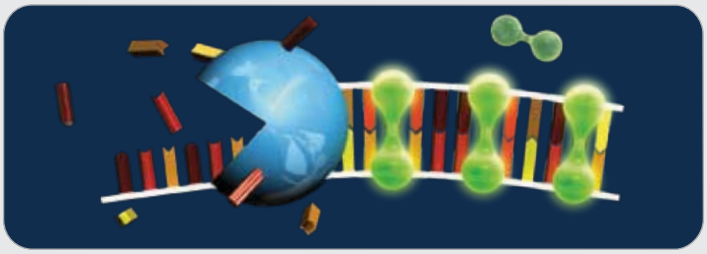
Real-Time PCR for Systems Biology:

A Review on Real-Time PCR-Related Technologies & Their Applications in the Post-Genomic Era



Introduction

Systems biology takes the integrative approach to study whole systems and systems interactions. The fields of genomics and proteomics research have revolutionized our understanding of systems biology. No single technology has made more of an impact on systems biology research than the polymerase chain reaction (PCR). In the 25 years since its invention, this transforming technology has been influential in diverse areas of science, but has been particularly useful in addressing the central questions relavant to transcriptional and epigenetic regulations of gene expression. This article will review various PCR-based technologies, describing their strengths and weaknesses in addressing these questions. We will highlight how some of these PCR-based assays are revolutionizing methodologies in the life science and biomedical industries.

Scientific Impact of Real-Time PCR Technology

The ability to magnify trace nucleic acids using PCR amplification, a process developed more than two decades ago, has made important contributions to many scientific disciplines [1].

The applications for PCR technology were further enhanced with the development of real-time PCR. Upon introduction of the reverse transcriptase enzyme to the amplification process, mRNA transcripts from the whole genome can now be converted to cDNA. Combined with real-time PCR analysis, the quantity of DNA copies being amplified can be accurately measured.

As the availability of PCR instruments has become widespread, researchers from a wider range of disciplines are discovering the value of nucleic acid analysis in their respective fields [1]. While accurate detection of gene transcription using qPCR is now routine, the approach continues to evolve in response to demands for ever-more specific, sensitive, convenient and cost-effective methods.

Real-Time PCR Product Detection Technologies

Detection Strategies and Chemistries:

1- SYBR® Green

This dye preferentially binds to double-stranded DNA and results in a strong fluorescence emission signal. The intensity of this signal is proportional to the amount of double-stranded DNA present; therefore this dye can be utilized to monitor the accumulation of amplified products resulting from qPCR amplification (Figure 1A). SYBR® Green is widely used both because of its high detection sensitivity and its relatively low cost. This dye does require high-quality primer designs to avoid primer dimers that could cause non-specific increases in the signal. Advances in the understanding of the PCR process and the development of sophisticated primer design algorithms have greatly helped in addressing this issue. Under carefully controlled conditions, SYBR® Green PCR works extremely well. SABiosciences Corporation leads the field of SYBR® Green PCR, featuring advanced SYBR® Green technologies including PCR Arrays, the unique hotstart Taq polymerase, and > 60,000 genome-wide certified primer assays.

2- DNA Hydrolysis Probes

Another major detection method for PCR-based amplification is based on the 5´ to 3´ exonuclease activity of Taq polymerase. This exonuclease activity is used to cleave a fluorogenic hybridization probe - TaqMan® Probe - which consists of an oligonucleotide recognizing an internal region of the target sequence. The probe is labeled with a 5´ fluorescent reporter dye and a 3´quencher dye in close proximity. A signal is generated when the Taq polymerase hydrolyzes the 5´ end of the probe while polymerizing the target DNA, and releases the reporter dye from the quencher (Figure 1B). The primary advantage of the DNA hydrolysis PCR assay is that it will not detect non-specific products such as primer dimers. A drawback is that it requires a specific probe for every gene target, thereby increasing the cost. In 2008, SABiosciences introduced the first cost-effective DNA Hydrolysis Array solution featuring this technology and optimized Probe Master Mixes for all RT-PCR instruments.

