Scholars Journal of Applied Medical Sciences (SJAMS) ISSN 2320-6691 (Online)
Sch. J. App. Med. Sci., 2014; 2(1D):485-488 ISSN 2347-954X (Print)

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com DOI: 10.36347/sjams.2014.v02i01.0104

Review Article

Internal Controls for the Quality Assessment of Polymerase Chain Reaction Methods for the Diagnosis of Infectious & Autoimmune Diseases

Narotam Sharma[1*], Duli Chand[2], Anurag Shukla[2], Man Singh[2], Ravi Kumar Govil[2], Banke Bihari[2], Aditya Saxena[2], A.K. Bhatia[2], R.K. Singh[1]

[1]Central Molecular Research Laboratory, Department of Biochemistry, Shri Guru Ram Rai Institute of Medical & Health Sciences, Patel Nagar, Dehradun-248001, Uttarakhand, India
[2]GLA University, Mathura, Uttar Pradesh, India

***Corresponding author**
Dr. Narotam Sharma
Email: sharmanarotam5@gmail.com

Abstract: Evaluation and interpretations of the results findings in Molecular diagnosis in patients care as well as in clinical research plays a vital role. The usage of various controls; positive, negative, internal controls are very significant for concluding the diagnostics assay's validity. The current review summarize about the different internal controls and the disease/disorder in which they can be utilized.

Keywords: Internal Control, Polymerization, Amplicon contamination, Molecular diagnosis

Molecular Diagnosis

Molecular diagnostics club up all the tools & technique used to analyze biological markers, genes variations in DNA sequences in the genome and proteome-the individual's genetic code and how their cells express their genes as proteins-by applying molecular biology to medical testing. The field of molecular biology grew in the late twentieth century, as did its clinical application. In 1980, Yuet Wai Kan *et al*. suggested a prenatal genetic test for Thalassemia that did not rely upon DNA sequencing-then in its infancy-but on restriction enzymes that cut DNA where they recognized specific short sequences, creating different lengths of DNA strand depending on which allele (genetic variation) the fetus possessed [1]. In the 1980s, the phrase was used in the names of companies such as Molecular Diagnostics Incorporated and Bethseda Research Laboraties Molecular Diagnostics [2]. 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. It is an in vitro, enzymatic & exponential amplification of target DNA sequence under controlled thermal conditions. 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.

Introduction to contamination control

A molecular diagnostic laboratory that plans on using one or more in-vitro amplification reaction(s) (IVAR) should also be evaluating measures to control a contamination problem which parallels the use of these procedures [1]. Historically, the concept of contamination in a biomedical laboratory has been associated with the unintentional disbursement of radioisotopes (e.g. ^{32}P, ^{35}S, etc.) in areas not designated for their use. Removal of these radioactive species was typically straightforward, rapid, and effective. Indeed, trace amounts of these radioactive species have usually been removed without problem and the laboratory restored to its original non-contaminated condition. However, the molecular genetics laboratory that has become contaminated with a biological species faces a much more difficult problem. Since the advent of the PCR, the molecular genetics laboratory has possessed an experimental capability of enormous sensitivity. Unfortunately, the PCR's exquisite capacity for amplification was accompanied by its extreme sensitivity to the presence of its own product as a feedback contaminant [2]. Because of the need for some molecular diagnostic laboratories to routinely detect less than 100 copies of certain target templates (e.g., viruses), this susceptibility of the PCR to trace amounts of its own product means the laboratory has a continual requirement to control a species it cannot easily detect, see or readily removed [3].

