Aqueous Two-Phase Systems for the Extraction of Cell-Free DNA from Plasma

Process Development and Application in Liquid Biopsy

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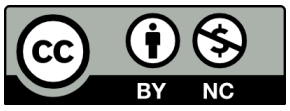
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„Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.“

Marie Skłodowska Curie

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Abstract

Molecular diagnostics based on circulating biomarkers have become increasingly important in precision medicine, especially in oncology, where there is an ongoing need for minimally invasive methods of disease detection and monitoring. In this context, liquid biopsy has gained significant attention as a technique that enables analysis of tumor-related material directly from body fluids. Unlike conventional tissue biopsies, it allows repeated sampling and facilitates the monitoring of disease progression and treatment response over time. Among the accessible analytes, circulating cell-free DNA (cfDNA) is particularly relevant, as it reflects tumor-associated genetic alterations and supports various clinical applications, including early diagnosis, therapeutic decision-making, and longitudinal disease monitoring. In addition, its rapid turnover in circulation provides a dynamic snapshot of disease status, making it suitable for real-time clinical assessment.

However, the analytical use of cfDNA is associated with several challenges. The concentration of cfDNA in plasma is typically low, rarely exceeding a few nanograms per milliliter, and varies by cancer type and stage. Furthermore, cfDNA consists of short, fragmented molecules with a characteristic length of approximately 167 bp and often exhibits a nucleosomal structure. These molecules are found within a complex biological environment with a high abundance of proteins, as well as high-molecular-weight genomic DNA (gDNA) potentially released during blood processing. These factors complicate sample preparation for sequencing-based analytical methods, such as droplet digital PCR and next-generation sequencing (NGS), which require highly efficient and selective extraction techniques. Therefore, the isolation of cfDNA is considered a crucial step in the pre-analytical phase of liquid biopsy procedures.

Current state-of-the-art cfDNA extraction predominantly relies on solid-phase adsorption to silica membranes or functionalized magnetic beads using strong chaotropes. Although these methods are widely adopted for their robustness and high purity, they present several drawbacks. Most kits involve time-consuming bind-wash-elute protocols with repeated manual handling and vacuum or centrifugation steps, which can increase the risk of sample loss and cross-contamination. Automated platforms can mitigate these risks and improve throughput and ease of use, but high reagent costs and the need for specialized equipment remain limitations that may restrict flexibility and scalability. Furthermore, incomplete adsorption or elution and contamination with gDNA can reduce the detection of rare tumor-derived variants. Short DNA fragments may also be recovered less efficiently, introducing a size bias that can affect downstream analysis. These issues highlight the need for alternative cfDNA extraction methods that can simplify workflows while maintaining high performance.

Aqueous two-phase systems (ATPS) represent a promising liquid–liquid extraction method for biomolecule separation and purification. These systems consist of two immiscible aqueous phases formed by mixing polymers and salts above a critical concentration threshold, within which biomolecules distribute according to their physicochemical properties. This enables selective separation without the need for denaturing agents. Besides their mild operating conditions and cost-effectiveness, ATPS offer advantages in scalability and process simplicity, as phase separation can be achieved quickly by centrifugation. Although ATPS are well established for a variety of biomolecules, including proteins and nucleic acids, their application to short DNA fragments relevant to liquid biopsy has not been systematically studied. In particular, the partitioning behavior of cfDNA-like fragments in polymer–salt systems remains poorly understood, especially in the presence of plasma, a complex biological matrix that may influence phase properties. Furthermore, the integration of ATPS capture into a complete extraction workflow compatible with downstream molecular analyses has only been superficially addressed to date. Therefore, linking partitioning behavior to process design is essential for the development of an efficient ATPS-based separation strategy.

Supported by relevant literature, Chapter 1 offers a comprehensive overview of the theoretical background, current research status, and remaining challenges within the field. As outlined in Chapter 2, this thesis presents the development of an ATPS-based extraction workflow for cfDNA-like fragments from plasma, integrating fundamental insights into phase partitioning with a practical, analytically compatible process. The three presented studies progress from investigations in well-defined model systems to application in a complex biological matrix and, ultimately, the creation of an integrated workflow. To enable systematic investigation, PCR-generated 160 bp DNA fragments were used as cfDNA-like surrogates in most experiments. The results provide new insights into the partitioning behavior of short DNA fragments in ATPS and demonstrate that the developed process yields sequencing-compatible DNA.

The first study (Chapter 3) examined the partitioning behavior of 160 bp DNA fragments in well-defined polymer–salt ATPS, addressing the limited availability of systematic data for molecules in the size range relevant to cfDNA. Considering the complex interaction of factors affecting solute distribution, a comprehensive experimental approach was used to investigate how key system parameters, including polymer molecular weight, salt type, tie-line length, phase volume ratio, and the addition of neutral salts, influence DNA partitioning and recovery. High-throughput screening with a liquid-handling platform and automated routines for binodal and tie-line determination enabled efficient testing of many system compositions. The results revealed consistent trends in DNA distribution and identified conditions that allowed high recovery. More generally, it was demonstrated that short DNA behaves similarly to plasmid DNA in PEG/phosphate ATPS, with overall partitioning trends mainly driven by PEG molecular weight. For example, DNA fragments were primarily found in the top phase when PEG was below 600 g/mol, whereas they accumulated in the bottom phase with PEG 600 and 1000. Additionally, it was confirmed that adding a neutral salt, such as NaCl, to a PEG 400 system shifted the DNA distribution from the top to the bottom phase. Preliminary experiments in a simplified synthetic plasma matrix containing human serum albumin and immunoglobulin G showed that partitioning behavior could, to some extent, be transferred to more complex systems. These experiments also indicated that the NaCl-induced phase shift is selective for DNA, achieving 88% recovery while removing 99% of proteins. These findings improved the understanding of short DNA partitioning in ATPS and laid the groundwork for selecting optimal conditions for future extraction studies.

