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Quantitative Real-Time Polymerase Chain Reaction for the Core Facility Using TaqMan and the Perkin-Elmer/Applied Biosystems Division 7700 Sequence Detector

Deborah S. Grove

Nucleic Acid Facility, Life Science Consortium,
The Pennsylvania State University,
University Park, PA 16802

Quantitative real-time polymerase chain reaction (PCR) using the Perkin Elmer/Applied Biosystems Division 7700 Sequence Detector provides an accurate method for determination of levels of specific DNA and RNA sequences in tissue samples. It is based on detection of a fluorescent signal produced proportionally during amplification of a PCR product. Turn-around time for data acquisition and analysis by real-time PCR with the 7700 model is short, and results are more reliable than by traditional PCR methods. This technology can be successfully offered as a service in a core faculty setting. (J Biomol Tech 1999;10:11–16)

KEY WORDS: quantitative real-time polymerase chain reaction, Perkin Elmer/Applied Biosystems 7700 Sequence Detector, nucleic acid sequence quantification.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO Deborah S. Grove, Nucleic Acid Facility, Life Science Consortium, Pennsylvania State University, University Park, PA 16802.

Quantitative real-time polymerase chain reaction (PCR) using the Perkin Elmer/Applied Biosystems (PE/ABD) (Foster City, CA, USA) Prism 7700 Sequence Detector System is a relatively new technology that provides a broad dynamic range (at least five orders of magnitude) for detecting specific gene sequences with excellent sensitivity and precision. DNA and RNA can be quantified using this detection system without laborious post-PCR processing.[1,2] This technology can be performed in a core facility environment because of the costs associated with instrument purchase, setup, and maintenance.

THE DETECTION SYSTEM

Quantitative real-time PCR using the PE/ABD 7700 is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product. The chemistry is the key to the detection system (Fig. 1). A probe (ie, TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome (usually 6-carboxy-fluorescein [6-FAM]) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) added at any T position or at the 3' end.[3] The probe is designed to have a higher T_m than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay. As long as both fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as *Taq* polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* degrades the probe, releasing the reporter fluorochrome.[4] The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.

In brief, the 7700 detection system consists of a 96-well thermal cycler connected to a laser and charge-coupled device (CCD) optics system. An optical fiber inserted through a lens is positioned over each well, and laser light is directed through the fiber to excite the fluorochrome in the PCR solution. Emissions are sent through the fiber to the CCD camera, where they are analyzed by the software's algorithms.

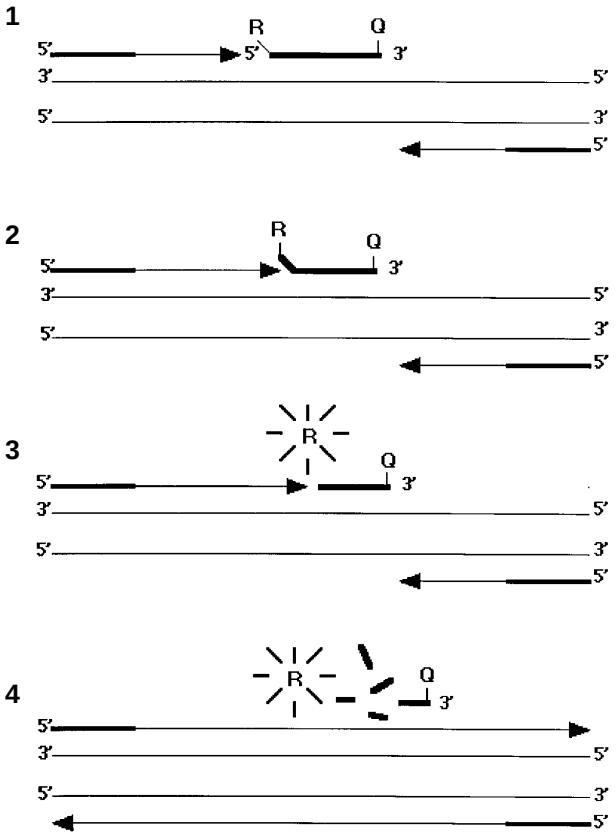

FIGURE 1

Fluorogenic 5' nuclease chemistry. **(1)** Forward and reverse primers are extended with *Taq* polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes attached anneals to the gene sequence between the two primers. **(2)** As the polymerase extends the primer, the probe is displaced. **(3)** An inherent nuclease activity in the polymerase cleaves the reporter dye from the probe. **(4)** After release of the reporter dye from the quencher, a fluorescent signal is generated.

