

## MULTIPLEX PCR - A DIAGNOSTIC METHOD FOR MICROBIAL PATHOGENS

### Abstract

PCR assays are now a days most popular tool of molecular plant pathology. It is the most practical and economical way for target amplification of diseased plant sample with very little DNA. DNA denaturation, annealing, and extension steps are repeated in cycles in PCR amplification. The use of PCR technology in disease diagnostic is occasionally constrained by the cost incurred and the small sample volume. A version of PCR known as multiplex PCR (mPCR) has been described to address these drawbacks while simultaneously enhancing the diagnostic capability of PCR. Multiplex PCR can amplify two or more target sequences of the sample by using more than one primer pairs in a single PCR reaction. Simultaneous amplification of multiple target sequences in a single mPCR reaction can save the time and effort significantly. Multiplex PCR is now a days becoming a quick and efficient diagnostic assay in both the pathology and research labs.

**Keywords:** Polymerase Chain Reaction, Multiplex PCR, Primer

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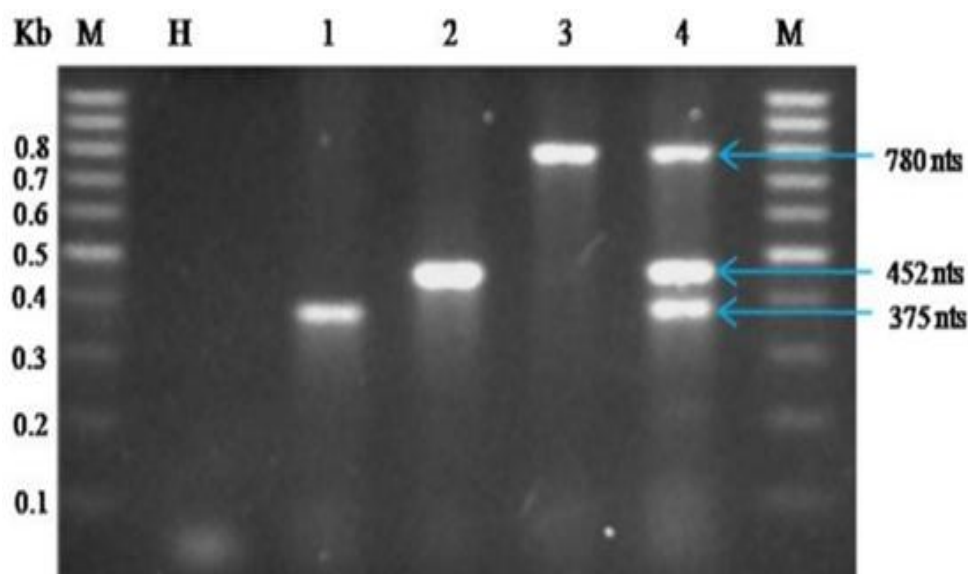
## I. INTRODUCTION

PCR assay is an important part of molecular biology and it constitutes the most convenient and cost-effective method for DNA amplification in very small DNA samples. The advancement in PCR technology has brought ease in understanding of several aspects of molecular biology. Multiplex polymerase chain reaction (mPCR) is a modification in PCR method where two or more than two target sequences can be amplified. The amplification is possible by using more than one primer pairs in one PCR reaction. Multiplex PCR can potentially reduce the hard work and time consumed in single PCR process. mPCR has already been successfully applied in many areas like, detection and diagnosis of pathogen, mutation and polymorphism analysis, reverse-transcription PCR, and quantitative analysis.

## II. MULTIPLEX POLYMERASE CHAIN REACTION

In mPCR, several primer sets in a single PCR reaction are used to generate the specific amplicons of desired sizes of different DNA sequences. Therefore multiplex PCR helps in detection and diagnosis of different pathogens at same time (James et al., 2006), and consequently, a single PCR test run is utilised rather than many PCR tests for every pathogen. It also saves time and reagents. The target sequence size or their base pair length, should be diverse enough to create discrete band when observed by agarose gel-electrophoresis, and annealing temperatures for each primer pair should be adjusted to operate properly in a single PCR reaction. Multiplex PCR is a crucial method for doing cost-effective high-throughput analysis.

An example is shown in Figure 1, where the simultaneous amplification of three different target sequences of different sizes has been carried out in a mixture of DNAs extracted from Cotton leaf curl virus infected leaf sample.



**Figure 1: Agarose Gel Electrophoresis Picture Showing Simultaneous Amplification of Three Different Target Sequences of Different Sizes**

### III. PRINCIPLE

mPCR is the simultaneous amplification of multiple target sequences utilising multiple primer pairs in a single PCR reaction tube. The compatibility of the PCR primers employed in the PCR reaction is crucial for this co-amplification of two or more than two targets in a single PCR reaction. Each PCR amplification can take place at the chosen temperature if all of the primers in the reaction have similar melting temperatures ( $T_m$ ), which allow them to anneal to and dissociate from complementary DNA sequences at roughly the same temperatures. If one primer set is annealing while another is dissociating from its target at any temperature, this technique could not be carried out. All primer pairs must be chosen so that their melting temperatures are only a few degrees ( $^{\circ}\text{C}$ ) apart from one another. As long as none of the reagents are present in rate-limiting quantities, each amplification occurs independently of the others, and each distinct amplification product or amplicon is produced in an unhindered manner. Choosing primers that define amplicons with around the same size range (100-600 bp) will ensure that each is synthesised effectively and at a similar rate. Every M-PCR test needs a detection step that can recognise each amplicon. This can be accomplished via gel electrophoresis and visual recognition of distinct amplicons of various sizes.

