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REVIEW

Controls in Real-Time Polymerase Chain Reaction Based Techniques

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From its discovery in the 1980s, Polymerase chain reaction was further developed and is nowadays used as the foundation for the various PCR-based techniques used in molecular diagnosis across different species, and numerous types of samples. Real-Time PCR enables the user to monitor the amplification of a deoxyribonucleic acid (DNA) or complementary DNA (cDNA) target during the PCR run, in real-time, and not at the end, as it is the case in conventional PCR. The most frequent types of applications include gene expression analysis, gene silencing, variant analysis, and fusion temperature analysis. Given its vast field of application, a key question remains, and it is related to the controls (negative controls, positive controls, internal exogenous and endogenous controls) and their purpose in a Real-Time PCR experiment. In this paper, we set out to find how and when to use them, and which type of controls are suitable for certain experiment types, since the use of appropriate controls during Real-Time PCR experiments will reduce the effects of variables aside from the independent variable within the sample, therefore yielding accurate results, be it in research or diagnostic purposes.

Keywords: real-time PCR, negative control, positive control, internal exogenous control, internal endogenous controls

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From its discovery in the 1980s [1], Polymerase chain reaction (PCR) was further developed and is nowadays used as the foundation for the various PCR-based techniques used in molecular diagnosis across different species, and numerous types of samples.

Real-Time PCR enables the user to monitor the amplification of a deoxyribonucleic acid (DNA) or complementary DNA (cDNA) target during the PCR run, in real-time, and not at the end, as it is the case in conventional PCR. This process is made possible by the use of several fluorescent dyes and quenchers, either attached to the primers themselves or found within the reaction mix [2]. The most frequent types of application include: *Gene expression analysis*, enabling the determination of gene expression patterns at the genetic transcription level; *Gene silencing*, or the study of gene expression suppression at transcriptional or translational levels, starting from a messenger ribonucleic acid (mRNA) target; Variant Analysis studying the existence of at least two genotypes at the same gene locus, ranging from a Single Nucleotide Polymorphisms (SNPs) to large nucleotide sequence modifications; Fusion temperature analysis, or the use of the melting temperature (Tm) to discriminate between different DNA fragments or amplicons.

Given its vast field of application, in both diagnosis and research, for human and non-human investigation (animal, bacterial, and plant to name just a few) a consensus was needed for accurate, reproducible, repeatable, and correctly interpreted results. These objectives were materialized in the form of the MIQE Guidelines [3], containing the least amount of information, that should be reported for a Real-Time PCR experiment, guidelines that were later reiterated [4] in the context of new PCR-based techniques being developed.

However, a key question remains, and it is related to the controls and their purpose in a Real-Time PCR experiment. In this paper, we set out to find how and when to use them, and which type of controls are suitable for certain experiment types.

Negative controls

A negative control is a Real-Time PCR reaction with no amplification [5] (no amplicons will be generated), interpreted as a true negative, a reaction that is also called *no template control* or NTC. Regularly, each PCR experiment should contain at least one no template control [6].

Conventionally, negative controls contain the PCR reaction mix and molecular grade water [7], which substitutes the volume of genetic material that is added to the other experiment wells or tubes. This kind of reaction checks whether there is any contamination within the reaction mix, and that there are no primer-dimers forming. Water was used as a negative control in the study of Buzard et al. that focused on the detection of *Francisella tularensis, Burkholderia mallei, Brucella melitensis*, and *Bacillus anthracis* using ten different mastermixes from several manufacturers, and three different Real-Time PCR platforms [8], as well as in the study of Czurda et al. that investigated fungal contamination during Real-Time PCR experiments either by airborne spores and particles or by contaminated reagents available from different suppliers [7].

Although dimers should be avoided since the design stage of the primers [9], negative control reactions can also be used to check for primer self-dimers (also referred to as homo-dimers). In this respect, two separate negatives

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should be set up, each containing the forward or the reverse primer, respectively, as well as the reaction mix.

Positive controls

A positive control will be evaluated as a true positive during the Real-Time PCR experiment. Positive controls are often used to verify that all reagents are working (also called PCR mix), primer annealing temperatures are correct, extension times are sufficient, and there are no PCR inhibitors within the experiment. When using the same positive control during comparable (or the same type of) experiments, the cycle threshold should be very similar, thus indicating consistency between multiple PCR runs.