Building on this foundation, the second study (Chapter 4) investigated the use of ATPS to capture cfDNA-like fragments from human plasma. Unlike the model systems described in Chapter 3, the presence of proteins and other plasma components introduces additional interactions that can affect phase behavior and solute partitioning. Therefore, the study assessed the recovery and distribution of these fragments, as well as phase formation, in plasma-containing PEG/phosphate ATPS. The selective NaCl-induced shift observed in PEG 400 systems proved less effective due to the formation of an interphase consisting of reversibly aggregated plasma proteins present in excess. Consequently, PEG 1000 ATPS were preferred, enabling DNA recoveries of up to 90% in the salt-rich bottom phase while reducing protein concentrations from 60–80 mg/mL in plasma to below 3 mg/mL, corresponding to over 95% removal. This study also confirmed the robustness of the ATPS capture step across plasma samples from different donors and collection tubes, both of which introduce variability. To address practical limitations caused by sample dilution from phase-forming components pre-dissolved in water, a system was designed in which solid PEG and salts were added directly to the plasma. This modification increased the plasma input from 38% (w/w) to 67% (w/w) without affecting DNA or protein partitioning. The scalability of this approach was verified by processing up to 4 mL of plasma at once, which is equivalent to the volume obtainable from a single blood tube. Additionally, reverse elution was implemented as a purification step to desalt and increase protein removal to above 99%, while reducing flow-through volume and thereby concentrating the DNA-rich extract, ultimately improving suitability for downstream sequencing-based analyses. The results confirmed that DNA can be efficiently recovered from plasma while separating it from the main protein fraction, yielding extracts with substantially reduced matrix complexity. This work established key process elements required for an integrated workflow and provides a basis for further process optimization.

The third study (Chapter 5) focused on integrating individual process steps into a cohesive, optimized ATPS-based extraction workflow and evaluating its compatibility with downstream analyses. First, the effect of different lysis methods on DNA recovery and protein removal was examined. The results indicated that a lysis step might not be necessary, as the high salt content of the DNA-containing bottom phase may be sufficient to separate DNA from most proteins and possibly dissociate the nucleosomal complex, streamlining the entire process. The system composition was then optimized to increase DNA concentration while minimizing losses by adjusting the phase volume ratio and tie-line length. In addition, the potential of serial ATPS steps for further enrichment was evaluated. Although each ATPS step increased DNA concentration and allowed additional plasma to be added to the previous bottom phase, it also led to cumulative DNA losses, highlighting a trade-off between concentration and overall yield. Practical process parameters such as centrifugation time, mixing temperature, PEG form, and tube filling ratio were evaluated and carefully optimized for simplicity, speed, and robustness, revealing that proper mixing and complete dissolution of the components before phase separation are key to reproducible DNA recovery. The performance of the developed workflow was evaluated by its ability to recover a wide range of input DNA, with as little as 2 ng/mL input detectable by quantitative PCR, even in a 1000-fold-diluted extract. Fragment-size selectivity and potential PCR inhibition were tested and compared with a widely used adsorption-based extraction method. The extracts showed no signs of inhibition and exhibited a size cutoff of ∼750 bp, indicating selective enrichment of shorter fragments. This feature may benefit liquid biopsy applications by separating contaminating high-molecular-weight gDNA from the cfDNA fraction. Although the total yield of 65% sequencing-ready DNA was slightly lower than that of the reference adsorption-based kit, the ATPS workflow significantly shortened processing time to approximately 10 minutes while reducing equipment requirements and costs.

Finally, the applicability of the extraction process was demonstrated by isolating cfDNA reference material from DNA-free plasma and subjecting it to NGS analysis. The successful generation of a sequencing library and the detection of the spiked mutations confirmed that the extracted DNA meets the requirements of downstream molecular assays, thereby providing proof of concept for a simplified ATPS-based cfDNA extraction approach.

In summary, this thesis presents a systematic study of ATPS for extracting cfDNA-like fragments from plasma, combining mechanistic research with process development and evaluation. The results indicate that ATPS are an effective and simple platform for capturing and concentrating these fragments. Additionally, this work offers new insights into the factors governing short DNA partitioning in polymer–salt systems, particularly in the presence of a complex biological matrix. Although further studies with clinical samples are needed to fully assess its applicability in a diagnostic setting, the presented approach establishes the foundation of an alternative cfDNA extraction method. Overall, these findings highlight the potential of liquid–liquid extraction strategies to complement existing methods and support the development of more efficient and scalable sample preparation workflows for liquid biopsy applications.

Zusammenfassung

Die auf zirkulierenden Biomarkern basierende Molekulardiagnostik gewinnt in der Präzisionsmedizin zunehmend an Bedeutung, insbesondere in der Onkologie, wo ein anhaltender Bedarf an minimalinvasiven Verfahren zur Erkennung und Überwachung von Erkrankungen besteht. In diesem Zusammenhang hat sich die Flüssigbiopsie als vielversprechender Ansatz etabliert, da sie die Analyse tumorassoziierten Materials direkt aus Körperflüssigkeiten ermöglicht. Im Gegensatz zu konventionellen Gewebebiopsien unterstützt sie wiederholte Probenahmen und eröffnet damit die Möglichkeit, Krankheitsverläufe sowie Therapieansprechen über die Zeit hinweg zu verfolgen. Unter den verfügbaren Analyten nimmt die zirkulierende zellfreie DNA (cfDNA) eine zentrale Rolle ein, da sie tumorassoziierte genetische Veränderungen widerspiegelt und für verschiedene klinische Anwendungen wie Früherkennung, Therapieauswahl und Verlaufskontrolle genutzt werden kann. Darüber hinaus liefert der schnelle Kreislaufumsatz eine dynamische Momentaufnahme des Krankheitsstatus, wodurch sich cfDNA zur klinischen Beurteilung in Echtzeit eignet.