**1. Designing multiplex PCR primer:** A popular method for amplification of several small sample of genomic DNA is multiplex PCR. Targeted amplification-based sequencing has a number of significant advantages over whole genome sequencing, as well as targeted sequencing using a hybrid capture technique. It is time and cost-efficient, allows for the use of specimens with less-than-ideal DNA quality, requires a minimal amount of DNA (10–200 ng) as the starting template, and offers high-depth sequencing and simple data interpretation. When progressively more PCR primers and reactions are pooled, serious performance problems appear. Specifically, heat damage to genomic targets occurs during high temperature cycling, which modifies the native nucleic acid sequence, and amplification artefacts are created as a result of polymerase editing errors during annealed oligomer extension. As a result of substrate competition, formation of primer-dimer, sequence-dependent variations in PCR efficiency and reaction biases also start to appear.

Target DNA denaturation, primer-DNA annealing and DNA sequence extension are repeated in cycles in PCR amplification. A known genomic sequence of sample DNA is intended to be complementary to the oligonucleotide primers. The  $T_m$  (melting temperature) of the primer pairs should be similar or within 1-2 $^{\circ}\text{C}$  of difference. The right GC content (50–55%), and the absence of primer cross-complementarity are all important considerations when creating amplification primers for multiplex PCR. Additionally, because they may impair PCR amplification efficiency and lead to amplification biasness, regions with repetitive sequences, single nucleotide polymorphisms (SNPs), and regions with high homology should be avoided.

To ensure that primer binding sites do not contain variable SNPs, the developed primers should be compared to SNP databases in NCBI website. Primers should be altered to accommodate a number of potential nucleotide changes in the primer design if binding site sequence variation cannot be prevented. To assess the primer specificity to the region of interest, sequence databases must also be examined at <http://blast.ncbi.nlm.nih.gov/> against the primers. By doing this, pseudogenes and other

sections with high sequence homology will not be amplified, which could lead to incorrect sequence alignment and the production of false positive calls. A variety of software tools are available to help in primer design (e.g., Primer3 and PrimerBLAST).

## 2. Components of multiplex PCR

- **Amount of primer:** The multiplex PCR uses equimolar primer concentrations of 0.1–0.5 mM each. The primer concentration for DNA with low copy numbers or high levels of complexity should be 0.3 to 0.5 mM. The primer concentration for DNA with a high copy number or low complexity should be 0.04–0.4 mM.
- **dNTP and MgCl<sub>2</sub> concentrations:** While the dNTP concentration is gradually raised from 0.5 to 1.6 mM, the dNTP MgCl<sub>2</sub> concentration is maintained at 2 mM. Above 200–400 mM each dNTP levels, the amplification is quickly inhibited and yields the best results. PCR amplification was still possible at a lower dNTP concentration (100 mM for each dNTP), but with noticeably fewer results. Stocks of dNTP are susceptible to thawing and freezing cycles. Multiplex PCRs frequently failed to function efficiently after three to five of these cycles. Small aliquots of dNTP can be prepared and stored refrigerated at -20°C to prevent such issues. When single loci are amplified, this "poor stability" of dNTP is less visible.
- **MgCl<sub>2</sub>:** Since Taq DNA polymerase is a magnesium-dependent enzyme, Mg<sup>2+</sup> must be optimised. The template DNA primers, dNTPs, and Taq DNA polymerase all bind Mg<sup>2+</sup>. Therefore, the dNTP content, particular template DNA, and sample buffer make up will all influence the ideal Mg<sup>2+</sup> concentration. The apparent Mg<sup>2+</sup> optimum may be changed by the presence of chelators like EDTA or EGTA in primers and/or template DNA. A high Mg<sup>2+</sup> concentration prevents DNA from fully denaturing, stabilising the double strand and lowering yield. Additionally, excessive Mg<sup>2+</sup> might stabilise primer annealing to false template sites, reducing specificity. The amount of product is decreased by insufficient Mg<sup>2+</sup> concentration, on the other hand.
- **PCR Buffer concentration:** The efficiency of the multiplex reaction is increased when the buffer concentration is increased to 2X. This performed better than all of the adjuvants that were evaluated. Longer amplification products in primer pairs are more effective at lower salt concentrations, while shorter amplification products in primer pairs are more effective at higher salt concentrations.
- **Amount of template DNA and Taq DNA polymerase:** Further reducing the annealing temperature will enable effective and targeted amplification when the template DNA concentration is extremely low. We tried several Taq DNA polymerase concentrations. Around 2.5 U/50 ml of reaction fluid seems to be the optimal enzyme concentration. The stock solution's high glycerol content may have contributed to the excess enzyme production, which led to an imbalanced amplification of different loci and a minor rise in background. Specific primer-target hybrids' extension rates are influenced by the enzyme's activity, the accessibility of necessary materials like MgCl<sub>2</sub> and dNTPs, and the make-up of the target DNA. Therefore, the bulk of

changes made to enhance PCR performance must target the variables influencing annealing and/or extension rates.