Principles of assay validation for nucleic acid detection tests

Validation is the evaluation of a diagnostic assay for the purpose of determining how fit the assay is for a particular use. When performing analyses of clinical material it is important to produce data of good quality. For this, some key criteria have to be fulfilled. The establishment of quality assurance (QA) and quality control (QC) systems is required, i.e. a set of quality protocols, including the use of control samples that ensure that the system is working properly and confirms data reproducibility and quality. QA and QC systems, together with trained and competent personnel, have already been established in many laboratories world-wide. Assay validation is another essential factor for assuring that test results reflect the true status of the samples. To predict the diagnostic performance of a diagnostic assay, it is necessary to use a validation methodology to document the expected performance of the assay is question. To diagnose infection when antibody levels are so low that previous exposure cannot be confirmed by an antibody test (e.g. enzyme-linked immunosorbent assay [ELISA] repeatedly in the 'gray zone' during the bovine leukaemia eradication programmes) [4, 5]. To discriminate between infection and maternal immunity in young animals (e.g. young calves in eradication programmes).To detect viral or bacterial nucleic acid when the diagnostic specimen is not suitable for virus isolation due to toxicity (e.g. semen, exam of mummified fetus). In the final stage of eradication programs, when thorough investigation of single cases is necessary (e.g. herpes virus latency and single reactor animals during the Aujeszky's disease eradication programmes) [6-8]. To discriminate vaccine strains from field viruses (DIVA [differentiating infected from vaccinated animals] approaches). To determine phylogenetic relationship of viruses and use this information for molecular epizootiology. To enable fast and safe first diagnosis in outbreak situations (e.g. the 2006 outbreaks of highly pathogenic, avian influenza).To determine the viral load (e.g. in porcine circovirus type 2 infections). Rapid monitoring of vaccinated animals that appear to have clinical signs. Detection of drug resistant mutants of pathogens, etc. To demonstrate freedom of infection in live animals or animal products. However, it has to be noted that some infected animals may have no detectable nucleic acid in the tissues being examined [9-11].

Internal Controls for the assessment of PCR protocols

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. Using house-keeping genes also complete with the target PCR but only for reagents that are in vast excess such as the polymerase and nucleotides. Minimal competition implies that the level of the true target is known, which cannot be the case for field samples. Armoured RNAs allow the mimic to be added in the extraction process, which is a step towards knowing that the extraction has worked. A true internal control provides more confidence that the extraction has been performed correctly. The down side of using housekeeping genes as internal controls is that they can be present in greater amounts than target pathogens [12, 13].

Table1. Various Genes Used as Internal Controls for the Validation of PCR

Sl. No.	Gene	Disease/Disorder/Syndrome
1	Human growth hormone (HGH)	HLA
2	*α-keratin*	Meningitis
3	*β-actin*	Cervical cancer
4	*β_2-microglobin*	HLA-B27/ Ankylosing spondalytis
5	Barlonella *rib(315bp)*	Barlonella
6	Flu BMA-R_2	Influenza B matrix
7	Flu ANA-F_5	Influenza disease
8	Ad_5 EIA-F3	Adenovirus5 EIA
9	*β-globin*	Cervical cancer
10	Quantification standard (QC)	Hepatitis C Virus/ Hepatitis B Virus
11	RnasP	Swine Flu
12	16sRNA & 18sRNA	Brain Diseases
13	Glyceraldehyde-3-phosphate dehydrogenase (GAPD)	Cervical cancer

Precautions taken to avoid false-positive results

False-positive results (negative samples showing a positive reaction), may arise from either laboratory related issues, such as cross-contamination, or assay-related factors, such as inefficient optimization or assay performance. Product carry-over from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments is a possible source of error, and various practices and tools have been applied to prevent false-positive PCR results [14-17]. Samples and reagents should be handled in separate laminar air-flow hoods, which are regularly

decontaminated using UV light (the use of UV-light demands very careful maintenance to be effective) and bleach. Constructing and using special tube-holders and openers can also help to prevent false-positives. In addition, good laboratory practices should be applied, i.e. to perform the basic steps (DNA extraction, mix and primer preparation, sample preparation, agarose gel, electrophoresis of amplification products, etc.) in separated laboratory areas or rooms. Different sets of pipettes should be used for each of the steps. The use of positive displacement and filtered tips is advisable. It is also, if possible, advisable to have different persons perform the different steps, who are restricted to the respective laboratory areas. Precautions should be taken to prevent the introduction of amplified material from potentially contaminated laboratories into 'clean' laboratory areas by movement restrictions on samples, papers, equipment, persons or any other potential method of contamination. Movement in the opposite direction should only occur after surface decontamination of equipment and tubes etc. and changing of laboratory coats and gloves. If the sample is expected to have a high amount of agent or target nucleic acid, it is preferable to dilute it prior to introducing it into 'clean' laboratory areas [18].

