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Review Article

RT-qPCR Protocol Optimization Assessment with Propidium Monoazide for Viable and Non-Viable Pathogen Detection

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ABSTRACT

Real time PCR or RT-qPCR (including reverse transcriptase PCR) is a gold standard in diagnostic testing for pathogens such as bacteria and viruses that contain RNA or DNA for amplification. As a validated molecular assay technique, RT-qPCR has been instrumental in microbial detection because of its high throughput, quantification of DNA or RNA samples as well as quick turnaround especially when compared with culturing methods that can take multiple days to generate result. While the advancement of PCR has had significant impact for diagnosis of diseases such as COVID-19through its high sensitivity and specificity, the test also brings with it its own challenges of false positive and false negative results. This is normally attributed to PCR's susceptibility to contamination or inhibitors. However, there are other conditions like DNA extraction techniques and protocols in PCR methodology that can impact the accuracy of the PCR results as well. This paper, therefore, aims to synthesize factors that contribute to false positive and negatives in PCR results, improvements to protocols that includes the use of DNA intercalating dyes such as Propidium Monoazide (PMA) in PCR assay to improve DNA extraction and discuss the importance of an optimized PCR assay for diagnostic testing efficacy from a social and economic standpoint.

INTRODUCTION

The COVID-19 pandemic provided a lot of lessons for epidemiologists and the scientific community at large on how to manage a pandemic. One of the keys determining factors in controlling the spread and impact of the disease is in diagnostic testing[1,2]. The quicker one is able to accurately diagnose a person with the infection, can determine how quickly the spread of the infection can be contained in a particular population through treatment and disease management protocols[3].

During the pandemic many lateral flow devices such as rapid antigen test kits[4] and antibody serology tests were optimized for population wide screening. These types of tests offer shorter run time of around 10-15 minutes and are useful in differentiating those with active infections compared to those who have recovered[5]. However, their low sensitivity and specificity leads the confirmatory diagnostics such as a Real Time PCR (RT-qPCR) to remain the gold standard. Polymerase chain reaction (PCR) is a molecular assay that aims to detect specific genetic material from pathogens such as bacteria or virus through DNA detection. Using the process of amplification, PCR replicates the small sample or fragments found of the test sample (virus or bacteria) and amplifies to a level that it becomes detectable as a signal. The overall testing accuracy is determined by how sensitive and specific the assay is. Sensitivity refers to the ability of the test to accurately detect infected individual while specificity is whether the uninfected test samples come up negative. For a laboratory performance, a PCR test is usually 95% sensitive and specific. However, in reality this performance does drop down because of external factors that include how the assay is handled, potential risk of contamination and other varying conditions in PCR protocols, all of which can determine the likelihood of false positive and false negative results appearing[6].

False Negative and False Positive Results:

False negative results, if we take the example of COVID-19 test samples, would indicate that a person who has viral infection is showing a negative result on the PCR instead of a true positive[7]. With an assumed 95% sensitivity, there is a 5% chance of false negative results appearing. This could be attributed to not just errors in handling of testing, cross contamination but also

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low viral load that isnot enough for the PCR to amplify and detect. One study has suggested that up to 58% COVID-19 patients may have false negative results on their initial RT-PCR[7]because of PCR testing protocols that include how early a person in their infection period is tested.

On the other hand, a false positive[8,9] indicates that a person does not have an active viral infection while the PCR result shows up as positive rather than a true negative. False positives are less common compared to false negatives and yet both types of results can potentially cause harm in high and low prevalence population setting, of a disease state in question. Prevalence indicates how common a disease, for example COVID-19, is in a specific population at a specific point in time. And this prevalence (or disease burden) plays a role on the positive predictive value (PPV) and negative predictive value (NPV) of a diagnostic test.

A PPV is a probability that the testing samples showing up positive are truly positive, while an NPV is the probability that samples testing negative are truly negative. In a high prevalence area, the PPV will increase but the NPV will decrease indicating that in a high prevalent risk population, false negatives will become more of an issue. While in a low prevalence setting with a decreased PPV and an increased NPV, false positives will be a concern[10,11].