Collected data are subsequently sent to the computer. Emissions are measured every 7 seconds.

The sensitivity of detection allows acquisition of data when PCR amplification is still in the exponential phase. This is determined by identifying the cycle number at which the reporter dye emission intensities rises above background noise; this cycle number is called the threshold cycle (C_t) (Fig. 2). The C_t is determined at the most exponential phase of the reaction and is more reliable than end-point measurements of accumulated PCR products used by traditional PCR methods. The C_t is inversely proportional to the copy number of the target template; the higher the template concentration, the lower the threshold cycle measured.

Advantages

There are many advantages to quantifying gene sequences using this technology, foremost being sensitivity and precision. This precision exists because quantitation of the gene sequence is determined by the C_t, which is calculated during the exponential phase of the reaction. High specificity is conferred by the requirement of three oligos to anneal to the DNA before any data are collected.

Turn-around time for data acquisition and analysis by real-time PCR with the 7700 is short. Setup and thermal cycling require less than 3 to 4 hours and data analysis less than 10 additional minutes. Traditional PCR quantification may require several days.

Competitive PCR is another technique often used to quantify DNA or RNA. Optimization of competitive PCR is laborious and time consuming. Several dilutions of target sequences must be tested to achieve a suitable ratio of target to competitor, and efficiencies

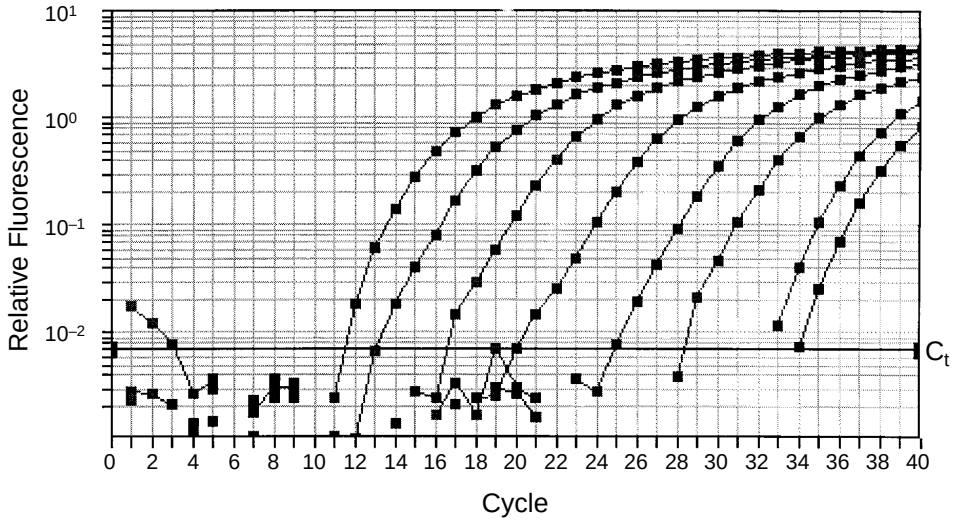

FIGURE 2

One of the views available after completion of the run is an amplification window. This window shows the amount of fluorescence obtained in each amplification cycle for each reaction. The threshold cycle (C_t) is shown by the darker horizontal line.

of target and competitor must be similar. This assay is linear only over a very short range compared with quantitation with the 7700. The number of samples that can be processed is also a limiting factor.

An advantage of providing the quantitative real-time PCR technology in a core facility setting is reduction of labor time and costs. One half-time employee can easily process 96 samples per day, including setup and data analysis. The use of robotics often found in core facilities allows preparation of samples with speed and accuracy.

