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Rapid Multiplex PCR for Species Authentication: Current Prospects and Challenges

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Abstract: Multiplex PCR is a powerful technique that allows simultaneous amplification of multiple DNA targets in a single reaction. It has been widely applied for species authentication in various food matrices, such as meat, fish, dairy, and honey products. Species authentication is important for ensuring food safety, quality, traceability, and compliance with religious, ethical, and environmental standards. However, multiplex PCR also faces some challenges and limitations, such as primer design, optimization, validation, interference, and interpretation of results. This review summarizes the current state of the art of multiplex PCR for species authentication, highlighting its advantages, applications, challenges, and future directions. It also provides some practical guidelines and recommendations for designing and performing multiplex PCR assays for reliable and accurate species identification.

Keywords: Multiplex PCR, Species authentication, Meat products; Species-specific DNA; Food fraud, Food quality.

Introduction

Food authentication is the process of verifying the identity, origin, and quality of food products and ingredients. It is essential for ensuring food safety, traceability, and compliance with religious, ethical, and dietary preferences of consumers (Safdar & Juenjo, 2015; Fanelli et al., 2021). However, food authentication is also challenging due to the complexity, diversity, and adulteration of food matrices, as well as the lack of standardized and reliable methods for detection and differentiation of food species (Safdar et al.,2013; Cichna Markl et al.,2023).

Among the various analytical techniques available for food authentication, multiplex PCR has emerged as a powerful and versatile tool that allows simultaneous amplification of multiple DNA targets in a single reaction. Multiplex PCR can provide rapid, sensitive, specific, and cost-effective identification of food species from raw and processed samples. It can also detect adulteration, substitution, or mislabeling of food products with undesired or prohibited animal species (Safdar et al.,2013; Fanelli et al., 2021).

However, multiplex PCR also faces several technical and practical challenges that limit its widespread application and acceptance in food authentication. These challenges include designing optimal primers, selecting appropriate annealing temperatures, balancing primer concentrations, avoiding primer-dimer formation, and ensuring specificity and sensitivity of the reaction. Moreover, multiplex PCR requires validation,

standardization, and harmonization of methods and protocols across different laboratories and regions (Ali et al., 2014;Ruslan et al., 2022).

In this review, we aim to provide a comprehensive overview of the current status and future prospects of multiplex PCR for food authentication. We will discuss the methods, and applications of multiplex PCR for species identification in various food matrices. We will also highlight the challenges and solutions of multiplex PCR for food authentication. Finally, we will suggest some directions for future research and development in this field.

Principles and Methods of Multiplex PCR

The principle of multiplex PCR is similar to that of conventional PCR, except that more than one pair of primers is added to the reaction mixture. Each primer pair anneals to a specific region of the template DNA and directs the synthesis of a complementary strand by a thermostable DNA polymerase. The resulting amplicons can be detected by various methods, such as gel electrophoresis, capillary electrophoresis, hybridization, or fluorescence (Markalatos et al., 2022; Perkel, 2012).

The methods of multiplex PCR vary depending on the type and number of targets, the choice of probes, and the detection system. Some of the common methods are:

- **TaqMan® probes:** These are hydrolysis probes that consist of an oligonucleotide with a fluorophore at the 5' end and a quencher at the 3' end. During PCR, the probe anneals to a specific target sequence between the primers. When the polymerase extends the primer and reaches the probe, it cleaves the probe and releases the fluorophore, which emits fluorescence. The fluorescence signal is proportional to the amount of target DNA. Different probes can be labeled with different fluorophores for multiplexing (Markalatos et al.,2022; Perkel, 2012).
- **Molecular beacons:** These are hairpin-shaped probes that have a fluorophore at one end and a quencher at the other end. In the absence of target DNA, the probe forms a stem-loop structure that brings the fluorophore and the quencher close together, preventing fluorescence. When the probe binds to a complementary target sequence during PCR, it opens up and separates the fluorophore from the quencher, allowing fluorescence. Different probes can be labeled with different fluorophores for multiplexing (Markalatos et al.,2022; Perkel, 2012).
- **FRET (fluorescence resonance energy transfer) probes:** These are pairs of probes that hybridize to adjacent regions of the target DNA during PCR. One probe is labeled with a donor fluorophore and the other with an acceptor fluorophore. When the probes are in close proximity, the donor fluorophore transfers energy to the acceptor fluorophore, which emits fluorescence at a different wavelength. The fluorescence signal indicates the presence of target DNA. Different pairs of probes can be labeled with different combinations of fluorophores for multiplexing (Markalatos et al., 2022; Perkel, 2012).
- **SYBR® Green:** This is a dye that binds to double-stranded DNA and fluoresces when excited by light. During PCR, as more amplicons are generated, more dye binds and more fluorescence is produced. The fluorescence signal is proportional to the amount of total DNA in the reaction. However, this method cannot distinguish between specific and nonspecific products, so it requires additional analysis such as melting curve analysis or gel electrophoresis to confirm the identity and size of the amplicons (Safdar et al., 2015).

