Streamlined ABO Genotyping with High-Speed Droplet Allele-Specific PCR

Background

ABO genotyping is used in many fields, including but not limited to forensic criminal investigation, victim identification in mass disasters, and medical diagnosis (Parthipan et al., 2022). Traditional PCR (polymerase chain reaction) techniques are relatively simple, but are expensive, labor-intensive, and time-consuming. The need to extract and purify DNA from samples is time-consuming and limits efficiency of genotyping (Lee et al., 2009). Access to rapid and reliable ABO genotyping is coveted, and many methods have been developed to attempt to accomplish this. These techniques involve PCR coupled with fragment length restriction (PCR-RFLP) (Tun et al., 1996), mutagenically separated polymerase chain reaction (MS-PCR)(Lee et al., 1996), methods that include nucleotide substitution present in certain alleles but not others (Olsson et al., 1995), and allele-specific PCR (Taira et al., 2018), as well as many others.

As new technology emerges in the field of microfluidics, droplet-based digital PCR (DDPCR) has become an increasingly sought after technology to implement in clinical diagnosis (Hou et al., 2023; Yin et al., 2021). The wide application of this simple, refined technology provides several advantages over its predecessor digital PCR technology. DDPCR offers higher target concentration, easier detection, less conflict with amplification efficiency and PCR inhibitors, and does not require digital calibration (Hou et al., 2023). This has led to the conception of the high-speed droplet allele-specific PCR (droplet-AS-PCR), which has many advantages over traditional PCR including very high accuracy, low sensitivity, and could be completed in under 10 minutes (Taira et al., 2018). As new technologies are created, AS-PCR may become more mainstream in clinical settings as specificity of SNP and ABO genotyping is improved. To improve the specificity of AS-PCR for SNP detection, potential enhancements

include designing allele-specific primers with additional mismatches, selecting allele-specific primers compatible with either the forward or reverse primer, implementing gel-free real-time AS-PCR analysis, and utilizing fluorescence probes (Parthipan et al., 2022). In addition, creating an approach that is able to amplify all alleles accurately would make AS-PCR a more reliable technique (Lee et al., 2009).

The traditional PCR has been reliable for years but as technology advances, droplet-AS-PCR extends those advantages (Nyaruaba et al., 2019;Quan at al., 2018). Starting with smaller reaction volume causing an increase in the concentration of the target alleles, which also leads to less detection elements required and the results having less influence from the thermal cycler amplification (Zhao et al., 2022). For blood samples, droplet-AS-PCR works by utilizing droplet PCR technology and referencing positive or negative amplification of blood group allele at certain single nucleotide polymorphism sites. There are many benefits to the advancements of PCR and would have a great impact on other fields of science as well (Borland and Kading, 2021).

Research Objectives

This project aims to develop and evaluate a streamlined genotyping approach that eliminates the need for purified DNA, providing a fast and reliable method for ABO blood group determination. We aim to improve AS-PCR SNP specificity and make AS-PCR ABO genotyping a more reliable technique. By validating the accuracy, reliability, and turnaround time of this novel genotyping method, the research will offer researchers a faster, more accessible, and cost-effective approach for ABO genotyping. This advancement can improve transfusion safety and enhance compatibility testing in transplantation medicine.

The research objective of “Rapid ABO genotyping by high-speed droplet allele-specific PCR using crude samples" by Chiaki Taira displays the development and assessment of an expedited approach for ABO genotyping utilizing droplet-AS-PCR. The issue at hand is that there needs to be a quicker more efficient method to determine the ABO genotype in crude samples such as blood or tissue. To address this issue, the article explores the use of droplet-AS-PCR along with SNP-specific primers and probes tailored for the ABO gene (Hou et al., 2023).

To ensure the success of the project, several considerations need to be taken into account. These include the availability of a diverse range of ABO genotype samples for the evaluation, including rare variants, optimization of the droplet-AS-PCR protocol, and the use of appropriate statistical analysis to validate the accuracy and reliability of the results. Additionally, collaboration with blood banks and clinical laboratories will be essential to validate the applicability and feasibility of the novel genotyping method in real-world settings.

The hypothesis of this study is that by optimizing high-speed droplet-AS-PCR through designing allele-specific primers and using fluorescence probes, a method for ABO genotyping using crude samples can be as reliable as traditional methods, while being more rapid as well as cost effective. The hypothesis behind high-speed droplet-AS-PCR is that it can accurately and rapidly detect specific genetic variations (alleles) in a given sample.