Applications

The applications for quantitative real-time PCR are innumerable. Detection of genomic or viral DNA in tissues can be a valuable diagnostic tool. Gene expression can be measured after extraction of total RNA and preparation of cDNA by a reverse transcription (RT) step. Setup and analysis are simple and can more easily be extended to the clinical environment than traditional PCR techniques.

One problem with developing applications is that most research conducted with this technology is done by pharmaceutical companies. Because their work remains proprietary, successful TaqMan primers and probes, as well as optimization conditions, are often not available. As a result, de novo design and optimization still require an investment of time and money.

Examples of gene sequences that have been optimized for detection are shown in Table 1. My colleagues and I also have optimized detection systems for prolactin, prolactin receptor, perforin, five isozymes of plant 1-aminocyclopropane-1-carboxylic acid synthase (ACS), type X collagen, cartilage matrix protein, occludin, JC virus, and CD19 (unpublished results).

Although I have focused on gene detection in this article, another application is an allelic discrimination assay that can detect single-base nucleotide mutations and polymorphisms. These assays require two separate probes that differ only by one base mismatch. One probe labeled with 6-FAM represents one allele, and the other probe labeled with the fluorochrome VIC represents the other allele. A mismatch leads to a less efficient amplification. Fluorescence spectra are collected after the run, and using multicomponent analysis, the software extracts the contribution of each component dye to the observed spectrum. Homozygotes for FAM show an increase in the FAM signal but no increase in the VIC signal, and homozygotes for the VIC probe show an increase in that signal. Heterozygotes show intermediate increases of FAM and VIC signals. All three groups are clearly distinguishable,[28] and the sensitivity is similar to that for the quantitative PCR application.

TABLE 1

Reported Real-Time Polymerase Chain Reaction Gene Sequences

Target Gene	Reference
DNA-dependent protein kinase	5
β-Actin, interleukin (IL)-2, IL-4, IL-10, interferon-γ	6,7
Tumor necrosis factor-α	6
IL-12	7
Leptin receptor	8
J_H and V_H genes of B cells	9
Muscle-specific genes	10
MMLV-based proviruses	11
erbB2 oncogene	12
Bcr-Abl gene	13
CDR3 sequences for multiple myeloma clones	9
SRY fetal protein	14
Herpes simplex thymidine kinase	15
AML1/ETO leukemia protein	16
Philadelphia chromosome–positive leukemia	17
Plasma simian immunodeficiency virus	18
Hepatitis C	19
E. coli toxin genes	20
Listeria	21
Salmonella	22
Papillomavirus	23
Aspergillus fumigatus	24
Mycobacterium tuberculosis DNA	25
Yersinia	26
Swine fever virus	27

GENE DETECTION

Optimization

Optimization of the PCR reaction is required for each primer and probe set. The optimal Mg^{2+} concentration is usually between 4 and 6 mM but sometimes can be as low as 2 mM. Optimal primer concentrations are usually between 100 and 800 nM. Optimization requires varying the concentration of one primer relative to the other, because the optimal concentration may not be the same for both. The optimal probe concentration may be as low as 50 nM or as high as 200 nM. The optimal Mg^{2+} concentration and reverse primer concentration must also be validated for the RT step.

Potential Contamination

The detection system is so sensitive that fewer than 10 copies of DNA can be detected. Aerosol contamination of primers and probes is a potential problem if samples are prepared in the laboratory where DNA is being

extracted. Probes synthesized by outside companies are delivered directly to us. We synthesize and cartridge-purify our own primers, and we prepare all PCR or RT-PCR reactions in a laminar flow hood. After thermal cycling, the sample plate is kept closed until analysis to minimize the chance of releasing PCR products.

Sample Preparation

For determination of gene expression, total or polyA RNA is isolated. A specific cDNA can be produced by using the same reverse primer used in the PCR reaction or by using random hexamer primers to produce a range of cDNA products. RNA can easily be prepared using kits such as RNAEasy from Qiagen (Valencia, CA, USA) and Triazol from Life Technologies (Gaithersburg, MD, USA).