Impact of PCR Testing Errors:

The adverse effects linked with false positive and false negative results, in disease infections, cannot be underestimated. In USA, Centers for Disease Control and Prevention (CDC) states that roughly 1 million healthcare associated infections and 90,000 deaths occur each year. Among the hospital acquired infections *Staphylococcus aureus* and Methicillin resistant *S. aureus* (MRSA) are the prevailing pathogens with approximately 5-10% prevalence in hospitals compared to 2-6% in general population. Adding to this, a study states that MRSA related false negative results can go from 6 to 30%, and false positive results around 20% through RT-PCR testing[12]. Looking at the existing burden of hospital infections on hospital resources and cost, the additional impact that diagnostic testing error is causing has to be given importance. With the RT-PCR already an established standard in testing, the optimization of its testing protocols to reduce its error rate is the next step.

Outside of contamination, poor techniques, low quality sampling and reagents and general handling of the test[13](Table-1), looking into PCR methodology can also aim to reduce errors[14]. One of the causes for false positive or negatives tests can be attributed to PCR machine being unable to differentiate between live and dead pathogen DNA. Given that DNA from dead bacteria can remain in clinical samples for months[15] even after a person has been cured of infection, therefore can tamper with the PCR results.

Contamination during
Sampling, DNA extraction, PCR amplification, developing and handling of lab reagents, equipments being used,
Cross contamination
with other viruses, sample mix ups,
Errors in
Sample labelling, data entry,
Non specific reasons

Table-1: PCR Testing Error Factors

The main principle of PCR is to detect DNA in the test sample and begin to amplify it through various rounds of thermal cycling[16], generating enough DNA sample that the fluorescent dye in the PCR is able to bring up a signal determining whether the pathogen DNA is present (positive) or not (negative). Quantification of the DNA is also done by measuring how many cycles it takes for the PCR to amplify the DNA at the threshold level. Lower the number of cycles, higher the number of genetic material present in initial sample which thereby indicates the severity of the infection.[17] However, if DNA from dead bacteria or virus is still present in the sample from previous infections, that DNA can also be amplified as the PCR is unable to discriminate between live and dead DNA thus overestimating the presence of active infection in a sample i.e., false positive[18].

Propidium Monoazide Dye:

Give then importance of viable and non-viable nucleic acid distinction in samples whether in a clinical setting or environmental, standardized protocols have still not been fully developed. Several viability assessments have been considered to distinguish live vs dead bacterial samples such as cultivation where live samples are detected using population growth methods such as gram positive/ gram negative bacterial cultures, flow cytometry and more. This process is limited

by the cultivatable species as well as time constraints. Another assessment can be done through a metabolism-based approach such as Molecular Viability Testing. The principal behind this technique looks at metabolic activities carried out by viable (live) samples such as changes in cellular energy (ATP production), respiratory measurements and more. Limitation of this approach is linked with risks of false positives due to presence of active extracellular enzymes that can show up in non-viable samples[19]. The third assessment is using a membrane-based method that looks at the integrity of membranes in live vs dead samples using different stains, dyes and treatments. For this approach, the viability PCR[20] uses Ethidium Monoazide (EMA) or Propidium Monoazide (PMA) as a membrane binding dye. While both EMA and PMA follow the same process flow, some studies have shown that EMA is not as effective in distinguishing samples by penetrating both dead and live nucleic acid samples[21,22].

Propidium Monoazide (PMA) is a DNA and RNA binding photo reactive dye that is membrane impermeant, which means that the dye is not able to penetrate a cell membrane allowing them to only bind with dead cells. Once they enter the dead cell, through the broken membrane, they bind to the DNA or RNA covalently once made reactive by light. This binding mechanism modifies the nucleic acid thus preventing it from being amplified during the amplification process in a PCR. The remaining PMA that is unbound and free-floating becomes inactivated upon light exposure as well therefore eliminating the risk of it attaching to DNA during the DNA extraction phase for example (Fig-1).

