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REVIEW ARTICLE

MOLECULAR TARGETS USED IN VALIDATION AND CORRECTIONS IN ARTIFACTS OF PCR BASED INFECTIOUS AGENTS DIAGNOSIS

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ABSTRACT

With the advents of Nucleic Acid amplification Technologies (NAAT), utilized for the molecular diagnosis of infectious agents, it become very significant for the validation of the protocols. Present review focuses on the use of genes, analyte, markers for the validation and interpretation of the molecular assays and its significance.

KEYWORDS: Spike, Exogenous control, Cyclophilin mRNA, Amplicons, Quantitative PCR.

INTRODUCTION:

It is an in vitro, enzymatic & exponential amplification of target DNA sequence under controlled thermal conditions (1). PCR is performed in a microprocessor controlled machine, the thermal cycler, which provides controlled temperature conditions under an automatic monitoring system (2). When the reaction is allowed to take place under most appropriate and congenial conditions, it is rapid, sensitive, specific, reliable, and reproducible and reduces the reporting time to as short as 24 hrs or less. Repeated cycles of varying define temperatures first denature the DNA target, then allow the primers to specifically anneal to their complementary sequences, and finally extend the primers (synthesize new DNA strands) with a thermo-stable DNA polymerase (3, 4). The result is the exponential accumulation of the specific nucleic acid sequence – the synthesis of potentially billions of copies of the nucleic acid sequence from a single starting copy, which can be easily detected in the laboratory by various analysis systems-Gel electrophoresis, ELISA etc.

ARTIFACTS AND RECTIFICATION IN CONVENTIONAL PCR:

From the isolation of specific genes to the sequencing of entire genomes, the polymerase chain reaction (PCR) has become one of the most widely used technologies for conducting biological research (5). Advances have led to the development of specific and sensitive high-throughput PCR methods for the detection of a variety of microorganisms, and these methods are increasingly being applied to analysis of environmental samples. The successful application of PCR requires the proper use of techniques and interpretation of results. Many PCR methods offer a level of sensitivity equal to, or greater than, more traditional environmental microbiological methods. Due to the ability to amplify small amounts of nucleic acid, PCR can be used to detect organisms that are difficult to culture in vitro or that cannot be cultured. However, the advantages of these techniques can be offset by the demanding assay protocols and the need to follow quality assurance/quality control (QA/QC) procedures carefully. These QA/QC procedures are necessary because the ability of PCR to produce many copies of target DNA creates the possibility of contamination by previously amplified products, which can lead to false-positive results. In addition, environmental samples may inhibit the PCR, which can lead to false-negative results. As efforts are made to standardize PCR protocols for analyses of environmental samples, it is essential to establish standardized QA/QC procedures (6,7).

PROBLEMS WITH PCR:

PCR is used to detect the proverbial "needle in the haystack", the few HIV particles in a blood sample or Mycobacterium tuberculosis bacilli in sputum that other conventional tests might fail to detect. PCR is more sensitive, specific & selective in diagnosis of infectious diseases than culture, serology, microscopic and histological based methods. But PCR has its own inherent problems. Ironically, false positive reactions are the Achilles' heel of PCR and stem from its greatest strength, namely the incredible sensitivity of enzymatic

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amplification. False positive results occur because PCR may amplify "contaminating DNA" that finds its way into a sample, even when that DNA is present in infinite small amounts. DNA contaminates sample through several means by the organisms or amplicons (amplified DNA sequences) being present in aerosols, workers clothing & body surfaces (8, 9).

QUALITY ASSURANCE/QUALITY CONTROL GUIDANCE:

Commonly used standards can be House keeping genes which includes; RNase P, Glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPD), β-actin Mrna, β-globin gene, MHC I (major histocompatability complex I) mRNA, Cyclophilin mRNA, mRNAs for certain ribosomal protein, E.g. RPLP0 (ribosomal protein, large, P0; also known as 36B4, P0, L10E, RPPO, PRLP0, 60S acidic ribosomal protein P0, ribosomal protein L10, Arbp or acidic ribosomal phosphoprotein P0), 28S, or 18S rRNA, Actin2, UBQ10.

NEGATIVE CONTROLS:

Negative controls using each primer set should be analyzed to verify that no contaminating nucleic acid has been introduced into the master mix or into samples during sample processing. These negative controls are considered acceptable if no amplification of nucleic acids is detected.