Applications of Multiplex PCR for Species Authentication

Species authentication is the process of verifying the identity and origin of animal species in food products and ingredients. It is important for ensuring food safety, quality, traceability, and compliance with religious, ethical, and dietary preferences of consumers. It is also essential for preventing food fraud, adulteration, substitution, or mislabeling of food products with undesired or prohibited animal species (Safdar et al., 2013; Safdar & Juenjo, 2015). It is a valuable technique for species authentication, as it can provide rapid, sensitive, specific, and cost-effective identification of multiple animal species in a single reaction. Multiplex PCR can detect DNA from different animal species in various food matrices, such as raw and processed meat, fish, seafood, dairy, and honey products. Multiplex PCR can also discriminate between closely related species, such as sheep and goat, or chicken and duck.

Multiplex PCR has been applied successfully for species identification in foods of various origins, such as meat (Lopez Andreo et al.,2006; Izadpanah et al., 2018), fish , and seafood products (Santaclara et al.,2006; Safdar&

Juenjo, 2015; Vakshiteh et al.,2016). Some practical examples of multiplex PCR methods for species authentication are:

- A multiplex PCR method using TaqMan probes for the detection of sheep/goat, bovine, chicken, duck and pig DNA in meat products (Izadpanah et al.,2018). The method was able to detect 0.1% of adulteration in binary mixtures and 0.01% in ternary mixtures.
- A multiplex PCR method using molecular beacons for the detection of horse, donkey, beef, pork, chicken and turkey DNA in meat products (Lopez Andreo et al.,2006). The method was able to detect 0.01% of adulteration in binary mixtures and 0.001% in ternary mixtures.
- A multiplex PCR method using FRET probes for the detection of Atlantic salmon (Salmo salar), rainbow trout (Oncorhynchus mykiss), European sea bass (Dicentrarchus labrax) and gilthead sea bream (Sparus aurata) DNA in fish products. The method was able to detect 0.1% of adulteration in binary mixtures (Santaclara et al.,2006; Vakshiteh et al., 2016; Verez Bagnis et al.,2017).
- A multiplex PCR method using SYBR Green for the detection of shrimp (Penaeus monodon), crab (Scylla serrata), squid (Loligo duvauceli) and cuttlefish (Sepia pharaonis) DNA in seafood products. The method was able to detect 0.01% of adulteration in binary mixtures (Santaclara et al.,2006;Safdar& Juenjo, 2015; Vakshiteh et al., 2016).
- **Foodborne pathogen detection:** Multiplex PCR can be used to detect and differentiate various foodborne pathogens, such as bacteria, viruses, fungi, and parasites, in food samples. Multiplex PCR can provide rapid and accurate identification of the causative agents of foodborne diseases, such as Salmonella, Escherichia coli, Listeria, Campylobacter, Vibrio, Norovirus, Hepatitis A virus, Aspergillus, Fusarium, Cryptosporidium, and Giardia (Malorny et al., 2003; Wang et al.,2014;Wang et al., 2015). Multiplex PCR can also be used to detect antimicrobial resistance genes or virulence factors in foodborne pathogens (Wang et al.,2015).
- **Food allergen detection:** Multiplex PCR can be used to detect and quantify food allergens, such as peanuts, soybeans, milk, eggs, wheat, fish, shellfish, and nuts, in food products (Vakhshiteh et al.,2016). Multiplex PCR can provide sensitive and specific detection of food allergens to prevent allergic reactions in consumers. Multiplex PCR can also be used to detect cross-contamination or mislabeling of food products with allergenic ingredients (Holzhauser et al.,2009; Roder et al.,2011).
- **Food authenticity detection:** Multiplex PCR can be used to detect and verify the origin and quality of food products and ingredients. Multiplex PCR can provide reliable identification of animal species, such as meat, fish, seafood, dairy, and honey products (Vakhshiteh et al.,2016). Multiplex PCR can also be used to detect adulteration or substitution of food products with cheaper or inferior materials (Safdar& Juenjo,2015). Multiplex PCR can also be used to detect genetically modified organisms (GMOs) or organic products in food products (Holst Jensen et al.,2003; QIAGE,n.d.).
- **Food mycotoxin detection:** Multiplex PCR can be used to detect and quantify mycotoxins, such as aflatoxins, ochratoxins, fumonisins, trichothecenes, and zearalenone, in food products. Multiplex PCR can provide rapid and accurate detection of mycotoxins to prevent toxic effects in consumers. Multiplex PCR can also be used to detect the presence of toxigenic fungi or molds in food products (Li et al.,2011; Li et al.,2012).