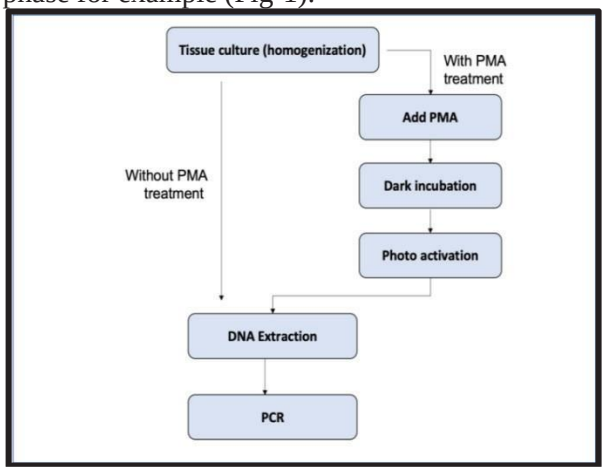

Fig-1: PMA Flow Chart

For PMA to work in optimal conditions there are several factors that need to be considered such as the PMA concentration being used, time periods for its dark incubation and the time period for photo activation process during which it binds with dead cells. All these parameters need to be tested to find the conditions necessary to give accurate results. Askar et al. (2019), in their study looked at these parameters when assessing affects of PMA on PCR performance for detection of periprosthetic joint infections[23] (*Staphylococcus aureus*) using tissue samples. Looking at cycle threshold difference(*ΔCt*), between viable and non-viable bacterial suspensions, *ΔCt* viable and *ΔCt* dead were calculated. *ΔCt* viable being the difference in Ct value of viable sample with and without PMA dye and same for ΔCt dead.

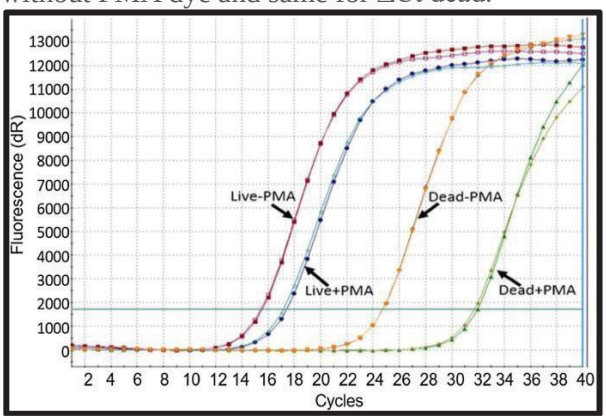

Fig-2: Optimization of PMA: Amplification plot of live (viable) and dead (non-viable) samples with and without PMA dye.Askar etal.(2019).

The Ct difference in live(viable) samples with and without PMA showed to be less than one cycle difference. While the Ct difference in the dead(non-viable) samples with and without PMA were significantly different, showcasing that the inclusion of PMA dye on non-viable samples increased the cycle numbers before reaching cycle threshold. In another paper (PanY.et al), the quantification of viable *Listeria monocytogenes* in food products was tested using PMA conditions. In that paper both EMA and PMA conditions were tested where EMA was found to be toxic to viable cells compared to PMA regardless of the varying conditions provided, further validating the shift to PMA from EMA dyes in DNA extraction[24]. Further, the paper lists out PMA concentrations and incubation temperatures as key conditions that impact the C_T values of PMA-PCR assay. Similarly (Vesper, 2008) found that optimizing the PMA conditions during DNA extraction for their fungal infection samples contributed to viable sample distinction and also suggested altering the PMA conditions for different species to get best outcomes[25]. This was discovered when the optimized conditions for PMA treatment which led

to effective distinction of viable samples in cyanobacterial species did not provide the same distinction when same conditions were applied in diatom species analyses[26].

PMA RT-qPCR Results in Laboratory and Clinical Settings:

In a laboratory setting with bacterial suspensions, PMA dye shows good discrimination in live and dead bacteria. However, the question arises on whether similar results can be seen in clinical setting where samples are more complex in nature through presence of other material that can hinder PMA efficacy. When looking at tissue suspension samples (ex vivo) the Ct difference of viable cells in tissue suspension compared with viable cells in bacterial suspension showed a delay in amplification. This could indicate PCR inhibition by other tissue materials in the sample. When Ct difference in viable tissue suspension samples was measured with PMA dye for amplification, the delay in amplification was seen to be less in PMA samples compared to the previous example, indicating that PMA could also be playing a role in reacting with various organic materials in the tissue sample that inhibit PCR amplification. While there are other factors that also need optimization such as improved techniques for DNA extraction that can reduce presence of human DNA and other homogenates that inhibit the PCR activity; the presence of PMA as a pre-treatment PCR technique does work to improve PCR sensitivity as well as specificity. As indicated in the study PCR efficacy increased from 71.7 to 89.1% for viable pathogen detection.