1. **Optimization of Droplet Digital PCR Parameters**

To optimize high-speed droplet-AS-PCR for ABO genotyping using crude samples we will fine-tune reaction conditions, including annealing temperature, primer concentration, and amplification cycles. Further statistical analysis of these parameters can be conducted by examining the impact of reaction conditions on resolution analysis in digital PCR, the resulting Poisson confidence limits in digital PCR, and the probability of error in relation to sample

compartmentalization (Lievens et al., 2016). These analyses have been utilized by preliminary studies in the efficiency of digital PCR. We will design allele-specific primers with additional mismatch and compatibility with either the common forward or reverse primer. Droplet generation will be optimized for size and concentration, ensuring optimal amplification efficiency. Amplification products will be detected and analyzed using ethidium bromide and SYBR Green staining (Lee et al., 2009; Parthipan et al., 2022).

2. **Assessment of Genotyping Accuracy and Reliability**

We will compare streamlined genotyping results with traditional methods using purified DNA samples. A diverse set of ABO genotypes, including rare variants, will be analyzed from consenting volunteers. Genomic DNA extraction from peripheral blood will be carried out using the QIAamp DNA Blood Mini Kit, followed by ABO genotype identification using conventional PCR (Taira et al., 2018). Comparisons will be made between genotypes from purified DNA and crude samples subjected to high-speed droplet-AS-PCR. To ensure the accuracy and reliability of the results, statistical analysis, including tests such as Fisher's exact or chi-square tests, will be conducted.

3. **Evaluation of Turnaround Time and Cost-effectiveness**

We will assess the turnaround time and cost-effectiveness of the streamlined genotyping approach by comparing it with traditional methods. Traditional PCR methods take between two and six hours to achieve results; the run times of the droplet-AS-PCR will be compared against this. As tests are run, the equipment expenses and cost of labor will be recorded and then compared to traditional PCR costs. This will involve analyzing a larger set of samples and calculating the time and cost savings achieved with the novel method.

The experiment will span three years and involve key milestones such as literature

review, primer design, and sample collection in the first year. The second year will focus on analyzing PCR products, comparing results with traditional methods, and refining the methodology. In the final year, there will be further improvements to the ABO genotyping method, broader sample testing, and data analysis for the preparation of a research paper.

Intellectual Merit & Impacts

In recent years, droplet PCR has earned its fair share of intellectual merit taking the advantages and applying them to various experiments. For example, it has earned merit in identifying high mortality disease such as sepsis by providing rapid accurate etiology diagnosis which is critical in the management of the sepsis (Lee et al., 2011). The knowledge provided by the accuracy and rapidness of the droplet PCR is helping sepsis cases and plenty of other disease related conditions that require fast therapy. Rapid ABO genotyping by droplet-AS-PCR can be used as a non-invasive method genetic fetal screening (Parthipan et al., 2022; Muro et al., 2012), as well as in disease genotyping and diagnosis (Parthipan et al., 2022), and can also be used in the process of identifying missing individuals and victims of criminal activities and catastrophic events (Muro et al., 2012).

Successful preliminary testing and cost reduction efforts can enable wide-scale adoption of droplet-AS-PCR technology for more efficient genotyping in the medical field. This advancement in genotyping, coupled with increased speed and reliability, can lead to automation in ABO genotyping (Muro et al., 2012), benefiting medical professionals in early disease detection and decreasing the burden on overcrowded clinics. Rapid ABO genotyping can also be highly valuable in large-scale disasters, aiding first-responders in identifying bodies when only degraded or low-quality DNA samples are available (Parthipan et al., 2022).

References

Borland EM, Kading RC. Modernizing the toolkit for arthropod bloodmeal identification. *Insects*. 2021;12(1):37.

This literature review examines the significant progress made in the past decade in molecular methods for identifying the vertebrate host of blood-engorged arthropods in field collections. The review discusses advancements in diagnostic markers, techniques, and technologies, highlighting the potential for rapid and accurate blood meal source identification in biomedical research.

Hou Y, Chen S, Zheng Y, Zheng X, Lin JM. Droplet-based digital PCR (Ddpcr) and its applications. *TrAC Trends in Analytical Chemistry*. 2023;158:116897.

The integration and miniaturization of droplet-based digital PCR equipment is based in microfluidics. Droplet-based digital PCR is a promising new technology in PCR that has abilities to detect trace nucleic acids present in complex backgrounds. This new technique shows how the field of genotyping using PCR is continuously developing and gives insight into new technologies.

Lee JC, Tsai LC, Chen CH, Chang JG. ABO genotyping by mutagenically separated polymerase chain reaction. *Forensic Sci Int*. 1996;82(3):227-232.

This research introduces a simplified approach for ABO genotyping, called mutagenically separated polymerase chain reaction (MS-PCR), which involves two sets of PCR reactions targeting specific nucleotide positions to differentiate A, B, and O alleles. The genotypes are then determined by comparing the PCR products obtained.

Lee HY, Park MJ, Kim NY, Yang WI, Shin KJ. Rapid direct PCR for ABO blood typing. *J Forensic Sci*. 2011;56 Suppl 1:S179-182.