Challenges and Limitations of Multiplex PCR

Optimization of reaction conditions: Multiplex PCR requires careful optimization of various parameters, such as primer design, primer concentration, annealing temperature, magnesium concentration, and cycle number, to achieve efficient and specific amplification of multiple targets in a single reaction. Optimization of multiplex PCR is often laborious, time-consuming, and empirical, and may require trial-and-error experiments or software programs to find the optimal conditions for each target. In addition, optimization of multiplex PCR may vary depending on the type and quality of the food sample, the presence of inhibitors or contaminants, and the detection system used (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Separation and detection of products: Multiplex PCR requires a reliable and sensitive method for separating and detecting the amplified products. Traditional methods, such as agarose gel electrophoresis or capillary electrophoresis, may not be suitable for separating multiplex PCR products that differ by only a few base pairs or have overlapping sizes. Moreover, these methods may not be able to quantify the products accurately or distinguish between specific and nonspecific products. Therefore, multiplex PCR often requires more advanced methods, such as real-time PCR with fluorescent probes or melting curve analysis, or high-resolution

electrophoresis with automated systems (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Quality control and validation: Multiplex PCR requires rigorous quality control and validation procedures to ensure the accuracy and reliability of the results. Quality control measures include using appropriate controls, such as positive controls, negative controls, internal controls, and external controls, to monitor the performance of the reaction and detect any errors or contamination. Validation procedures include verifying the specificity, sensitivity, reproducibility, robustness, and limit of detection of the multiplex PCR method for each target and each food matrix. Validation procedures may also require comparison with other methods or reference standards (Perkel,2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Strategies to Overcome the Challenges of Multiplex PCR

Strategies to overcome the challenges of multiplex PCR in food science are:

Primer design: Primer design is crucial for successful multiplex PCR, as it affects the specificity, sensitivity, and efficiency of the reaction. Primers should be designed to have similar melting temperatures, lengths, and GC contents, and to avoid complementarity or cross-hybridization with each other or with nonspecific regions of the template DNA. Primers should also be designed to amplify products of different sizes or with different fluorescent labels for easy separation and detection. Primer design can be facilitated by using software programs or databases that can check for primer properties and potential interactions (Perkel,2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Primer concentration: Primer concentration is another important factor for optimal multiplex PCR, as it affects the balance and yield of the products. Primer concentration should be adjusted empirically or using a universal primer concentration to ensure equal amplification of all targets. Primer concentration should also be optimized to avoid primer-dimer formation or nonspecific amplification. Primer concentration can be optimized by using gradient PCR or serial dilutions ((Perkel,2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Annealing temperature: Annealing temperature is a critical parameter for specific and efficient multiplex PCR, as it affects the hybridization of primers to their target sequences. Annealing temperature should be optimized to ensure optimal binding of all primers without compromising specificity or sensitivity. Annealing temperature can be optimized by using gradient PCR or empirical testing (Perkel,2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Magnesium concentration: Magnesium concentration is a key component for multiplex PCR, as it affects the activity and fidelity of the DNA polymerase. Magnesium concentration should be optimized to ensure optimal enzyme performance without causing nonspecific amplification or primer-dimer formation. Magnesium concentration can be optimized by using gradient PCR or serial dilutions (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Cycle number: Cycle number is a variable parameter for multiplex PCR, as it affects the quantity and quality of the products. Cycle number should be optimized to ensure sufficient amplification of all targets without causing saturation or degradation of the products. Cycle number can be optimized by using standard curves or empirical testing (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al.,2018).