Precautions taken to avoid false-negative results

PCR has proven to be a very effective method of detecting nucleic acids, such as viral genomes in clinical specimens. However, an infected animal in the later phases of infection may no longer have viral nucleic acid in the tissues being examined. Consequently, in such cases the negative PCR results should be considered as one part of a complex diagnostic examination. False-negative results (samples containing the agent of interest but tested as negative) occur mostly due to inhibitory effects and/or pipetting errors; however, issues attributable to sample handling can also yield false negative results [19]. Therefore, internal controls can be used as indicators of PCR assay efficiency. PCR internal controls may include foreign DNA added to the sample or ubiquitous DNA naturally occurring in the sample [20]. Foreign DNA added to the sample, may include DNA or RNA mimics. DNA mimics, manufactured oligonucleotides, have the same primer-binding sequences as the PCR target, but flank a heterologous DNA fragment of a different size. The identical primer-binding nucleotide sequences allow co-amplification of the target and the mimic in the same tube with minimal competition. The size differences provide easy discrimination by Southern blot analysis. Armored RNA®, an identical concept to DNA mimics, uses a control RNA fragment packaged in bacteriophage coat proteins to protect or stabilize the RNA for control or standardization of RT-PCR assays (further details on internal controls, see above). With real-time PCR assays, it is also possible to use internal controls, a naturally occurring housekeeping gene, a selected fragment of the host animal's genome such as beta-actin, GAPDH, or ribosomal RNA. By multiplexing such an intrinsic control with a specifically coloured reporter fluorophore, it is possible to check the sample quality and confirm PCR efficiency, as the target agent and intrinsic DNA are simultaneously detected. Internal controls (for example 'mimics') increase the reliability of diagnostic PCR. Caution must be used when designing and validating internal controls. Extensive testing is necessary to ensure that PCR amplification of the added internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity. Internal controls are used in concentrations slightly higher than the detection limit of the diagnostic PCR to ensure the test's performance. It should also be remembered that internal controls have a disadvantage, similar to spiked samples, in not being representative of target nucleic acid and can lead to false-negative results. Human leukocyte antigens-B27 is a class I surface antigen encoded by B locus in the major histocompatibility complex (MHC) on the short (p) arm of chromosome no. 6 at position 21.3 from base pair 31,429,845 to base pair 31,432,923 and presents microbial antigen to T cells. The disease ankylosing spondylitis (AS) has been associated to the human leukocyte antigen (HLA) class I allele HLA-B27, and the relationship of HLA-B27 with AS is the strongest for any HLA locus. Almost all nucleated cells in the body, except neurons and striated muscle cells containsclass I HLA molecules on it. The strongest evidence of the involvement of HLA-B27 in AS came from studies done with mice and rats that had been given HLA-B27 as a transgene. They developed diseases such as ankylosing enthesopathy, and rats with a high copy number of HLAB* 2705 developed axial and peripheral arthritis, gut inflammation, and lesions on the skin [21]. Rats and mice that were kept in sterile environments did not develop joint disease or gut inflammation. Development of the disease was dependent on the presence of gut bacteria and a high copy number of HLA-B27 in cells of bone-marrow lineage. Major histocompatibility complex (MHC) class I molecules presents endogenous peptides derived from intracellular proteins to the b T cell receptor on CD8? cytotoxic T lymphocytes in infected cells. Ankylosing spondylitis is a disease that predominately affects males and usually appears in young adulthood. It usually beginswith unilateral pain at the sacroiliac joint, progressing to bilateral pain that radiates down the legs, and up the spine, The internal b-globulin control, which was detected in all HLA-B27 negative samples, demonstrated the correct PCR condition and the presence of sufficient sample DNA, thus preventing false negative results.