3- Universal Probe Library

This approach for DNA detection through qPCR analysis utilizes locked nucleic acids (LNA), which are chemically modified to increase thermodynamic stability. The Universal Probe Library (UPL) for humans consists of 90 oligomers of 8-9 nucleotides that recognize the most prevalent sequences in the transcriptome. Through selection of the appropriate probe, any gene can be detected. A fluorescent reporter and a quencher are attached to the probe, then operate as in the DNA hydrolysis assay (Figure 1C). The advantage of the UPL is that the 90-probe library is sufficient to cover the entire human transcriptome, instead of requiring a specific probe for each gene as with the TaqMan® probes. However, the price of the Universal Probe Library considerably inflates the total cost of the qPCR assay when compared with the SYBR® Green detection method.

4- Molecular Beacon

Like the aforementioned hydrolysis probes, the Molecular Beacon is a dual-labeled single-stranded oligonucleotide probe configured with a labeled fluorophore and a quencher linked on opposing ends. It forms a stem-loop structure (Figure 1D). This is the result of each side of the probe containing complementary sequences that anneal, bringing the fluorophore and quencher in close proximity. The loop contains a probe sequence that is complementary to a target sequence. The Molecular Beacon remains dark when free in solution and in its stem-loop form. However, when it hybridizes to a nucleic acid strand containing a target sequence, it undergoes a conformational change and fluoresces.

5- LightCycler® HybProbe

The hybridization-based probe Lightcycler HybProbe also utilizes the principles of fluorescence resonance energy transfer (FRET) to emit signals when bound to target sequences. Two sequence-specific oligonucleotide probes are labeled with different dyes, one having a donor dye at the 3' end and the other an acceptor dye on its 5' end. During the annealing phase of PCR, the HybProbe hybridizes to adjacent target sequences on the amplicon in a head-to-tail fashion, thereby bringing the two dyes closer together. The donor dye is excited by light at a specific wavelength and passes its energy to the acceptor dye through FRET, which in turn emits fluorescent light at a different wavelength. This fluorescence is directly proportional to the amount of target DNA generated during PCR. Careful selection of the acceptor and donor dyes is necessary to find those with efficient FRET between the dyes.

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6- LUX (Light Upon eXtension) Primer

In the LUX system, one of the PCR primers is labeled with a fluorophore at its 3' end. This fluorogenic primer has a short sequence (4-6 nucleotides) at the 5'end that is complementary to its 3'end, resulting in a hairpin structure that effectively quenches the fluorophore. In addition, the fluorescence level remains low when the primer becomes single stranded during the denaturation step. Upon incorporation into the double-stranded PCR product, the fluorophore is no longer quenched and a 10-fold increase in fluorescent signal is observed. Multiplexing is possible by using different fluorescent dyes. Unfortunately, melt curve analyses may be necessary to distinguish true amplicons from primer-dimer artifacts.