Additives or enhancers: Additives or enhancers are optional components for multiplex PCR, as they can improve the reaction efficiency and specificity. Additives or enhancers can include synthetic additives, such as Factor MP (QIAGEN), that promote stable and efficient annealing of multiple primers; buffer components, such as betaine, DMSO, or formamide, that reduce secondary structure formation or increase primer melting; or enzyme stabilizers, such as BSA, that prevent enzyme inhibition or degradation(Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Quality control measures: Quality control measures are essential for reliable and accurate multiplex PCR results, as they can monitor the performance and validity of the reaction. Quality control measures can include using appropriate controls, such as positive controls, negative controls, internal controls, and external controls, to detect any errors or contamination; using validation procedures, such as verifying the specificity, sensitivity, reproducibility, robustness, and limit of detection of the multiplex PCR method for each target and each food

matrix; and using comparison methods or reference standards to confirm the results (Perkel, 2012; Multiplex PCR,n.d.; QIAGEN n.d.; Izadpanah et al., 2018).

Validation the Specificity and Sensitivity of a Multiplex PCR Assay

Some strategies to validate the specificity and sensitivity of a multiplex PCR assay for food allergen detection are:

- **Specificity:** Specificity is the ability of the multiplex PCR assay to detect only the intended food allergens and not other food components or contaminants. Specificity can be validated by testing the multiplex PCR assay with various food matrices, both positive and negative for the target allergens, as well as with potential cross-reactive or interfering substances, such as other allergens, plant or animal species, or DNA inhibitors. Specificity can also be validated by comparing the multiplex PCR assay with other methods, such as ELISA or mass spectrometry, or with reference standards, such as certified reference materials or proficiency testing samples (Garber et al.,2020; Waiblinger et al., 2022).
- **Sensitivity:** Sensitivity is the ability of the multiplex PCR assay to detect low levels of the target food allergens in food samples. Sensitivity can be validated by testing the multiplex PCR assay with serial dilutions of known concentrations of the target allergens in various food matrices, both raw and processed. Sensitivity can also be validated by determining the limit of detection (LOD) and the limit of quantification (LOQ) of the multiplex PCR assay for each target allergen in each food matrix. LOD and LOQ can be calculated by using statistical methods, such as probit analysis or linear regression, based on the signal-to-noise ratio or the standard deviation of the blank (Garber et al., 2020; Waiblinger et al., 2022).

Multiplex PCR for Simultaneous Detection of Multiple Foodborne Pathogens

We can use multiplex PCR for simultaneous detection of multiple foodborne pathogens in complex matrices by following these steps:

- **Selecting the target genes:** The target genes should be specific and conserved for each foodborne pathogen, and preferably located on the chromosome rather than on plasmids or phages. The target genes should also be related to the virulence or pathogenicity of the foodborne pathogens, such as toxin genes, invasion genes, or resistance genes (Garrido Maestu et al.,2019; Chen et al.,2021).
- **Designing the primers and probes:** The primers and probes should be designed to have similar melting temperatures, lengths, and GC contents, and to avoid cross-hybridization or primer-dimer formation. The primers and probes should also be designed to amplify products of different sizes or with different fluorescent labels for easy separation and detection. The primers and probes should be tested individually and in combination before multiplexing (Garrido Maestu et al.,2019; Chen et al.,2021).
- **Optimizing the reaction conditions:** The reaction conditions, such as primer concentration, annealing temperature, magnesium concentration, cycle number, and additives or enhancers, should be optimized to ensure efficient and specific amplification of all targets in a single reaction. The reaction conditions should also be optimized to overcome the inhibitory effects of complex matrices, such as food components or contaminants. The reaction conditions should be optimized by using gradient PCR, serial dilutions, standard curves, or empirical testing (Garrido Maestu et al.,2019; Chen et al.,2021).
- **Separating and detecting the products:** The products should be separated and detected by using a reliable and sensitive method, such as real-time PCR with fluorescent probes or melting curve analysis, or high-resolution electrophoresis with automated systems. The products should be quantified by using standard curves or internal controls. The products should also be verified by using quality control measures, such as positive controls, negative controls, external controls, or validation procedures (Garrido Maestu et al.,2019; Chen et al.,2021).