Die analytische Nutzung von cfDNA ist jedoch mit mehreren Herausforderungen verbunden. Die Konzentration von cfDNA im Plasma ist in der Regel gering, nur selten wenige Nanogramm pro Milliliter überschreitend, und variiert je nach Krebsart und -stadium. Gleichzeitig liegt cfDNA überwiegend in Form kurzer, stark fragmentierter Moleküle mit einer charakteristischen Länge von etwa 167 bp vor, die häufig eine nukleosomale Struktur aufweisen. Diese Fragmente befinden sich in einer komplexen biologischen Matrix mit hohem Proteingehalt sowie potenziell vorhandener hochmolekularer genomischer DNA (gDNA), die während der Probenverarbeitung freigesetzt werden kann. Diese Eigenschaften stellen hohe Anforderungen an die Probenvorbereitung, insbesondere für sequenzierungsbasierte Analysemethoden wie Droplet Digital PCR und Next-Generation Sequencing (NGS), die eine effiziente und selektive Aufreinigung der Zielmoleküle erfordern. Entsprechend gilt die Extraktion von cfDNA als entscheidender Schritt in der präanalytischen Phase der Flüssigbiopsie.

Der derzeitige Stand der Technik für die cfDNA-Extraktion basiert überwiegend auf der Festphasenadsorption an Silikamembranen oder funktionalisierten Magnetpartikeln unter Verwendung chaotroper Salze. Obwohl diese Methoden aufgrund ihrer Robustheit und hohen Reinheit weit verbreitet sind, weisen sie mehrere Einschränkungen auf. Die meisten kommerziellen Kits erfordern zeitaufwändige Protokolle mit Bindungs-, Wasch- und Elutionsschritten sowie wiederholten manuellen Eingriffen und Vakuum- oder Zentrifugationsschritten, was das Risiko von Probeverlusten und Kreuzkontaminationen erhöhen kann. Automatisierte Plattformen können diese Risiken reduzieren und den Durchsatz sowie die Anwenderfreundlichkeit verbessern; jedoch stellen hohe Reagenzienkosten und der Bedarf an spezialisierter Ausstattung weiterhin limitierende Faktoren dar, die Flexibilität

und Skalierbarkeit einschränken. Darüber hinaus können unvollständige Adsorption oder Elution sowie Kontamination mit gDNA den Nachweis seltener tumorabgeleiteter Varianten beeinträchtigen. Insbesondere kurze DNA-Fragmente werden häufig mit geringerer Effizienz wiedergewonnen, was zu einer Fragmentlängenverzerrung führen und die nachgelagerte Analyse beeinflussen kann. Insgesamt verdeutlichen diese Aspekte den Bedarf an alternativen Strategien zur cfDNA-Extraktion, die einfachere Prozessabläufe begünstigen und zugleich eine hohe Leistungsfähigkeit gewährleisten.

Wässrige Zweiphasensysteme (ATPS) stellen ein vielversprechendes Flüssig-Flüssig-Extraktionsverfahren zur Trennung und Aufreinigung von Biomolekülen dar. Diese Systeme bestehen aus zwei nicht mischbaren, wässrigen Phasen, die durch das Mischen von Polymeren und Salzen oberhalb einer kritischen Konzentrationsschwelle gebildet werden. Dabei verteilen sich Biomoleküle entsprechend ihren physikochemischen Eigenschaften zwischen den Phasen, was eine selektive Trennung ohne den Einsatz denaturierender Agenzien ermöglicht. Neben den milden und kostengünstigen Prozessbedingungen bieten ATPS Vorteile hinsichtlich der Skalierbarkeit und der Prozessvereinfachung, da die Phasentrennung durch Zentrifugation schnell erfolgen kann. Obwohl ATPS für die Aufreinigung verschiedenster Biomoleküle, darunter Proteine und Nukleinsäuren, etabliert sind, ist ihre Anwendung auf kurze DNA-Fragmente im Kontext der Flüssigbiopsie bislang nur begrenzt untersucht. Insbesondere ist das Verteilungsverhalten cfDNA-ähnlicher Fragmente in Polymer-Salz-Systemen noch nicht umfassend verstanden, vor allem unter dem Einfluss von Plasma, einer komplexen biologischen Matrix, die das Phasenverhalten und die Verteilung der Komponenten maßgeblich beeinflussen kann. Die Integration von ATPS als Capture- und Konzentrationsschritt in einen vollständigen Extraktionsworkflow, der mit nachgelagerten molekularen Analysen kompatibel ist, ist nach wie vor von deutlichen Forschungslücken geprägt. Daher ist die Verknüpfung des Verteilungsverhaltens mit der Prozessgestaltung für die Entwicklung einer effizienten ATPS-basierten Trennungsstrategie essenziell.

Gestützt auf die maßgebliche Fachliteratur bietet Kapitel 1 einen umfassenden Überblick über den theoretischen Hintergrund, den aktuellen Stand sowie die verbleibenden Herausforderungen in diesem Forschungsfeld. Wie in Kapitel 2 dargelegt, befasst sich diese Arbeit mit der Entwicklung eines ATPS-basierten Extraktionsworkflows für cfDNA-ähnliche Fragmente aus Plasma, wobei grundlegende Erkenntnisse zur Phasentrennung in einen praktisch umsetzbaren und analytisch kompatiblen Prozess überführt werden. Die drei vorgestellten Studien bauen aufeinander auf und reichen von Untersuchungen in definierten Modellsystemen über die Anwendung in einer komplexen biologischen Matrix bis hin zur Entwicklung eines integrierten Workflows. Um eine systematische Untersuchung zu ermöglichen, werden im Rahmen dieser Arbeit PCR-generierte 160 bp DNA-Fragmente als cfDNA-ähnliche Modellfragmente eingesetzt. Die erzielten Ergebnisse liefern neue Einblicke in das Verteilungsverhalten kurzer DNA-Fragmente in ATPS und zeigen, dass mit dem entwickelten Verfahren die Gewinnung sequenzierbarer DNA möglich ist.