The value of the PMA technique is dependent on its versatility in different microbial diagnostic testing. Outside of *Staphylococcus aureus*, it is important to consider how PMA interacts with other bacteria and viruses. *Mycobacterium tuberculosis*[27] or *Pseudomonas aeruginosa* that come under the category of viable but not culturable bacteria (VBNC), present issues with public health providers as culturing is not only time-consuming (taking an average of 5-7 days to generate results) but such VBNC pathogens are difficult to culture altogether due to their low metabolic activity. In environmental sampling, detection of such bacteria in open bodies of water such as swimming pools is time sensitive, which is where a RT-qPCR with a PMA dye will not only speed up the time taken for detection of sample but can also differentiate whether sample contains viable or non-viable bacteria. One study[28] showcased that using PMA in water samples for detection of bacteria helped to reduce false positives in RT-PCR[29] testing due to the presence of damaged bacteria in the samples while not impacting the viable DNA from being amplified, or being affected by environmental materials that could otherwise hinder efficacy.

PMA RT-qPCR Testing Protocol Modifications:

One study looked at the effect of PMA RT-qPCR for UTI infection detection in urine samples. In most cases, with a prolonged infection, clinical symptoms of a UTI infection can persist even if bacterial cultures turn up negative. This can be attributed to long antibiotic courses that are able to still give false negatives in cultures. While the PMA dye inclusion in RT-qPCR can bring the distinction between live and dead bacteria and improving accuracy over a conventional PCR, the efficacy of RT-qPCR when reacting with urine components is impacted as PMA efficiency in binding with live vs dead cells is reduced. This again highlights the need to augment the PMA RT-qPCR protocols depending on the external conditions of the samples provided. In this study (Zeng, D. et al.,2016). PMA efficiency increased once the bacterial samples were centrifuged, sediments collected and suspended in phosphate buffered saline (PBS).

Similar to modification of PMA RT-qPCR by suspending or removing organic material in target sample, there are other modifications to PMA for different pathogens that have yielded improved results. Such as dithiothreitol cotreatment in *Bacillus subtilis* spores that helped PMA penetration in inactivated spores compared to viable spores[31]. Other strategies include the already discussed parameters such as incubation time for light and dark periods, concentration but also temperature for incubation that can help to increase dye penetration in non-viable cells.

As PMA can work with any organism that has its nucleic acid enclosed in a PMA impermeable membrane or structure, it should also function on viral organisms. An effective method to detect infectious viral particle is not only important from a diagnostic clinical perspective but also from assessing disinfection treatments. To that effect, viral culturing or virus induced plaques are the gold standard methods in determining viral infectivity. In virus culturing, a virus is injected in laboratory cell lines to observe whether cell damage and death occurs or not and if the virus

reproduces. While this is effective, culturing is time consuming, has a long turn-around time for results, needs biosafety level protocols and is expensive. A PCR approach is still preferable but the distinction of the virus being active vs inactive remains crucial as every PCR result cannot be sent for a viral culture confirmation afterwards.

The SARS-CoV-2 is an enveloped protein virus that upon damage to its membrane can allow PMA to penetrate and bind. One study showed that the addition of a surfactant sodium dodecyl sulfate (SDS) helped the PMA dye penetration[32] in damaged cells as it is a membrane destabilizing agent. The study found that that SDS-PMA assisted q-RTPCR led to a quicker distinction between the viable and non-viable samples. This is because PMA is able to react to the free RNA released or inside damaged virions but is unable to penetrate active (live) virus and bind with its RNA, when used with SDS (0.0005%). The RNA that does bind to PMA therefore inhibits the PCR amplification, allowing for only live virions to be detected[33].