Positive controls can have a variety of sources. The most hassle-free way to obtain one is acquiring a commercially available positive control, for a specific type of experiment. This kind of positive control sample, available with the MammaTyper PCR assay, was successfully used in the study of Laible et al. that focused on Erb-B2 Receptor Tyrosine Kinase 2 *ERBB2*, Estrogen Receptor 1 *ESR1*, Progesterone Receptor *PGR* and Marker Of Proliferation *Ki-67 MKI67* genes in breast cancer [10]. Recently, to facilitate the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or COVID-19 diagnosis, several manufacturers included positive control samples in their COVID-19 CE-IVD (In-vitro Diagnostic Medical Devices) Real-Time PCR kits sold in Europe [11-13].

However, mainly in research situations, positive controls can be obtained either by sequencing, and thus confirming, a sample that was previously diagnosed as positive, or by designing a plasmid with the required sequence, information that is usually available in genetic data banks. For example, Picard-Meyer et al. used the fixed rabies virus challenge standard strain to evaluate lyssaviruses by six different Real-Time PCR kits using several commercially available master mixes and thermocyclers [14]. Plasmid DNA for the human cytomegalovirus was used as a positive control in the study of Pavsic et al. that assessed three Real-Time PCR platforms during quantification experiments [15]. In a paper published by Lion, RNA derived from cell lines that expressed certain gene fusions were proposed as a positive template control in the investigation of leukemia-related gene fusion transcripts, taking into consideration that a certain dilution must be applied since cell lines contain larger RNA quantities, as compared to the patients' malignant cells [16].

Internal controls

Internal controls are widely used in Real-Time PCR experiments and are often crucial for the correct interpretation of the results. Although the term *housekeeping genes* is broadly used, the appropriate term should be *reference genes*, according to the MIQE guidelines [3].

In Real-Time PCR experiments, internal controls can serve various purposes, such as: an indicator for proper nucleic acid purification, reverse-transcription control, normalization reference [17], as well as PCR inhibition supervision [18]. Traditionally, internal controls will be amplified together with the target sequence during the same PCR reaction, or within the same PCR run. It is therefore crucial for the internal control not to compete with the target region of interest, and to be easily distinguishable (i.e. different fluorophore marking) from the target of interest. Additionally, there should be a difference of maximum 12 amplification cycles between internal controls and the target gene (or genes), within the experiment [19].

The two types of internal controls used in everyday PCR experiments are *Exogenous controls* and *Endogenous controls*, each with its use indications, regarding the type of experiment.

Exogenous controls are pipetted directly into the raw sample, or added to the isolated nucleic acid before the PCR reaction[20], and will target a different species [21] to the studied one. They are mainly used for absolute quantification (i.e. number of gene copies per examined sample) and for the control of the relative amplification from a set of specific primers [22]. Another use for exogenous controls is the discrimination between negative samples (where the control will still amplify) and PCR inhibition (when no amplification will be observed) [21]. Liu et al. used the Phocine Herpesvirus-PhHV as an exogenous control that was added to the samples during DNA extraction, in their study on 30 viral and bacterial diarrhea-causing species [23]. Another exogenous control, namely Bacteriophage MS2 is also used in the COVID-19 diagnosis, by the TaqPath COVID-19 CE-IVD RT-PCR Kit (Applied Biosystems, USA), a product that is certified for in vitro diagnostic use [13].

On the other hand, a synthetic noncompetitive exogenous control was proposed by Aralar et al., that was suitable to be used with existing Real-Time PCR microbial detection methods, as it was noncomplementary with the 16S ribosomal RNA bacterial hypervariable regions, thus making it potentially universally suitable for the investigation of diverse pathogens (viruses, fungi, and bacteria) [24].