The primary aim of this study is to develop an innovative method for ABO genotyping using crude samples, eliminating the need for DNA extraction. This research contributes valuable insights into PCR instruments, optimized polymerases, and allele-specific primers, with the potential to reduce run times.

Lee SH, Park G, Yang YG, Lee SG, Kim SW. Rapid ABO genotyping using whole blood without DNA purification. *Korean J Lab Med*. 2009;29(3):231-237.

Background on PCR genotyping in ABO genotyping and usage of crude samples, as a precursor to digital droplet PCR.

Lievens A, Jacchia S, Kagkli D, Savini C, Querci M. Measuring digital pcr quality: performance parameters and their optimization. *PLoS One*. 2016;11(5):e0153317.

A previous study that has a standardized approach for determining accuracy and ideal parameters for digital PCR technology. This analysis can be utilized to provide accuracy and preferred conditions for allele specific ABO genotyping.

Muro T, Fujihara J, Imamura S, et al. Determination of ABO genotypes by real-time PCR using allele-specific primers. *Leg Med (Tokyo)*. 2012;14(1):47-50.

This study primarily investigated the rapid ABO genotyping technique utilizing real-time PCR and allele-specific primers. The findings provide valuable insights into streamlining ABO genotyping methods and highlight essential SNPs for accurate ABO genotyping.

Nyaruaba R, Mwaliko C, Kering KK, Wei H. Droplet digital PCR applications in the tuberculosis world. *Tuberculosis (Edinb)*. 2019;117:85-92.

This article goes into the third generation of ddPCR and how it accurately detects and quantifies low abundant targets and does so by applying the technology to tuberculosis diagnosis. The technique offers a significant amount of advantages from high precision and high accuracy in real time.

Olsson ML, Chester MA. A rapid and simple ABO genotype screening method using a novel B/O2 versus A/O2 discriminating nucleotide substitution at the ABO locus. *Vox Sang*. 1995;69(3):242-247.

A newly identified nucleotide substitution (G1096A) exclusively present in B and O2 alleles was employed in the development of an innovative ABO genotype screening method. This technique utilizes a rapid and dependable single-tube approach, incorporating multiplex PCR and restriction enzymes. Validation on a Swedish blood donor population of 150 individuals proved its cost-effectiveness and informational superiority, establishing it as the most efficient ABO genotyping method available.

Parthipan S, Sandrasagran M, Ishar S. A review of mitochondrial snp determination using allele-specific pcr in forensic identification. *Sains Malaysiana*. Published online 2022.

The different fields in which ABO genotyping is utilized. This article compares different allele-specific PCR techniques utilized in research from 2010-2022. Information found in this publication gives valuable insight into different techniques that have already been tested, as well as the advantages each has over others. This collection of results and methods provides variables that may be changed, combined, and tested in order to further streamline run times and decrease cost of rapid ABO genotyping.

Quan PL, Sauzade M, Brouzes E. Dpcr: a technology review. *Sensors (Basel)*. 2018;18(4):1271.

Here in this study, it goes into the fundamental differences between droplet PCR versus traditional PCR. Comparing the results to see which technique has the better advantage. Using both techniques in the analysis of quantification of nucleic acids.

Taira C, Matsuda K, Takeichi N, et al. Rapid ABO genotyping by high-speed droplet allele-specific PCR using crude samples. *J Clin Lab Anal*. 2018;32(1):e22196.

A background on allele specific rapid ABO genotyping. This is the prerequisite research that established the technique and provided base levels of efficient parameters in which we are basing our study.

Tun Z, Honda K, Nakatome M, et al. Rapid and clear detection of ABO genotypes by simultaneous PCR-RFLP method. *J Forensic Sci*. 1996;41(6):1027-1030.

Background on PCR with restriction of fragment length as a comparative and precursor technology to digital PCR.

Yin H, Wu Z, Shi N, et al. Ultrafast multiplexed detection of SARS-CoV-2 RNA using a rapid droplet digital PCR system. *Biosens Bioelectron*. 2021;188:113282.

In this study, they go into depth of combining droplet digital and rapid PCR techniques for the detection of SARS-CoV-2 RNA. Goes into detecting specific targets and reference genes.

Also demonstrates how a rapid digital PCR system gives a consistent accurate result with the use of low concentrated samples to pull from.

Zhao Y, Lin K, Zhang H, et al. Evaluation of droplet digital PCR rapid detection method and precise diagnosis and treatment for suspected sepsis (Progress): a study protocol for a multi-center pragmatic randomized controlled trial. *BMC Infect Dis*. 2022;22(1):630.

The PROGRESS trial aims to assess the efficacy of a novel diagnostic tool, known as digital droplet PCR (ddPCR), in the diagnosis of sepsis. The trial will compare the outcomes of patients assigned to the ddPCR group with those assigned to the control group. This study will provide important information on the usefulness of ddPCR in sepsis diagnosis.