Facility Design

Contamination between samples and from previous PCR amplicons generated in the laboratory is a significant potential source of invalid PCR results. Thus, the separation of work space is critical. A laboratory performing PCR analyses on environmental samples should be divided into at least three physically

separate rooms [22]. Reagent preparation (using positive pressure to prevent the introduction of contamination) Sample preparation (using negative pressure to keep template nucleic acids in the room) Amplification and product detection (using negative pressure to keep amplified nucleic acids in the room). The diagnosis of infectious diseases is performed by direct and/or indirect detection of infectious agents. By direct methods, the particles of the agents and/or their components, such as nucleic acids, structural or non-structural proteins, enzymes, etc., are detected. The indirect methods demonstrate the antkibodies induced by the infections.

Acknowledgment

The authors are grateful to Honorable Chairman, Shri Guru Ram Rai Education Mission for his kind support and guidance.

REFERENCES

1. Cheung MC, Goldberg JD, Kan YW; Prenatal diagnosis of sickle cell anemia and thalassaemia by analysis of fetal cells in maternal blood. Nature genetics, 1996;14(3):264-268.
2. Ferrara J; Personalized medicine: challenging pharmaceutical and diagnostic company business models. McGill Journal of Medicine: MJM, 2007; 10(1):59.
3. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M *et al*.; The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry, 2009; 55(4): 611-622.
4. Cines DB, Bussel JB, McMillan RB, Zehnder JL; Congenital and acquired thrombocytopenia. Hematology Am Soc Hematol Educ Program, 2004: 390-406.
5. Glenn SJ, Post KW; Veterinary microbiology: bacterial and fungal agents of animal disease. 1st edition, Elsevier Health Sciences, 2004.
6. Belák S1, Thorén P; Molecular diagnosis of animal diseases: some experiences over the past decade. Expert Rev Mol Diagn., 2001; 1(4): 434-443.
7. Yaeger MJ; Disorders of Pigs. In Njaa BL; Kirkbride's Diagnosis of Abortion and Neonatal Loss in Animals, 4th edition, 2011: 89.
8. Buxton D, Rodger SM; Toxoplasmosis and neosporosis. Diseases of Sheep, 2008; 4: 112-118.
9. Stocker, Bruce AD; Novel non-reverting shigella live vaccines. U.S. Patent 5,077,044, issued December 31, 1991.
10. Luckey TD; Germfree life and gnotobiology. Elsevier, 2012.
11. Plummer M, Plummer DT; Introduction to Practical Biochemistry. Tata McGraw-Hill Education, 1988.
12. Olivier T, ElMoualij B, Heinen E, Zorzi W; A decade of improvements in quantification of gene expression and internal standard selection. Biotechnology Advances, 2009; 27(4): 323-333.
13. Burd EM; Validation of laboratory-developed molecular assays for infectious diseases. Clinical Microbiology Reviews, 2010; 23(3): 550-576.
14. Neumaier M, Braun A, Wagener C; Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics. Clinical Chemistry, 1998; 44(1): 12-26.
15. Parry JV, Mortimer PP, Perry KR, Pillay D, Zuckerman M, Health Protection Agency HIV Laboratory Diagnosis Forum; Towards error-free HIV diagnosis: guidelines on laboratory practice. Communicable Disease and Public Health, 2003; 6(4): 334-350.
16. Weissenborn SJ, Wieland U, Junk M, Pfister H; Quantification of beta-human papillomavirus DNA by real-time PCR. Nature Protocols, 2010; 5(1): 1-13.
17. Hoffmann B, Beer M, Reid SM, Mertens P, Oura CA, van Rijn PA *et al*.; A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Veterinary Microbiology, 2009; 139(1-2): 1-23.
18. Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA *et al*.; Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clinical Microbiology Reviews, 2006; 19(1): 165-256.
19. Postgate JR; The sulphate-reducing bacteria. CUP Archive, 1979.
20. Burgess JW, Schwan WR, Volk TJ; PCR-based detection of DNA from the human pathogen Blastomyces dermatitidis from natural soil samples. Medical Mycology, 2006; 44(8): 741-748.
21. Ivanyi P; Immunogenetic basis of the seronegative spondyloarthropathies. Current Opinion in Rheumatology, 1992; 4(4): 484-493.
22. Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N *et al*.; Genetic analyses from ancient DNA. Annu Rev Genet., 2004; 38: 645-679.