- **Use of adjuvants: DMSO, Glycerol, bovine serum albumin:** A PCR additive, such as DMSO, glycerol, formamide, or betaine, can greatly enhance even the most challenging multiplex PCR operations by relaxing DNA and facilitating easier template denaturation. DMSO and glycerol produced inconsistent findings in the multiplex PCR. Therefore, it is necessary to evaluate these adjuvants' value in each situation. More than DMSO or glycerol, BSA doses up to 0.8 mg/ml significantly improved the PCR's efficiency.
- 3. Advantages of MPCR:** The prime advantage of mPCR is the ability to detect more than one target samples in a single PCR test. For samples such as different plant viral diseases from which several different viruses can be identified and diagnosed. This advantage of mPCR offers potential cost and time savings. The second benefit of M-PCR is its high level of sensitivity and capacity to identify both viruses that are noncultivable and viruses that have been killed in antigen-antibody complexes. Another advantage of mPCR is the effective sequencing of DNA and RNA with subpar quality by amplifying relatively short genomic areas (80–150 base pairs). However, a cross-reaction between primer pairs can be occurred in multiplex PCR due to primer overlap when sequencing large consecutive genomic regions; as a result, it may be necessary to separate closely spaced primers into different multiplex pools and consider whether a capture-based method would be more appropriate for the analysis. Target quantification, which is currently making its way into commercial PCR tests, will also be added into M-PCR assays for a number of drugs as technology develops. The quantitative PCR applications are still developing.

Multiplex PCR has a few small flaws, but nothing major. Due to cheaper thermal cyclers, cost, which was once a key disadvantage, is now becoming less significant as more laboratories purchase equipment and competition becomes more significant in the market. The requirement for efficient anticontamination procedures and the length of time needed to develop and analyse novel assays are two additional drawbacks of multiplex PCR that are comparable to those of conventional PCR. These problems are somewhat due to less working knowledge on mPCR among the researchers and as each laboratory gains proficiency in PCR, these limitations will diminish in importance.

#### IV. CONCLUSION

PCR has completely changed how detection and diagnosis of plant pathogen is done. Multiplex PCRs for the detection of viral, bacterial, fungal and/or other infectious pathogens in one reaction tube have been created to overcome the inherent disadvantage of cost and to improve the diagnostic capabilities of the test. Numerous papers have been published about the use of multiplex PCR in the diagnosis of infectious agents, notably those which target viral nucleic acids, as a result of efforts to improve sensitivity and specificity as well as to facilitate automation. For practical reasons, it will become increasingly desirable to accomplish simultaneous detection of several microbial biotic agents that cause related or identical plant diseases and/or share comparable epidemiological aspects as the number of microbial agents detectable by PCR increases. The automation of DNA diagnosis techniques has a bright future.

## REFERENCES

- [1] Elnifro, E. M., Ashshi, A. M., Cooper, R. J., & Klapper, P. E. (2000). Multiplex PCR: optimization and application in diagnostic virology. *Clinical microbiology reviews*, 13(4), 559-570.
- [2] James B. Mahony, Max A. Chernesky, 10 - Multiplex Polymerase Chain Reaction, Editor(s): Danny L. Wiedbrauk, Daniel H. Farkas, *Molecular Methods for Virus Detection*, Academic Press, 1995, Pages 219-236, ISBN 9780127489209, <https://doi.org/10.1016/B978-012748920-9/50011-X>.
- [3] Marina N. Nikiforova, William A. LaFramboise, Yuri E. Nikiforov, Chapter 4 - Amplification-Based Methods, Editor(s): Shashikant Kulkarni, John Pfeifer, *Clinical Genomics*, Academic Press, 2015, Pages 57-67, ISBN 9780124047488, <https://doi.org/10.1016/B978-0-12-404748-8.00004-6>.
- [4] Markoulatos, P., Siafakas, N., & Moncany, M. (2002). Multiplex polymerase chain reaction: a practical approach. *Journal of clinical laboratory analysis*, 16(1), 47-51.
- [5] Palchoudhury, S., Khare, V. K., Balram, N., Bhattacharyya, U. K., Das, S., Shukla, P., Chakraborty, P. & Biswas, K. K. (2022). A multiplex polymerase chain reaction for the simultaneous detection of the virus and satellite components associated with cotton leaf curl begomovirus disease complex. *Journal of Virological Methods*, 300, 114369.