POSITIVE CONTROLS:

Positive controls are analyzed to verify that the method is capable of adequately recovering and amplifying the target. The concentration of the sequence of interest in these positive controls should be 10 to 100 times higher than the defined detection limit of the PCR. A positive control is considered to be acceptable if the DNA of interest was amplified by PCR, as determined by the same confirmation technique used for the analytical samples. Precautions should be taken to avoid contamination of field samples with the positive control template. Positive control preparation should be physically separated from field sample and negative control preparation, and positive control samples should be handled last.

PCR POSITIVE CONTROL:

PCR positive controls are used to verify that the PCR master mix and reagents were prepared correctly in order to produce amplification of the target nucleic acid. This type of positive control is run with each PCR batch. A PCR batch is defined as a group of samples that are processed and amplified at the same time under the same conditions, using the same PCR master mix, and in the same thermocycler. PCR positive controls are prepared by the addition of an exogenous control to the master mix (10, 12).

EXOGENOUS CONTROLS CAN BE:

A purified total nucleic acid extract from the organism containing the sequence of interest, the whole organism, which may be used when the nucleic acid target of interest can be released from the seeded organism by heating before or during PCR, a specific nucleic acid fragment containing the entire sequence to be amplified, including primer binding sites (e.g., a low concentration of a previously amplified, and/or a cloned DNA fragment that has been sequenced for confirmation), a cloned DNA fragment containing a modified form of the target sequence, a heterologous sequence that has been previously shown to be amplified with an efficiency that is comparable to the target sequence, RNA transcribed from a cloned DNA fragment containing the target sequence or a modified target sequence (for use in RT-PCR applications). For quantitative PCR methods, the PCR positive control is evaluated by determining the total amount of the target nucleic acid or organism in the control divided by the amount added to the reaction as a spike. For qualitative PCR methods, the PCR positive control typically is evaluated in terms of 'detect' or 'nondetect' (although a detection signal that is weaker than normal may still indicate a problem).

PCR INHIBITION POSITIVE CONTROLS:

Inhibition positive controls are used to verify that interfering constituents from an environmental matrix, which may be carried over during isolation of nucleic acids or organisms during sample processing, do not inhibit the PCR. Inhibition positive control templates can be prepared by adding any of the exogenous controls to a processed sample or by using an endogenous control. Endogenous controls are target sequences that are expected to always be present in the sample (e.g., ribosomal DNA or RNA). These controls should be used only if it is demonstrated that there are consistent occurrence and recovery of the endogenous control templates in different samples, and if the control and target assays show comparable susceptibility to inhibition in different matrices. They are commonly used for analyses of clinical samples, but are unlikely to be applicable for the analyses of most environmental samples.

EXOGENOUS CONTROLS CAN BE USED TO ANALYZE FOR PCR INHIBITION IN SEVERAL WAYS:

In separate aliquots of the same nucleic acid extract from the same sample; in extracts from separate, replicate samples processed in parallel, in the same aliquot

of the same sample using modified internal controls. The absence of detectable PCR product from this control signals PCR inhibition. For quantitative PCR methods, different degrees of inhibition can be assessed directly by comparing the results from the control with the results from the PCR positive control. However, this requires that both controls be amended with the same amount of the positive control template. For qualitative PCR methods, the PCR inhibition positive control is evaluated in terms of a detect or non-detect (although a detection signal that is weaker than that of the PCR positive control may still indicate a problem) (13, 14).

METHOD POSITIVE CONTROL:

The method positive control is used to verify that the entire method is performing properly. This control should be performed by analyzing a reagent water sample seeded with known quantities of the target organisms prior the start of sample processing. For quantitative PCR methods, recovery of the method positive control is determined as the total amount of the analyte found in the sample divided by the amount of the control analyte added into the sample as a spike.

MATRIX SPIKE:

The matrix spike is used to determine the effect of the matrix on the overall method recovery. This control can be performed by the analysis of a duplicate sample collected at the same time and location as the environmental sample and seeded with known amounts of the target organism prior to sample processing. The seeded sample should be processed at the same time and in the same manner as the unseeded environmental sample and the method positive control, if feasible. Laboratories should, however, be cautious when processing seeded matrix samples at the same time as unseeded environmental samples to prevent cross-contamination (15, 16).

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CONFLICT OF INTEREST: None

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