

# Real-time PCR applications in clinical research and diagnostics

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**Abstract** — Polymerase chain reaction (PCR) has enabled enormous progress in the field of molecular biology in the last twenty-five years. It became popular due to its high sensitivity and specificity and was introduced to numerous scientific fields. Today many different variants of PCR exist, with real-time PCR being the most common. This method is being successfully used at our institution for a variety of different applications, among which gene expression analyzes and development of protocols for clinical diagnostics currently dominate. But the extreme flexibility and customizability of real-time PCR, coupled with our team's expertise, ensures efficient application of this technique to various new research projects.

**Index Terms** — real-time PCR, SYBR Green, TaqMan, quantification, gene expression

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## 1 INTRODUCTION

### Principle of PCR reaction

**P**olymerase chain reaction (PCR) is a technique that has had a tremendous influence on development and research in molecular biology. Nowadays, it has become common and is an essential laboratory tool, on which the majority of molecular biological studies are based.

The primary purpose of PCR is rapid amplification of single or few copies of template DNA, generating thousands to millions of copies of the original (template) DNA sequence. Key components that enable this process are primers, deoxyribonucleotides, and a DNA polymerase. The first are short nucleotide sequences that specifically bind

to a region of the template DNA and enable selectivity of amplification and initiation of DNA synthesis. DNA polymerase is a thermostable enzyme that synthesizes a DNA strand complementary to the template. PCR reaction is composed of 30-40 cycles of alternated steps of heating and cooling, which are necessary for the separation of the two strands of DNA double helix, annealing of primers and for the DNA synthesis. Based on the detection method of PCR amplicons, two main PCR techniques exist: i) end point PCR, where the product is detected after the PCR reaction is completed, usually with gel electrophoresis, and ii) real-time PCR, where the product is detected during the reaction, as it is being

### Detection dyes in real-time PCR

Fluorescence-producing chemistries used in real-time PCR can be grouped into two main types: nonspecific and specific. The most commonly used nonspecific chemistry is SYBR® Green I, where the nonspecific dye emits fluorescence only when it reversibly binds to any double stranded DNA that is generated during PCR reaction. Therefore, distinguishing between specific and nonspecific products based only on the amplification curve is impossible. Post-amplification analyses are needed to confirm specificity of the amplicon with the use of dissociation-curve analyses.

The most commonly used specific chemistry is TaqMan® chemistry. What makes this chemistry appealing is that it integrates an additional level of specificity, obtained thanks to the use of specific oligonucleotide probes. A fluorescent reporter dye is bound to the 5'-end of the probe and a quencher dye is bound to the 3'-end. As long as the probe is intact, the quencher inhibits the fluorescence of the reporter dye. But when the probe hybridizes to its target sequence, the 5'-exonuclease activity of DNA polymerase degrades the probe, thereby separating the reporter and quencher dyes, which results in increased fluorescence of reporter dye. Since only specific products are detected that way, no post-amplification analyses are needed. Nevertheless, TaqMan® has a unique drawback. In order for the chemistry to function as desired, one must pay special attention at the choice of DNA sequences of both primers and probe. A poorly chosen probe or mismatched annealing temperatures of all three oligonucleotides in the reaction can severely handicap detection of amplicons, producing a false result.

Moreover, labeling specific TaqMan® probes with different reporter dyes enables detection and quantification of different target sequences in the same reaction tube (multiplexing), which significantly reduces the reaction costs. SYBR® Green chemistry also enables multiplexing, but distinguishing between two different products is much harder, since there must be apparent differences in dissociation-curve peaks of the different multiple amplicons. On the other hand, when using TaqMan® chemistry, multiplexing is limited only by the choice of dyes with emission spectra compatible with the detection spectra of the real-time PCR machine.

## 2 OUR EXPERIENCE WITH REAL-TIME PCR

Our research facility is equipped with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). It is being used for a variety of different applications. Because PCR is a very sensitive method, designated areas and separate pipetting tools are used for PCR reaction set-up.

Not only that PCR is very sensitive, it also has high specificity, thus it can be used to distinguish highly similar template DNA sequences. This means that closely related species or even subspecies and strains, depending on the variability of nucleotide sequence being amplified, can be identified. Thus choosing an appropriate template gene and a region in that gene is essential for successful identification. At our institution, both TaqMan® and SYBR® Green I chemistries are currently being used for species detection and identification.

SYBR® Green I chemistry is used for detection and identification of bacteria and viruses in a variety of different clinical samples. Samples of periprosthetic tissue from patients who had undergone revision arthroplasty were analyzed with the use of broad-range primers for the presence of the 16S rRNA bacterial gene, while identification was done by sequencing of the amplified products. In clinical samples from patients with malignant pleural mesothelioma, species-specific primers were used for detection and identification of SV40 virus, thus dissociation-curve analyses in combination with appropriate controls were sufficient for confirmation of specificity of the amplified products.

The use of species-specific primers and sequencing of amplicons for identification can be overcome with the use of TaqMan® chemistry. An example of user-friendly TaqMan® chemistry is 'Custom TaqMan gene expression assays' (Applied Biosystems), which include a validated and optimized mix of a fluorogenically labeled probe and primers. Besides analyzing gene expression (as the name implies), these assays can also be used for species identification, in our case bacteria. The advantage of these assays is that no post-amplification manipulation and analyses are needed for confirming the specificity of the fluorescence signal. No primer-probe validation needs to be performed by the user, which significantly reduces cost and labour.

Quantitative PCR analyses are also being performed at our institution. Currently, only TaqMan® chemistry is used for quantitative analyses. Gene expression was relatively quantified for analyzing transcriptional silencing of genes that are involved in cancer phenotype regulation. Residual amounts of therapeutic plasmids, containing the IL-12 gene, and used in electrogene therapy of canine skin tumors, were absolutely quantified using TaqMan® assays.

Standard detection and identification techniques in virology have already been replaced with modern molecular methods and used for diagnostic purposes enabling us to detect and subtype different viruses.

### 3 CONCLUSION

The use of the polymerase chain reaction (PCR) in molecular diagnostics has increased to the point where it is now accepted as the gold standard for detecting nucleic acids from a number of origins and it has become an essential tool in the research laboratory. Real-time PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. The technology has been applied to different areas of microbiology as well as studies of gene expression and genetic disease. Within our institution, real-time PCR method has been applied to various research fields, granting our team valuable expertise for implementation of this technique to several possible research projects in the future.

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