Endogenous controls are targets within the studied sample, other than the region/regions of interest, such as constitutively expressed genes [25] serving basal cell functions, and are therefore dependent on the type tissue being examined. They are often used in experiments that require data normalization [26] (i.e. study of gene expression profile), irrespective of the initial nucleic acid input quantity. For example, the study of Laible et al. used two endogenous control targets, namely for the Beta-2-microglobulin *B2M* and Calmodulin 2 *CALM2* genes in their study on breast cancer samples from formalin-fixed paraffin-embedded tissue [10]. In a study on colorectal cancer, Pharo et al. identified a panel of four genes, namely Synaptotagmin-10 *SYT10*, Pleckstrin Homology and FYVE Domain Containing 1 *PLEKHF1*, Kelch Repeat and BTB Domain Containing 4 *KBTBD4* and Ephrin type-A receptor 3 pre-

cursor *EPHA3* as suitable endogenous controls for droplet digital PCR methylation study on 34 malignant cell lines [27]. The human coagulation factor XIII (F13) gene was successfully used as an endogenous control for PCR typing of the human leukocyte antigen (HLA) Class I and Class II genes [28] by Neduvat et al. who managed to correlate the variation in expression of both, irrespective of the DNA concentration in the studied samples. In another research paper, C-C motif chemokine receptor 5 *CCR5* and T-cell antigen receptor complex, gamma subunit of t3 *CD3G* were found to be reliable endogenous control genes in the study on HIV diagnosis from peripheral blood mononuclear cells by Ruhanya et al., while Telomerase Reverse Transcriptase *TERT*, Beta-actin *ACTB*, Beta-globin *HBB*, and Glyceraldehyde phosphate *GAPDH* genes which were previously used, showed limited results regarding DNA to cell number equivalents [17].

It is typically necessary to determine the most appropriate reference gene or genes for each type of experiment or tissue being studied, as there is no "universal" gene to be used in this respect. Although Glyceraldehyde 3-phosphate dehydrogenase *GADPH* [29] for DNA experiments and 18S [2, 30] ribosomal RNA or r18s for RNA experiments have been arbitrarily used as reference genes, several other targets can be used, and dedicated software such as geNorm [31, 32], Bestkeeper [33], Normfinder [34], RefGenes[35] and Transcriptome Mapper-TRAM [29], to name just a few, can be useful in selecting the most appropriate ones, albeit an experiment validation step is generally indicated [26]. Regardless of the method of choice, the expression level of the target gene should be normalized to a number of reference genes (generally two to six reference genes), instead of a single one [36]. For example, the study of Leal et al. found the combination of two (TATA-Box Binding Protein *TBP* and Hypoxanthine Phosphoribosyltransferase 1 *HPRT1*) or three genes (*TBP*, *HPRT1*, and Beta-2-Microglobulin *B2M*) more suitable to be used as endogenous controls in the investigation of Collagen Type III Alpha 1 Chain *COL3A1* gene expression in patients with ruptured and non-ruptured rotator cuff tendons [37]. The reference genes used in their study were indicated by several dedicated software and were later experimentally verified [37]. In a study on stomach malignancy, Rho et al. used two software packages (namely geNorm and NormFinder) to identify and later validate the combination of *GAPDH* and *B2M* as suitable endogenous controls for the investigation of stomach malignant cells, as well as the use of *B2M* and Ribosomal subunit L29 *RPL29* for all stomach cells [38].

On the other hand, the study of Montero-Melendez et al. used NormFinder to compute the best model for reference gene selection from a panel of six candidates and indicated *GADPH* as the least suitable gene to be used as a control in Inflammatory Arthritis, as its expression was negatively influenced [39]. Similar results, namely improperness of *GAPDH* and Hypoxanthine-guanine phosphoribosyltransferase *HPRT* as endogenous controls were also demonstrated *in silico* and *in vivo* by Al-Sabah et al. in their study on osteoarthritis concerning Matrix metalloproteinase 3 *MMP3* and Aggrecan *ACAN* gene expression [40].

However, as suggested by several authors, data expression normalization performed with reference genes can be sidestepped, if the total amount of cDNA generated after RNA reverse transcription is precisely quantified, using several fluorochrome dyes [41-43].

Whether for diagnosis or research purposes, appropriate controls should be used in all Real-Time PCR experiments. On the one hand, negative and positive controls will permit the validation of the PCR run. On the other hand, the use of an internal control, be it exogenous or endogenous, is usually up to the user, as the appropriate genes should be selected to better suite each experiment.

Conclusion

The use of appropriate controls during Real-Time PCR experiments will reduce the effects of other variables aside from the independent variable within the sample, therefore yielding accurate results, be it in research or diagnostic purposes.

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Authors' contribution

EM: Concept, Data acquisition, Drafting, Critical revision, Final approval

VM: Concept, Design, Critical revision, Funding, Final approval

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