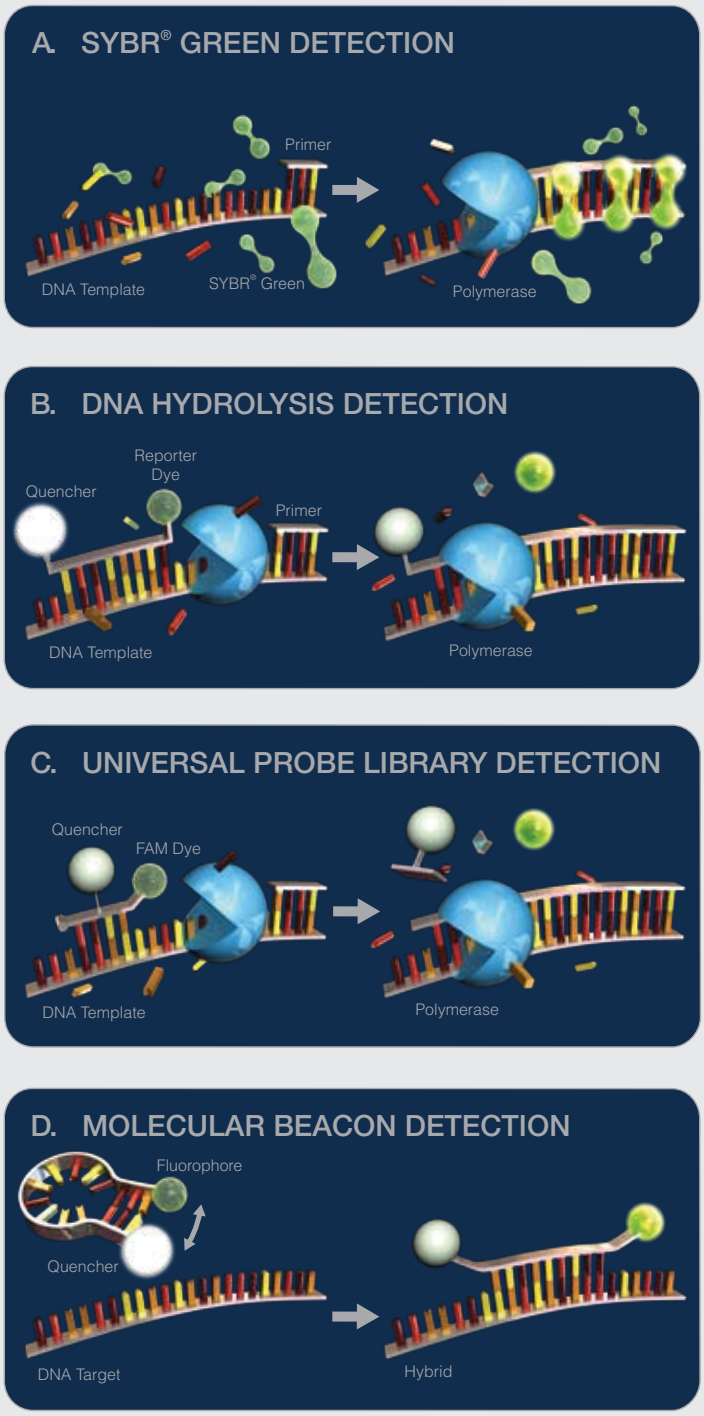

Figure 1: Popular Real-Time PCR Detection Methods.

Real-Time PCR Applications

1- Single Cell PCR

Technologies such as laser capture microdissection (LCM) and fluorescence-activated cell sorting (FACS) enable isolation of small cell populations for PCR gene expression analysis. The inherent sensitivity of the qPCR method allows this level of detection, since the limit of detection is just one copy.

One of the most difficult issues in measuring gene expression is the preparation of RNA from cell lysates to yield consistent results. Varying prepatory conditions even slightly can dramatically alter resulting RNA quality. Laser capture microdissection has been developed by Arcturus' Autopix™ instrument to make this process more uniform, and allows for the separation of different cell types from tissue samples. The process uses an infrared laser to deposit target cells onto a thermoplastic film, thereby ensuring the integrity of the RNA extracted from cells. This approach is particularly useful for cancer biopsy analysis, as it facilitates isolation of metastatic cells from normal tissue.

It has been observed that as cell numbers decrease, large variability between replicates are detected. Random, stochastic events can explain a part of this variation; however, these studies also reveal that mRNA production in cells is not constant, but occurs in "bursts". The timing between these bursts is long enough to degrade much of the mRNA, substantially changing the expression levels. When larger cell populations are analyzed, these variations are averaged out.