Controls

Multiplexing quantitative PCR reactions by using more than one fluorescent dye per tube was not an option when the 7700 was first released. Because we could not use internal tube controls, we used the ribosomal phosphoprotein (RPPO) as an external control and found the data obtained to be consistent. After multiplexing became available, internal tube controls could be established. Kits are available from PE/ABD for 18S ribosomal RNA or for glyceraldehyde-3-phosphate dehydrogenase as a control. We have found that 18S appears to work across many species, including plants. The probe used for the internal control has been labeled with the PE/ABD fluorochrome, JOE. A fluorochrome with a higher intensity, VIC, is also available. These two fluorochromes are preferred for use with FAM, the reporter used on the probe.

If copy number is required, standard curves of plasmid DNA can be constructed and assayed each time with samples containing the target gene sequence. If the starting molecule is RNA, cRNA can be prepared and used as a standard. Kits are available to prepare RNA from plasmids containing the gene sequence. T7, T3, or SP6 primers typically are used to prepare the cRNA. The cRNA produced must be validated in the RT and PCR reactions to determine if it is transcribed and amplified at the same efficiency as the sample RNA present in a mixture of extracted RNAs.

Other important controls are no-amplification controls (NACs) and no-template controls (NTCs). NACs test for contamination of RNA by genomic DNA. If the primers and probe set have been designed so that at least one spans an intron, this control is unnecessary. However, intron sites may not be known or may not be present. Nearly all RNA samples that we have received have had NACs with C_ts at least three cycles higher and usually much more than three cycles. This has been acceptable for quantification. NTCs test for the contamination of assay reagents.

Reaction Mix

Several types of reaction mixes are available from PE/ABD. The TaqMan PCR Core Reagent kit has been satisfactory for our use and allows flexibility in preparing the different master mixes that are needed for the wide variety of primer and probe sets used. We mix the kit components to create a customized master mix for two-step RT-PCR that is easy to use and allows rapid assay development. A similar kit, TaqMan Universal PCR Master Mix, contains the core reagents in an easy to use 2× solution. The TaqMan Gold RT-PCR kit allows one-step or two-step RT-PCR. The one-step option allows an investigator to set up the RT and PCR steps without opening the tube, whereas the two-step option separates the RT step from the PCR. The two-step protocol is used for assays for which multiple transcripts are being prepared from random hexamers or oligo-dT. The tube is opened after the RT step, and aliquots are removed and run with specific TaqMan primers and probes. The TaqMan EX RT-PCR kit specializes in applications that require a higher-temperature RT step to destabilize secondary structure.

Master mixes can also be assembled by purchasing the various components, such as NTPs, buffer, Mg^{2+}, and *Taq* polymerase, from many other companies offering molecular biology reagents. It is important to validate that only one specific PCR product is produced with the *Taq* polymerase selected.

ANALYSIS

Real-time monitoring of the release of fluorescence several times during each cycle allows collection of abundant data. After 40 cycles, data are processed by the software within seconds. Data can be viewed in an "amplification window" in the analysis program (see Fig. 2). This allows the operator to check the fluorescence from each reaction at each cycle. The linearity of the fluorescence response for each sample at each cycle and the baseline can be checked for each tube. An occasional problem tube can easily be identified and the data point discarded, or the amplification curve may indicate that a different baseline should be chosen for the experiment to generate more accurate C_ts.

PRIMER AND PROBE DESIGN

Primers and probes must be carefully designed because of the costs associated with producing probes with different dyes at 5' and 3' ends. We use PE/ABD Primer Express software, which is specifically designed to select the primers and probes. Considering the high numbers and varied backgrounds of potential users, our facility personnel designs primers and probes to ensure investigators' investment in expensive probes can lead to successful results.