The addition of SDS is an important augmentation as simply heat-treating the virus (in moderate conditions) does not damage its viral envelope. If a higher temperature (95°C) is used it would damage the envelope enough for PMA to bind but such conditions, as are not present in the environment, are not optimal to include as part of protocols.

PMA RT-qPCR In Viral Testing:

PMA pre-treatment for viral infections is still not completely validated since PMA is limited by its requirement of a damaged membrane for penetration. Not all inactivated viruses have a damaged capsid[34], for example, there are some viruses that can lose receptors that allow them to remain infectious while still having their capsid intact. In this scenario, the virus is inactive but PMA is unable to penetrate and block it from amplification in RT-qPCR thus retaining the risk of a false positive[35].Thus highlighting a critical note to membrane-based method for viable sample detection as the integrity of a membrane cannot fully determine the viability or active nature of a cell. One study[36] looked at T4 bacteriophage to determine the conditions suitable for effective PMA pre-treatment to distinguish between viable and non-viable (inactive) viruses. It determined that moderate temperatures and protease treatments might not be effective as it did not damage the capsid that protected the viral nucleic acid. Therefore, other additions to the PMA parameters are needed for further optimization in viral testing. For water treatments or general assessment of disinfectant efficacy, using extreme conditions of heat, chlorination can help to inactivate enteric viruses such as poliovirusand Hepatitis A[37]and damage their capsid. In that case, using a PMA RT-qPCR would be effective in determining whether such conditions worked in inactivating viruses from a particular environment[38]. However, further research into which type of conditional changes (for example susceptibility of Hepatitis A, E and other non-culturable viruses to temperate, surfactants etc[39,40]) are optimal for PMA dyes to penetrate and perform, is critical for the success of this PCR approach. While false negatives are often reported and investigated, false positive results though less common still need to be understood. High number of cases of false positives could be attributed to the PCR sensitivity in picking up dead viral DNA from previous infections. From a COVID-19 contagion standpoint, a false positive can have a psychological impact on a person by triggering panic and anxiety. Moreover, as part of contact tracing and isolation protocols that have been put in place, such a false positive report can create more harm whereby the person could be quarantined with other COVID-19 positive patients thus increasing likelihood of a getting an actual infection. In such scenarios, the DNA assays need to be able to assess pathogenic risk instead of the conventional PCR approach where a signal is detected from infectious or non-infectious viral DNA. With continuous research and development, the PCR methodology continues to be optimized and expanded allowing for changes in the protocols to accommodate for PCR efficacy.

CONCLUSION

Using intercalating DNA binding dye has been on an evolutionary journey for many years, where the previous dye Ethidium Monoazide (EMA) has now been replaced with Propidium Monoazide (PMA) because PMA is a more impermeant membrane than EMA. However, there are still limitations[41] to the PMA pre-treatment that needs to be further reviewed. The additions of surfactants or alterations in temperature, concentrations and other parameters can be customized to pathogen specific testing protocols. However, one limitation that does not have a clear-cut solution is the dyes inability to detect inactivation of pathogen that does not damage the

membrane. In that scenario, the only alternative approach that can be considered is a Molecular Viability Test (MVT)[42,43].

MVT uses a pre ribosomal RNA as a detection method to differentiate between viable and non-viable bacteria. In viable bacterial cells, pre rRNAs are intermediates in ribosomal RNA synthesis that are found abundantly in growing bacterial cells. Given a nutritional stimulus these pre-rRNA intermediates (pools) rapidly replenishes which can be observed as fluctuations that can be detected. This can help improve the sensitivity of PCR detection for bacterial viability discrimination since MVT does not require the membrane to be damaged for detection. However, the limitation with this approach is its being relatively new with little data and research behind it. It can therefore be considered as an alternative for scenarios where PMA with its alterations to protocol are not providing efficient RT-qPCR results, with culturing techniques as a secondary approach.

Overall, PMA is an elegant addition to pathogen detection through RT-PCR[44,45]. With population expansion and climate change, the spread of various diseases has been exponential in the past few years. For that, it is imperative that we work on improving our diagnostic testing as well as track and trace approaches through optimized molecular techniques. And the utility of PMA allows for such an expanded scope into various disease testing.

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