2- Genotyping with PCR

High-resolution melting (HRM) analysis has proven to be a highly sensitive method for SNP (single nucleotide polymorphism) detection. PCR is performed in the presence of a double-strand DNA binding dye, with the samples denatured and renatured over a defined temperature range. SNPs, small insertions and deletions can be detected by this method. Previous SNP analysis relied on fluorescently labeled oligonucleotides (e.g. Taqman® assays), but limited the number of analyses performed as a result of its considerable cost.

Although melting curve analysis is a capability of most PCR instruments, only two instruments (Rotor-Gene™ 6000 and Lightcycler® 480) on the market are capable of performing true HRM analysis. HRM requires acute well-to-well thermal and optical precision, as well as high speed data acquisition.

3- FFPE Samples

Millions of formalin-fixed, paraffin-embedded (FFPE) tissue samples are stored in labs worldwide. It is often desirable to obtain gene expression data on these stored samples in retrospective studies. Unfortunately, obtaining useful RNA for PCR analysis from these samples is a challenge because the proteins and nucleic acids are crosslinked.

A number of companies (including SABiosciences) have developed methods to extract RNA from these samples. High RNA yields suitable for qRT-PCR analysis from 5-10 year old tissue samples are possible. If this technique proves to be widely applicable, qPCR-based gene expression analysis could be performed on archived tissue samples, yielding potentially valuable gene expression data.

4- Proximity Ligation

This is a recently developed methodology that can be adapted to a variety of assays that probe protein-protein interactions. With proximity ligation, the challenge of detecting specific proteins is shifted to the analysis of corresponding DNA sequences (with femtomolar sensitivity). It is based on coupling two oligonucleotides to antibodies specific to target antigens. The two proximity probes ligate when brought within a few angstroms of each other, forming an amplifiable tag sequence using qPCR. The assay is not limited to the use of antibodies as probes, since any two molecules brought within a few angstroms can be detected. Single molecule sensitivity is possible, although issues such as background binding can complicate data interpretation. Attachment of the two

oligonucleotides to the molecules of interest can be accomplished easily with biotin/avidin linkages. This method holds great promise for the development of highly sensitive assays for molecular interactions.

Recent Promising Developments

Several new techniques currently in development have been engineered to significantly improve existing PCR-based analysis methods.

1- MNAzymes and Multiplexing

Mokany, E. and colleagues at Johnson & Johnson are working on a novel qPCR detection methodology based on multi-component nucleic acid enzymes (MNAzymes). These MNAzymes bind to accumulating amplicons and use a non-protein based enzymatic reaction to cleave a generic reporter probe, separating the fluorophore from its quencher. This design is based on the self-assembly of two parts of the MNAzymes on adjacent sequences of the amplicon. This allows the catalytic core to form, and provides a binding site for the generic reporter probe on the reporter arms of the MNAzyme. The subsequent cleavage of the bound generic reporter probe results in fluorescent signal generation.

The sensor arms of the MNAzymes are designed to discriminate between single base polymorphisms without bias. Their greatest promise resides in multiplex analysis applications. A multiplex analysis can be set up using generic reporter probes with different fluorophores binding to their respective MNAzyme reporter arms. Presently, five target transcripts have been detected without cross-talk [2].

2- REMS PCR

Restriction enzyme mediated selective PCR (REMS PCR) is a technique to selectively amplify related sequences of lower abundance. The problem with this procedure is that restriction enzymes are temperature sensitive and lose effectiveness after a few cycles, forcing the repetitive addition of fresh enzyme. Researchers at Diasorin have developed a thermostable endonuclease (PspG1) that can selectively cleave KRAS oncogenes and allow mutant sequences to be amplified. In a second approach, Diasorin also investigated KRAS mutations by using peptide nucleic acid (PNA) probes which form a hybrid with DNA, achieving greater clamping strength than DNA probes. When the KRAS gene has mutated, and a single-base mismatch occurs, the PNA primer is displaced by the polymerase, resulting in target amplification [2].