Die erste Studie (Kapitel 3) untersuchte das Verteilungsverhalten von 160 bp DNA-Fragmenten in definierten Polymer-Salz-ATPS und adressierte damit den Mangel an systematischen Daten zu Molekülen im für cfDNA relevanten Größenbereich. Angesichts der komplexen Wechselwirkungen zwischen den Einflussfaktoren auf die Molekülverteilung in ATPS wurde ein umfassender experimenteller Ansatz gewählt, um die Auswirkungen zentraler Systemparameter wie des Molekulargewichts des Polymers, der Salzart, der Konodenlänge, des Phasenvolumenverhältnisses sowie der Zugabe neutraler Salze auf DNA-Verteilung und -Rückgewinnung zu untersuchen. Ein Hochdurchsatz-Screening mithilfe einer Liquid-Handling-Plattform und automatisierter Routinen zur Bestimmung von Binodalen und Konoden bildete die Grundlage für die effiziente Untersuchung einer Vielzahl von Systemzusammensetzungen. Die Ergebnisse zeigten konsistente Trends in der DNA-Verteilung und

identifizierten Bedingungen, die eine hohe Rückgewinnung ermöglichen. Insgesamt wurde gezeigt, dass sich kurze DNA in PEG/Phosphat-ATPS ähnlich wie Plasmid-DNA verhält, wobei die Verteilung maßgeblich vom Molekulargewicht des PEGs beeinflusst wird. So wurden DNA-Fragmente überwiegend in der Oberphase gefunden, wenn PEG ein Molekulargewicht unter 600 g/mol aufwies, während sie sich bei PEG 600 und 1000 bevorzugt in der Unterphase anreicherten. Darüber hinaus wurde bestätigt, dass die Zugabe eines neutralen Salzes wie NaCl zu einem PEG 400-System die DNA-Verteilung von der oberen in die untere Phase verschiebt. Vorläufige Experimente in einer vereinfachten synthetischen Plasmamatrix, bestehend aus Humanserumalbumin und Immunoglobulin G, zeigten, dass sich die beobachteten Verteilungstrends zumindest teilweise auf komplexere Systeme übertragen lassen. Zudem wurde gezeigt, dass die durch NaCl induzierte Phasenverschiebung selektiv für DNA ist, wobei Rückgewinnungen von bis zu 88 % sowie eine gleichzeitige Entfernung von etwa 99 % der Proteine erzielt werden konnten. Diese Ergebnisse tragen zu einem verbesserten Verständnis des Verteilungsverhaltens kurzer DNA in ATPS bei und bilden die Grundlage für die gezielte Auswahl geeigneter Systembedingungen in weiterführenden Extraktionsstudien.

Aufbauend auf diesen Ergebnissen untersuchte die zweite Studie (Kapitel 4) die Anwendung von ATPS zur Capture von cfDNA-ähnlichen Fragmenten aus menschlichem Plasma. Im Gegensatz zu den in Kapitel 3 beschriebenen Modellsystemen führt das Vorhandensein von Proteinen und weiteren Plasmakomponenten zu zusätzlichen Wechselwirkungen, die sowohl das Phasenverhalten als auch die Molekülverteilung beeinflussen können. Entsprechend wurden die DNA-Ausbeute und -Verteilung sowie die Phasenbildung in PEG/Phosphat-ATPS mit Plasma untersucht. Die in PEG 400-Systemen beobachtete NaCl-induzierte DNA-Verschiebung erwies sich als weniger effektiv, da sich eine Interphase aus reversibel aggregierten, im Überschuss vorliegenden Plasmaproteinen bildete. Daher wurden PEG 1000-ATPS bevorzugt, da sie DNA-Ausbeuten von bis zu 90 % in der salzreichen Unterphase ermöglichten und gleichzeitig die Proteinkonzentration von etwa 60–80 mg/mL in Plasma auf unter 3 mg/mL reduzierten, einer Abreicherung von über 95 % entsprechend. Darüber hinaus bestätigte die Studie die Robustheit des ATPS-basierten Capture-Schritts gegenüber Variationen in Plasmaproben verschiedener Spender und Entnahmeröhrchen. Zur Vermeidung der Probenverdünnung durch vorab gelöste Phasenbildner wurde ein Systemdesign entwickelt, bei dem festes PEG und Salze direkt dem Plasma zugegeben wurden. Dadurch konnte der Plasmaanteil von 38 % (w/w) auf 67 % (w/w) erhöht werden, ohne die Verteilung von DNA oder Proteinen signifikant zu beeinflussen. Die Skalierbarkeit dieses Ansatzes wurde durch die Verarbeitung von bis zu 4 mL Plasma auf einmal demonstriert, was dem verfügbaren Volumen aus einem Blutröhrchen entspricht. Ergänzend wurde ein Aufreinigungsschritt auf Basis der Reverse Elution implementiert, um die DNA-haltige Phase zu entsalzen, die Proteinabreicherung auf über 99 % zu erhöhen und gleichzeitig das Extraktvolumen zu verringern. Dadurch wurde die DNA effektiv konzentriert und die Eignung der Extrakte für nachfolgende sequenzierungsbasierte Analysen verbessert. Insgesamt zeigen die Ergebnisse, dass DNA effizient aus Plasma gewonnen und zugleich von der Hauptproteinfraktion getrennt werden kann, wodurch Extrakte mit deutlich reduzierter Matrixkomplexität erhalten werden. Damit wurden zentrale Prozesselemente für einen integrierten Workflow etabliert und eine Grundlage für weiterführende Prozessoptimierungen geschaffen.

Die dritte Studie (Kapitel 5) konzentrierte sich auf die Integration der einzelnen Prozessschritte in einen zusammenhängenden und optimierten ATPS-basierten Extraktionsworkflow sowie auf dessen Bewertung hinsichtlich der Kompatibilität mit nachgelagerten Analysen. Zunächst wurde der Einfluss verschiedener Lysemethoden auf die DNA-Ausbeute und die Proteinentfernung untersucht. Die Ergebnisse deuteten darauf hin, dass ein zusätzlicher Lyse-Schritt möglicherweise nicht erforderlich ist, da der hohe Salzgehalt der DNA-haltigen Unterphase ausreicht, um DNA von