Future Perspectives and Recommendations

Increasing the multiplexing capacity: Multiplex PCR can be improved by increasing the number of targets that can be detected simultaneously in a single reaction. This can be achieved by using novel probe technologies, such as padlock probes, molecular inversion probes, or universal tag arrays, that can generate thousands of unique signals for different targets. Alternatively, multiplex PCR can be combined with other

techniques, such as microarrays, next-generation sequencing, or digital PCR, that can increase the throughput and resolution of the analysis (Mafra et al., 2008; Sint et al., 2012).

Enhancing the sensitivity and specificity: Multiplex PCR can be enhanced by increasing the sensitivity and specificity of the detection of low-abundance or closely related targets in complex matrices. This can be achieved by using novel amplification strategies, such as loop-mediated isothermal amplification, rolling circle amplification, or helicase-dependent amplification, that can amplify the target sequences without thermal cycling or primers. Alternatively, multiplex PCR can be combined with other techniques, such as immunomagnetic separation, aptamer-based capture, or CRISPR-Cas systems, that can enrich or edit the target sequences before or after amplification (Mafra et al.,2008; Sint et al., 2012).

Developing point-of-care devices: Multiplex PCR can be developed into point-of-care devices that can provide rapid and accurate detection of foodborne pathogens or allergens in situ. This can be achieved by using microfluidic platforms, such as lab-on-a-chip or paper-based devices, that can integrate sample preparation, amplification, and detection steps in a miniaturized and automated format. Alternatively, multiplex PCR can be combined with other techniques, such as lateral flow assays, electrochemical biosensors, or smartphone-based readers, that can provide simple and portable detection systems (Mafra et al.,2008; Sint et al., 2012).

Some ethical and social implications of using multiplex PCR for food safety are:

- **Consumer protection:** Multiplex PCR can provide consumer protection by ensuring the authenticity, quality, and safety of food products and ingredients. Multiplex PCR can prevent food fraud, adulteration, substitution, or mislabeling of food products with undesired or prohibited animal species, allergens, GMOs, or organic products. Multiplex PCR can also prevent foodborne diseases, allergic reactions, or ethical/religious violations by detecting foodborne pathogens or allergens in food products (Settani & Corsetti,2007; Ali et al.,2014).
- **Regulatory compliance:** Multiplex PCR can provide regulatory compliance by meeting the standards and requirements of food labeling and traceability. Multiplex PCR can provide accurate and reliable identification and quantification of food components or contaminants in various food matrices, both raw and processed. Multiplex PCR can also provide rapid and cost-effective detection of food components or contaminants in situ or in the field. Multiplex PCR can facilitate the enforcement of food regulations and the prevention of food fraud (Settani & Corsetti,2007).
- **Ethical responsibility:** Multiplex PCR can provide ethical responsibility by respecting the rights and preferences of consumers, producers, and animals. Multiplex PCR can inform consumers about the origin, quality, and safety of food products and ingredients, and allow them to make informed choices based on their dietary, ethical, or religious preferences. Multiplex PCR can also protect producers from unfair competition or false accusations by verifying the authenticity and quality of their products. Multiplex PCR can also reduce animal suffering by minimizing the use of animals for testing or experimentation (Settani & Corsetti,2007;Safdar& Juenjo,2015).

Conclusion

It is concluded that multiplex PCR is a powerful technique that can simultaneously amplify multiple target sequences in a single reaction, providing rapid, sensitive, and specific detection of various food components or contaminants. It has been widely applied in food analysis for species authentication, foodborne pathogen detection, food allergen detection, food authenticity detection, and food mycotoxin detection. However, it also faces some challenges, such as optimization of reaction conditions, separation and detection of products, quality control and validation of methods, and ethical and social implications of results. In addition, it can be improved by using novel probe technologies, amplification strategies, detection systems, microfluidic platforms, and point-of-care devices. Furthermore, it can also be combined with other techniques, such as microarrays, next-generation sequencing, digital PCR, immunomagnetic separation, aptamer-based capture, CRISPR-Cas systems, lateral flow assays, electrochemical biosensors, or smartphone-based readers. Finally, multiplex PCR has great potential for future applications in food safety and quality control.

Scientific Ethics Declaration

The author declares that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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