3- Real-time PCR at Microarray Densities

Microarrays have high-throughput but suffer from the quantification limitations of DNA hybridization. Limited dynamic range for detection and lower sensitivity are two main drawbacks of microarray analysis. Several companies have recently announced the introduction of miniaturized real-time PCR assays that can analyze thousands of samples simultaneously without loss of accuracy, and at greatly reduced reagent cost.

BioTrove has developed high-density arrays for 3,072 simultaneous PCR measurements on a microscope slide-sized plate. The fluidic challenges in these miniaturizing efforts are immense. One particular issue with such small volumes is prevention of losses due to evaporation, necessitating special liquid handling approaches. Biotrove uses polyethylene glycol and silanes to create hydrophobic and hydrophilic regions around and in the wells to prevent cross-contamination. Primer sets are dried into each well, and highly accurate dispensers deliver 33 nanoliters of reaction volume to each well. The greatly reduced reagent consumption per assay is one of the attractions of this approach. Potential applications include SNP genotyping, detection of pathogen RNA/DNA and real-time PCR quantification of gene expression. SABiosciences has partnered with Biotrove to offer pathway-focused gene profiling arrays. The successful resolution of this and other technical challenges will result in a real-time PCR assay with higher-throughput, greater accuracy, and much lower cost per assay.

4- DNA Methyl Profiling with qPCR

With the discovery that DNA hypo- and hyper-methylation of genes play key roles in cancers, interest in improved methods to study DNA methylation has considerably increased. The current method of identifying DNA methylation is based on bisulfite conversion of cytosines to uracil (the methylated cytosines are not converted), followed by sequence analysis. Although this procedure is considered the gold standard, time-consuming steps, low-throughput, and significant expense limit its use and adoption.

Several PCR-based methods have been recently introduced to overcome these limitations. The MethyLight procedure employs two primers and an interpositioned probe to differentially anneal to bisulfite converted DNA. This method claims to be specific and sensitive to the presence of uracils. The results obtained by MethyLight have been found to correlate with results obtained for the same samples through traditional bisulfite-sequence analysis [3].

SABiosciences has recently introduced the Methyl-Profiler™ detection method to determine the methylation profile of cancer genes or other genes of interest. The Methyl-Profiler assay uses two restriction endonucleases that are sensitive to DNA methylation to determine the methylation profile of the genes. One enzyme (Hhal) is methyl-sensitive and will not cleave methylated genes, whereas the other (McrBC) is methyl-dependent and cleaves only methylated genes. By quantifying the level of the target genes remaining after digestion using real-time PCR analysis, a profile of the methylation state can be determined. This new method is much easier, more economical and has a higher throughput capacity than current methods, making it ideal for cancer gene methylation studies (see page 7 for more details on this technology).

5- Histone Modification Studies with qPCR

In the epigenetics article on page 2, we reviewed the covalent modifications present on DNA and histone tails that recruit enzymes and binding proteins, as well as co-factors, which together regulate gene expression. Current methods to study histone modifications are based on chromatin immunoprecipitation (ChIP), with antibodies specific to transcription factors or RNA polymerase used to isolate associated DNA regions. The analyses of these immunoprecipitates (containing proteins as well as genomic fragments) provides valuable information about promoter regions. As with the Methyl-Profiler DNA methylation assay, the ChIP assay depends on the accurate determination of gene copy number brought down by the immunoprecipitation for interpretation of results. Successful ChIP assays can therefore ONLY be achieved with the reliability and reproducibility of real-time PCR.

The use of qPCR in ChIP assays also promises to provide a means to study histone modifications. By using antibodies specific to modified histones, such as specific methylation sites, it will be possible to study this challenging aspect of gene regulation. As additional specific antibodies become available, this approach may make the breaking of the histone code a reality.

References

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2. Genetic Engineering and Biotechnology News 28:7 (2008).
3. Eads, C.A., et al, Nucleic Acids Research 28(8) e32 (2000).

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