einem Großteil der Proteine zu trennen und den Nukleosomenkomplex potenziell zu dissoziieren. Dies würde die Voraussetzung schaffen, den Gesamtprozess zu kürzen und zu vereinfachen. Im weiteren Verlauf wurde die Systemzusammensetzung gezielt optimiert, indem das Phasenvolumenverhältnis und die Konodenlänge angepasst wurden, um die DNA-Konzentration zu erhöhen und gleichzeitig Verluste zu minimieren. Darüber hinaus wurde das Potenzial serieller ATPS-Schritte zur weiteren Anreicherung untersucht. Zwar erhöhte jeder ATPS-Schritt die DNA-Konzentration und ermöglichte die Zugabe weiterer Plasmamengen zur bestehenden Unterphase, jedoch traten dabei auch kumulative Verluste auf, die einen Zielkonflikt zwischen Konzentration und Gesamtausbeute verdeutlichen. Neben der Systemzusammensetzung wurden auch praxisrelevante Prozessparameter wie Zentrifugationszeit, Mischtemperatur, PEG-Form sowie Füllgrad der Reaktionsgefäße systematisch untersucht und hinsichtlich Einfachheit, Geschwindigkeit und Robustheit optimiert. Dabei zeigte sich, dass insbesondere eine ausreichende Durchmischung und die vollständige Auflösung der Phasenbildner vor der Phasentrennung entscheidend für eine reproduzierbare DNA-Rückgewinnung sind. Die Leistungsfähigkeit des entwickelten Workflows wurde anhand seiner Fähigkeit bewertet, ein breites Spektrum an DNA-Ausgangskonzentrationen zuverlässig zu extrahieren. Auch bei einer Ausgangskonzentration von 2 ng/mL war die DNA selbst in einem 1000-fach verdünnten Extrakt mittels qPCR eindeutig nachweisbar. Zudem wurden die Fragmentgrößenselektivität und mögliche PCR-Inhibition untersucht und mit einer etablierten adsorptionsbasierten Referenzextraktionsmethode verglichen. Die resultierenden Extrakte zeigten keine Hinweise auf eine Inhibition und wiesen einen Cut-off von etwa 750 bp auf, was auf eine selektive Anreicherung kürzerer Fragmente hindeutet. Diese Eigenschaft ist insbesondere im Kontext der Flüssigbiopsie vorteilhaft, da sie die Abtrennung hochmolekularer gDNA aus der relevanten cfDNA-Fraktion ermöglicht. Obwohl die Gesamtausbeute an sequenzierbarer DNA mit etwa 65 % leicht unter der des adsorptionsbasierten Referenzverfahrens lag, zeichnete sich der entwickelte ATPS-Workflow durch eine deutlich kürzere Prozessdauer von etwa 10 Minuten sowie geringere Anforderungen an Geräte und Verbrauchsmaterialien aus. Die Anwendbarkeit des Verfahrens wurde schließlich durch die Extraktion von cfDNA-Referenzmaterial aus DNA-freiem Plasma und dessen Analyse mittels NGS demonstriert. Die erfolgreiche Erstellung von Sequenzierungsbibliotheken sowie der Nachweis der zugesetzten Mutationen bestätigten die Eignung der gewonnenen DNA für nachgelagerte molekulare Analysen und lieferten damit einen Proof of Concept für einen vereinfachten ATPS-basierten Extraktionsansatz.

Zusammenfassend bietet die vorliegende Arbeit eine systematische Untersuchung von ATPS zur Extraktion cfDNA-ähnlicher Fragmente aus Plasma, in der mechanistische Fragestellungen mit prozessorientierter Entwicklung und Bewertung verknüpft werden. Die Ergebnisse zeigen, dass ATPS eine effektive und zugleich einfache Plattform zur Capture und Konzentration dieser Fragmente darstellen. Darüber hinaus liefert diese Arbeit neue Erkenntnisse über die Einflussfaktoren auf die Verteilung kurzer DNA in Polymer-Salz-Systemen, insbesondere bei Zugabe einer komplexen biologischen Matrix. Auch wenn für eine abschließende Bewertung weitere Untersuchungen mit klinischen Proben erforderlich sind, schafft der entwickelte Ansatz eine fundierte Grundlage für alternative Extraktionsstrategien im Kontext der Flüssigbiopsie. Insgesamt heben die Ergebnisse das Potenzial von Flüssig-Flüssig-Extraktionsverfahren als Ergänzung zu etablierten Methoden hervor und leisten einen Beitrag zur Entwicklung effizienterer, skalierbarer und stärker integrierbarer Probenvorbereitungsprozesse.

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1

Introduction

Molecular diagnostics based on circulating biomarkers have become a cornerstone of precision medicine, enabling minimally invasive disease characterization and longitudinal monitoring [1, 2]. Among these approaches, liquid biopsy has emerged as a promising tool in oncology, enabling the acquisition of tumor-derived genetic information from body fluids without the need for surgical tissue biopsies [3, 4]. Its ease of use and repeatability, along with its potential to capture spatial and temporal tumor heterogeneity, make liquid biopsy attractive for applications such as early cancer detection, treatment selection, therapy monitoring, and the detection of minimal residual disease [5].

A central biomarker in liquid biopsy is circulating cell-free DNA (cfDNA), consisting of short, double-stranded DNA fragments released into the bloodstream primarily through apoptosis and necrosis [6]. In healthy individuals, cfDNA originates largely from hematopoietic cells, whereas in cancer patients, a small fraction is derived from tumor cells and referred to as circulating tumor DNA (ctDNA) [7]. cfDNA exhibits a characteristic fragment-length distribution centered around 167 bp, corresponding to nucleosome-protected DNA, whereas ctDNA fragments are often even shorter [8, 9]. The low abundance of cfDNA in plasma, typically in the range of 1–40 ng/mL, combined with its high fragmentation and susceptibility to contamination by high-molecular-weight genomic DNA (gDNA), makes reliable extraction particularly challenging [10, 11].