The required parameters for well-designed primers and probe have been well documented and are built into the program.[3] These parameters include a T_m for the probe that is 10°C higher than the primers, primer T_ms between 58°C and 60°C, amplicon size between 50 and 150 bases, absence of 5' Gs, and primer length. If primers or probes are not found, these parameters can be modified with satisfactory results. Early versions of Primer Express designed primers and probe sets with longer amplicons, and some of these work very well.

The best design for primers and probes to use for the quantitation of mRNA expression requires positioning of a primer or the probe over an intron. The Primer Express software has a tool called Junction Annotation that allows a user to mark exon junctions. When the primers are calculated, at least one crosses an exon junction. Tools are also available to exclude certain regions or to choose certain regions for a probe or primer to be designed.

Primer and probe design is different for the allelic discrimination assays. The probes designed for each allele should be centered over the mismatched base, and the probes only have to differ by that base. Another major difference is that the probe for these assays has a lower T_m requirement than the TaqMan PCR assays.

COST

Cost has been the biggest factor in determining whether investigators use this technology. We charge $4.50 per reaction starting with DNA and $5.40 per reaction starting with RNA. Kits can be purchased from PE/ABD to use for the entire procedure for the PCR reaction or RT-PCR. This is the least labor-intensive option and ensures quality control. However, master mixes for the reaction can be assembled by purchasing less expensive components such as NTPs, buffer, Mg^{2+}, and *Taq* from other companies. We add RNAse inhibitor and reverse transcriptase to the Universal Master Mix to measure RNA, and this addition accounts for the higher price for reactions starting with RNA.

Labor costs vary among institutions. Our labor cost is about 10% of the final cost per reaction. Optical caps from PE/ABD, tubes, plates, and filtered tips comprise another 15% to 30% of the cost. The service contract cost (up to $8500 per year) can be as much as 10% to 20% of the cost per reaction, depending on number of samples submitted throughout the year. Cost-effective projects include those that involve medical or agricultural diagnostics and gene expression studies in clinical trials.

PREPARATION OF PROBES

We typically advise investigators to order their TaqMan probes from outside vendors. Although we can easily synthesize the 5' 6-FAM derivatives on a standard DNA synthesizer, the 3' TAMRA labeling is more complex. The additional steps in the synthesis and post-synthesis purification can take 7 to 14 days to complete.

A solid support (TAMRA CPG) became available recently, allowing preparation of 3' TAMRA-labeled oligonucleotides in high yield. This may allow more rapid and purified synthesis of dual-labeled probes.

ALTERNATIVE INSTRUMENTATION

Two similar quantitative PCR instruments are available from PE/ABD that also measure the presence of DNA. The 5700 detects all light from 500 to 700 nm with no spectral separation, eliminating multiplexing and allelic discrimination assays. The 5700 and 7700 models have a linear dynamic range of 5 logs. The 7700 functional specification is twofold resolution using the β-actin kit (5000 versus 10,000 copies), whereas the 5700 specification is fivefold resolution (2000 versus 10,000 copies). Both machines can use TaqMan or SYBR Green.

SYBR Green can be used as a "probeless" alternative to the TaqMan system. Because it binds to all double-stranded DNA, it is imperative to ensure that the PCR product being quantified is a clean, single product, because any primer-dimers or background smears are detected. It can be used as a prescreening assay before ordering a probe. If the results are satisfactory, the probe can be ordered and TaqMan reactions run with higher specificity.

The LightCycler from Boerhinger (Indianapolis, IN, USA) is another alternative. In a price range between the 7700 and 5700 models, it can do SYBR Green or TaqMan chemistry and perform analysis with hybridization probes based on fluorescent resonance energy transfer.

The LS-50B from PE/ABD is an end-point fluorescence detector used for allelic discrimination assays or "plus/minus" assays. The reactions are run in a thermal cycler and read in the LS-50B.

CONCLUSIONS

Quantitative real-time PCR using the PE/ABD 7700 Sequence Detector provides a rapid and accurate method for determining levels of specific DNA and RNA sequences in various tissue samples. This technology can be successfully offered as a service in a core faculty setting.

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