with any pair of primers for any target, making its use less expensive than that of a probe. However, specificity is diminished due to the risk of amplifying nonspecific PCR products. Indeed, SYBR Green I® binds to any dsDNA, detecting not only the specific target, but also nonspecific PCR products and primer dimers. This is a major disadvantage. There are a few ways of handling this problem, comparing melting curves being one of them. In this way, the fraction of fluorescence originating from the target can be distinguished from that originating from primer dimers or specific amplification products. Once the melting curves are established, it is possible to set the software to acquire fluorescence above the primer dimers' melting temperature but below that of the product. Second, a careful design of the primers and optimization of the reaction conditions can reduce the formation of primer dimers to a level that is important only for very low copy number detection. Furthermore, techniques such as Hot Start PCR can reduce primer dimer formation (1, 7, 13).

In the **Table** are presented the main characteristics and applications of the chemistries, widely used in real-time PCR assays.

Threshold cycle

The concept of the threshold cycle (Ct) is at the heart of accurate and reproducible quantification using real-time PCR. Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more templates present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background. This point is defined as the Ct, and will always occur during the exponential phase of amplification. *C*t values decrease linearly with increasing input target quantity. This can be used as a quantitative measurement of the input target (7).

Instrumentation

At present, it is possible to choose between a variety of competing instruments. All the instruments available run the reaction as a closed-tube system, diminishing the chanc-

TABLE

Chemistry	How it works	Quencher	Signal detection	Major applications
SYBR Green I®	Binds to the minor groove of dsDNA	No	Extension	Quantify and detect targets of interest. Melting curve analysis
Molecular Beacons	Hybridization Probe (Hairpin Shaped)	Yes	Annealing	SNP* detection Quantify and detect targets of interest
TaqMan	Hydrolysis Probe (5'-3' Exo)	Yes	Any step	SNP detection Quantify and detect targets of interest

*SNP – single nucleotide polymorphism

es of contamination. Recently, our laboratory has been equiped by the real-time system Opticon-2 (MJResearch). The system can be operated with molecular beacons, TaqMan probes, or SYBR Green I®. The optical system incorporates an array of 96 LEDs for excitation and a pair of sensitive PMTs for detection. A temperature gradient feature permits simultaneous incubation at 12 different temperatures to optimize reactions in a single run. Moreover, the 96 samples are tracked simultaneously, thereby providing a very fast assay. Besides, a compact footprint ensures that the Opticon system fits quite easily.

Another commercially available real-time PCR systems are ABI prism 7700 SDS, GeneAmp 5700 SDS, ABI Prism 7900 HT SDS (Applied Biosystems), iCycler iQ (Bio-Rad), Smart Cycler (Cepheid), Rotorgene (Corbett Research), LightCycler (Roche Molecular Biochemicals), Mx4000 Multiplex (Stratagene).

Application of real-time PCR in HPV diagnostics

Real-time PCR has been extremely useful for studying viral agents of infectious diseases and helping to clarify disputed infectious disease processes. The use of real-time PCR has provided insight into the role of human papillomavirus in the development of wide spectrum of disease ranging from benign warts to esophageal, laryngeal, and cervical carcinoma, as well as many carcinomas of the head and neck. Infection with high-risk (oncogenic) types of HPV (HPV-16, -18, -31, -33, -35, 39, -45,

-52, -56, -58, and -68) is a well-established risk factor for the development of cervical carcinoma (17), which is the second most common female malignancy worldwide. The most common oncogenic HPV type in cervical cancer is HPV type 16 (HPV16), which is detectable in more than 50% of the cases (16). Premalignant cervical lesions are commonly staged according increasing severity, as Cervical Intraepithelial Neoplasia I (CIN-I), CIN-II, and CIN-III, where CIN-III represents severe dysplasia or cancer in situ. Studies of Groff et al. (8) suggested that benign HPV lesions and low-grade intraepithelial lesions (CIN-I) mostly contain the viral sequences only as episomes. In contrast, viral DNA is integrated into the host genome in virtually all cases of cervical carcinomas and their derivate cell lines (3). Viral DNA integration into host cell DNA usually disrupts the E1 and E2 open reading frames (ORFs). In contrast, the E6 and E7 ORFs and LCR (long control region) generally remain intact (4). Initial heavy viral loads favor the probability of integration into chromosomal sites. There is increasing evidence that viral load is a critical determinant in patient prognosis (6).

Real-time PCR is a powerful tool for determination of both physical state and viral load of HPVs. Nagao et al. (15) have described a rapid method of quantitative real-time PCR to quantify copy numbers of E2 and E6 genes for analysis of the physical status of HPV16 DNA. Peitsaro et al. (16) have described also a system capable of identifying of whether HPV-16 is integrated at early stages of cervical carcinogenesis. With the help of both techniques, it is possible to calculate viral copy numbers for both episomal and integrated forms separately.

Hart et al. (9) constructed a technique for HPV typing that they have named viral evaluation using self-probing amplicons (VESPA). VESPA utilizes selfprobing amplicon primers known as "*scorpions*". VESPA is quick (<1 h), specific (single-base discrimination), and less laborious (single step) and is capable of estimating viral load. Viral load estimation can be successfully performed by fluorescent 5' exonuclease assay (11). The fluorescent 5' exonuclease assay, employed in TaqMan, was found to discriminate well between the HPV types investigated and can be used to quantify the HPV copy number over a wide range of HPV copy numbers. By means of TaqMan assay, Moberg et al., (14) have used the descried above assay to study the relationship between viral DNA amount and risk of cervical carcinoma for the HPV types most commonly found in cervical tumors. They were able to demonstrate that the titer of HPV16 in cervical smears can be used to predict the risk of development of cervical cancer *In Situ* (cervical intraepithelial neoplasia, stage III [CIN-III]). These results indicate that determination of the HPV titer present an opportunity to assess whether an infection will progress into cervical cancer or be cleared. Subsequently, based on other study designs or methods for quantification of the amount of HPV, it has been confirmed that a high HPV16 titer is associated with an increased risk of developing more se-

vere dysplasia and that reduction of the viral load may predict regression of CIN to normalcy.

For HPV genotyping and quantitation have been constructed systems using SYBR Green I® and molecular beacons (18). The sensitivities of the molecular beacons method and the TaqMan approach are similar, but molecular beacon probes have a higher degree of specificity compared to TaqMan probes.

Real-time PCR assay can be also used for differentiation between HPV16 and HPV18 using consensus primers and melting curve analysis (2). Because HPV16 and HPV18 product lengths are very similar, it is impossible to differentiate between individual HPV types by agarose gel electrophoresis after GP5+/6+ PCR amplification. In contrast, melting curve analysis can distinguish between products of the same length but different GC:AT ratios. In mixed samples, two separate peaks of distinct Tm are seen, even when there is a considerable difference in the copy number of each type. This approach has been confirmed with type specific primers (2).

The described real-time PCR assays are fast and simple to quantitatively detect and type HPV DNA. Besides, by means of RT-PCR can be defined the physical state of the virus, providing valuable information with regard to the progression of HPV infection. Thus, identification of integrated HPV and measurement of the viral load provides a new potential prognostic tool with which to estimate the risk of cervical cancer in individual patients.

In conclusion, we described the basic principles and advantages of real-time PCR and reviewed the literature as it applies to detection and research of human papillomavirus in the routine and research laboratory. However, the technology discussed has been applied to other areas of microbiology as well as studies of gene expression and genetic disease.

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