Despite major advances in downstream molecular analysis, cfDNA extraction remains a critical bottleneck in the liquid biopsy workflow. Commercial extraction kits predominantly rely on solid-phase adsorption to silica membranes or coated magnetic beads under chaotropic salt conditions [12]. While these methods provide high purity and are widely accepted as reference techniques, they present several limitations. Multi-step bind-wash-elute protocols increase processing time, handling complexity, and the risk of sample loss or cross-contamination [13]. Residual chaotropic salts may inhibit downstream enzymatic assays, and short cfDNA fragments are often recovered less efficiently, potentially introducing fragment-length bias [14, 15]. In addition, the use of specialized consumables and equipment can limit scalability and cost-effectiveness, particularly in high-throughput or routine clinical settings.

These limitations have motivated the exploration of alternative extraction strategies that better match the physicochemical properties of cfDNA and the practical requirements of clinical diagnostics. Aqueous two-phase systems (ATPS) represent a promising liquid–liquid extraction approach, enabling the partitioning of biomolecules between two immiscible, water-rich phases formed by combinations of polymers and salts [16, 17]. ATPS operate under mild, non-denaturing conditions, are readily scalable, and can be integrated into automated, high-throughput workflows through simple centrifugation-based phase separation [18, 19]. ATPS-based processes have been successfully applied to the separation and purification of a wide range of biomolecules, including proteins, viruses, cells, and nucleic acids [20, 21].

Previous studies on DNA partitioning in ATPS have largely focused on gDNA or plasmid DNA (pDNA), primarily in the context of large-scale bioprocessing and protein purification [22, 23]. However, short DNA fragments that resemble cfDNA differ substantially in size, conformation, and interaction behavior, and their partitioning characteristics may not be directly extrapolated from studies of longer DNA molecules. Moreover, most reported investigations have been conducted in well-defined or simplified systems. In contrast, the presence of complex biological matrices such as plasma can significantly affect phase formation, component distribution, and separation performance [24, 25]. In this context, developing alternative ATPS-based separation strategies for short circulating DNA fragments from plasma has the potential to advance process understanding and facilitate the practical implementation of liquid biopsy workflows.

The following subchapters present key aspects of liquid biopsy and the current state of the art in cfDNA extraction and molecular diagnostics (Section 1.1), the theoretical foundations of ATPS and biopartitioning (Section 1.2), and relevant DNA purification and quantification strategies (Section 1.3).

1.1 Liquid biopsy

Cancer is a genetic disease driven by molecular alterations that disrupt the normal regulation of cell growth, survival, and differentiation [26]. The progressive accumulation of these alterations leads to tumor formation, organ dysfunction, and ultimately life-threatening disease [27]. As cancer remains one of the leading causes of death worldwide, improved diagnostic and monitoring strategies are of major clinical importance [28]. The current gold standard for tumor characterization is tissue biopsy, defined as the removal of cells or tissue for histopathological and molecular analysis [3, 29]. Tissue biopsies enable tumor classification and guide personalized treatment decisions. However, they are invasive, may not be feasible for all tumor sites, and are difficult to repeat [30]. A single biopsy offers only a limited snapshot of a spatially and temporally heterogeneous disease [31].

Liquid biopsy has emerged as a complementary approach to overcome these limitations. It refers to the analysis of tumor-derived biomarkers, such as circulating cells, nucleic acids, proteins, or extracellular vesicles, from body fluids, most commonly blood [32–34]. The minimally invasive nature of blood sampling enables repeated sampling over time, allowing longitudinal monitoring of disease progression and therapeutic response [35, 36]. Figure 1.1 shows a schematic liquid biopsy workflow from blood collection to clinical decision-making. Although many methodological foundations of liquid biopsy originate from non-invasive prenatal diagnostics and have also been applied in organ transplantation medicine, the primary clinical momentum lies in oncology [37–40]. In this context, liquid biopsy holds promise for early cancer detection, molecular stratification, monitoring of treatment response, detection of minimal residual disease, and tracking clonal evolution [41–43].

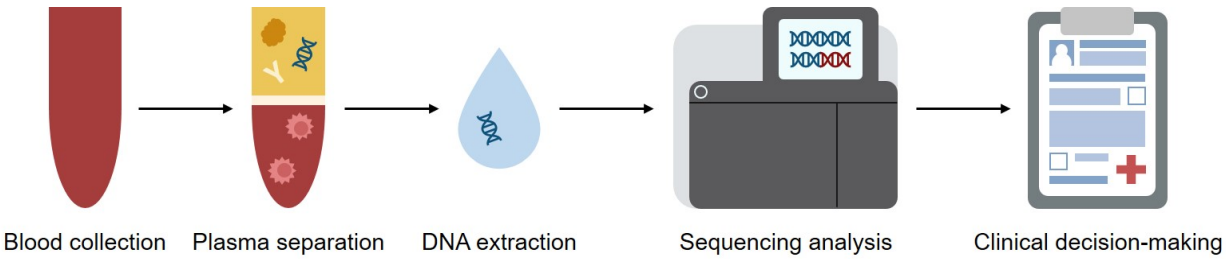


Figure 1.1 Schematic overview of the liquid biopsy workflow from blood collection to clinical decision-making. Following a blood draw, samples are processed to separate cellular components from plasma, which contains relevant biomarkers such as cell-free DNA. The target biomarker is subsequently extracted and purified to ensure compatibility with downstream sequencing-based analysis, enabling the detection of tumor-associated genetic alterations for informed cancer management.

Despite its potential, liquid biopsy faces significant challenges, including the low abundance of tumor-derived material, the requirement for highly sensitive analytical methods, and the need for standardized pre-analytical and clinical validation frameworks [44].

1.1.1 Circulating cell-free DNA

Among biomarkers suitable for liquid biopsy, circulating cell-free DNA (cfDNA) stands out as the most promising analyte for molecular diagnostics due to its relative stability in plasma and compatibility with established sequencing technologies [45]. It was first described in 1948 by Mandel and Métais [46]. Under physiological conditions, cfDNA originates predominantly from hematopoietic cells [47], and plasma levels in healthy individuals are typically well below 40 ng/mL [10]. Circulating levels are influenced by physiological and pre-analytical factors, including physical activity, and can increase with cancer progression [48, 49]. In cancer patients, a variable fraction of total cfDNA is tumor-derived and referred to as ctDNA [50]. This fraction is often small and strongly dependent on tumor burden, stage, and biology [51].

From an analytical perspective, ctDNA may occur at very low variant allele frequency, meaning that only a limited number of mutant fragments may be present in a given plasma sample [52]. Therefore, reliable detection requires highly sensitive and specific analytical technologies [53]. At the same time, scalable extraction methods that enable processing of larger plasma volumes can increase the probability of detecting rare variants [54].

Biologically, cfDNA is released predominantly during apoptosis and necrosis, with additional contributions from active secretion [55]. These mechanisms generate highly fragmented DNA with a characteristic size distribution dominated by mononucleosomal fragments of approximately 167 bp, corresponding to DNA wrapped around a histone octamer [56, 57]. This nucleosomal pattern and distinct fragmentation are defining structural features of cfDNA. Due to its short half-life in circulation (<2 hours), cfDNA provides a dynamic snapshot of the current tumor burden [58].

Clinically, cfDNA analysis provides both quantitative and qualitative insights. Total cfDNA levels can correlate with tumor burden and prognosis [59], while longitudinal measurements enable monitoring of treatment response and detection of relapse [60]. Genomic analyses identify tumor-specific mutations to guide therapy selection and track clonal evolution [61]. In addition, epigenetic

features, such as tissue-specific methylation patterns and emerging fragmentomic signatures, provide complementary diagnostic information [62].

Taken together, the intrinsic characteristics of cfDNA pose substantial analytical challenges and directly affect pre-analytical handling and extraction efficiency [7, 63]. Size-dependent losses, contamination with gDNA, and recovery bias can markedly reduce sensitivity [45, 64]. Because these factors are closely linked to the biochemical properties of the plasma matrix, a detailed understanding of plasma as the source material is essential for developing reliable isolation strategies.

1.1.2 Blood plasma

Whole blood consists of cellular components (erythrocytes, leukocytes, and platelets) and plasma, which accounts for approximately 55–60% of total blood volume [65]. Plasma is a complex aqueous solution composed of roughly 90% water, 7% proteins, and 1–2% dissolved solutes, including electrolytes, metabolites, hormones, and waste products [66, 67]. Blood typically has a physiological pH of about 7.4.

Proteins constitute the dominant macromolecular fraction. Human serum albumin (HSA) is the most abundant plasma protein (approximately 35–55 g/L), contributing to colloid osmotic pressure and serving as a carrier for fatty acids, hormones, and other small molecules [68, 69]. Immunoglobulins, particularly immunoglobulin G (IgG), form the second major fraction, with other globulins involved in transport, coagulation, and immune responses [70]. In addition, plasma contains lipoprotein particles of varying densities that transport cholesterol and triglycerides [71].

From a separation perspective, this high protein content is particularly relevant. Plasma proteins can interact non-specifically with nucleic acids, compete for binding surfaces, alter phase behavior in liquid–liquid systems, and contribute to co-precipitation or adsorption losses [72–74]. Dissolved salts that make up plasma's buffer systems can influence ionic strength and electrostatic interactions, thereby modulating DNA solubility and partitioning behavior [75–77]. The abundance of these macromolecules and solutes creates a challenging background for the selective enrichment of short DNA fragments present at very low concentrations (Table 1.1). Efficient isolation strategies must therefore discriminate between nucleic acids and a large excess of proteins and other plasma constituents while minimizing size-dependent losses and contamination [78].

Table 1.1 Representative plasma components and their physicochemical properties. The large excess of proteins compared to cfDNA illustrates the challenge of selective DNA extraction.

	HSA	**IgG**	**Apolipoprotein A-I**	**Transferrin**	**cfDNA**
Concentration in g/L	35–52	7–16	1–2	2–3.6	$1–40 \times 10^{-6}$
Molecular mass in kDa	66	160	28	80	∼110 (167 bp)
Isoelectric point	4.7–4.8	6.1–9.5	5.2–5.8	5.2–5.9	–
Net charge at pH 7.4	negative	variable	negative	negative	strongly negative

Although plasma is generally preferred over serum for liquid biopsy applications, it remains sensitive to handling conditions [79]. Cellular lysis during blood collection or storage can release gDNA and intracellular components, including hemoglobin, thereby substantially altering the composition of the plasma fraction [80]. Consequently, pre-analytical variables are critical to preserving sample integrity and ensuring reliable downstream analysis.

1.1.3 Pre-analytical considerations

Sample collection, handling, and storage are critical determinants of cfDNA yield, integrity, and background contamination. They must therefore be carefully controlled to match the intended downstream application [11, 81]. A standard blood draw typically provides about 10 mL of whole blood, yielding 4–5 mL of plasma after centrifugation. While 1–4 mL is sufficient for many molecular assays, larger input volumes generally increase total cfDNA yield and improve detection of low-frequency variants [82]. In clinical practice, however, sample availability may be limited [10].

The choice of blood collection tube strongly influences sample stability [83]. Conventional anticoagulant tubes containing EDTA, citrate, or heparin prevent clotting but do not inhibit cellular degradation beyond a few hours [84]. At room temperature, leukocyte lysis may begin within hours to a day, releasing high-molecular-weight gDNA and diluting the tumor-derived fraction [85]. In contrast, specialized cfDNA stabilization tubes contain preservatives that inhibit white blood cell lysis and enable room-temperature storage for extended periods, in some cases up to two weeks [86, 87]. These stabilizing tubes are particularly advantageous when immediate plasma processing is not feasible.

After blood collection, plasma separation must efficiently remove residual cellular components to minimize gDNA contamination [81]. Double-centrifugation protocols are commonly used and typically consist of an initial low-speed step (approximately 1600 × g for 10 minutes) to collect plasma, followed by a high-speed step (16000 × g for 10 minutes) to remove residual cell debris [88, 89]. Incomplete separation, disturbance of the buffy coat, or hemolysis can substantially increase background DNA and nucleosome levels and alter fragment size profiles [90].

Inadequate control of these pre-analytical variables can compromise the sensitivity and reproducibility of downstream molecular assays, underscoring the need for standardized workflows in liquid biopsy applications [91, 92].

1.1.4 Molecular diagnostics

The clinical value of liquid biopsy depends on the reliable detection of tumor-specific genetic and epigenetic alterations in ctDNA. Because tumor-derived fragments often constitute only a minor fraction of total cfDNA, molecular assays must detect rare variants against a substantial wild-type background [93]. The achievable limit of detection is therefore a central performance parameter that depends on both analytical sensitivity and sample quality [41].

Recent advances in genomic technologies and bioinformatic error-suppression strategies have markedly improved the sensitivity of ctDNA analysis [94]. Depending on the methodological approach, mutations can be detected at variant allele frequencies well below 1%, with highly optimized systems reaching even lower thresholds [95, 96]. However, each analytical platform has specific requirements for DNA input quantity, purity, and fragment integrity [41, 97].

1.1.4.1 PCR-based analysis

Methods based on polymerase chain reaction (PCR) remain widely used for targeted mutation analysis because of their specificity and rapid turnaround time. PCR relies on cyclic thermal amplification of a defined DNA target sequence. During each cycle, double-stranded DNA (dsDNA) is first denatured at high temperature, typically around 95°C, resulting in strand separation. Sequence-specific primers then anneal to complementary regions on the single-stranded template at lower temperatures. In the following elongation step, a thermostable DNA polymerase synthesizes

the complementary strand by incorporating deoxynucleotides in the 5'-3' direction [98]. Repeated cycling leads to exponential amplification of the target sequence.

Conventional PCR allows qualitative detection of specific sequence alterations, whereas quantitative PCR (qPCR) enables real-time monitoring of amplification via fluorescence-based signal detection [99]. However, both approaches rely on bulk amplification within a single reaction volume and are therefore limited in sensitivity by background amplification, signal noise, and variations in amplification efficiency [10, 82]. Droplet digital PCR (ddPCR) increases sensitivity by partitioning a DNA sample into thousands of microreactions [100]. Each partition ideally contains zero or one template molecule (Figure 1.2). After amplification with mutation-specific fluorescent probes, reactions are scored as positive or negative, enabling absolute quantification of mutant and wild-type alleles using Poisson statistics [101]. This method enables highly sensitive detection of rare variants but requires prior knowledge of the mutation of interest, limiting it to targeted analyses [102].

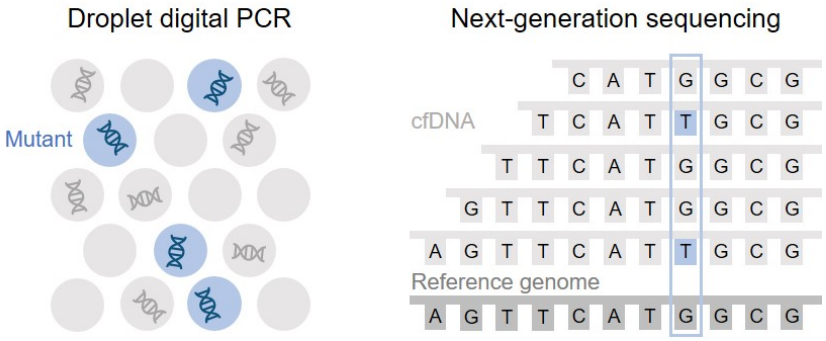


Figure 1.2 Schematic comparison of two molecular diagnostic approaches. Droplet digital PCR (left) partitions samples into thousands of microreactions, enabling highly sensitive detection of predefined mutations. In contrast, next-generation sequencing (right) analyzes cfDNA against a reference genome, enabling broader detection of genomic alterations, including de novo variants.

PCR-based assays are sensitive to inhibitors commonly present in plasma-derived samples, including proteins, salts, and residual extraction reagents [103]. Impurities can reduce amplification efficiency, skew quantification, and increase variability [104]. Consequently, extraction methods must yield DNA of sufficient purity and remove inhibitory substances to ensure reliable amplification.

1.1.4.2 Next-generation sequencing

Next-generation sequencing (NGS) enables high-throughput, parallel analysis of millions of DNA fragments and allows simultaneous interrogation of multiple genomic regions [105, 106]. Depending on the application, sequencing may target predefined gene panels, whole exomes, or entire genomes [107]. Compared with ddPCR, NGS provides broader mutational coverage and can identify previously unknown alterations (Figure 1.2) [108, 109].

Before sequencing, cfDNA samples must be converted into an NGS library of adapter-flanked DNA fragments compatible with the sequencing platform [110]. Although protocols vary, library preparation for cfDNA typically includes end repair, ligation of platform-specific adapters, optional size selection, and PCR amplification [111, 112]. Because each step affects fragment representation, library preparation imposes constraints on DNA concentration, fragment-length distribution, and chemical purity. Low input amounts, amplification bias, or co-purified inhibitors can reduce library

complexity, impair coverage uniformity, and limit sensitivity to detect low-frequency variants in cfDNA analysis [113, 114].

Advanced strategies, such as molecular barcoding and error-correction algorithms, have further enhanced detection of low-frequency variants by distinguishing true mutations from sequencing artifacts [94, 115]. Nevertheless, analytical sensitivity remains directly influenced by input DNA quantity and background contamination with high-molecular-weight gDNA, underscoring the importance of adequate cfDNA extraction and purification.

1.1.5 Cell-free DNA extraction

The majority of cfDNA extraction protocols rely on solid-phase adsorption, most commonly using silica membranes in spin-column formats or silica-coated magnetic beads [12]. Both systems are based on the selective adsorption of nucleic acids onto silica surfaces under chaotropic conditions (Figure 1.3). Silica surfaces are covered with silanol (Si–OH) groups, which are partially deprotonated at neutral to slightly alkaline pH and thus negatively charged [116, 117]. Under normal aqueous conditions, electrostatic repulsion between the negatively charged phosphate backbone of DNA and the silica surface prevents binding. In the presence of high concentrations of chaotropic salts, such as guanidinium isothiocyanate or guanidinium hydrochloride, the hydration shells of DNA and silica are disrupted, and the structured water network is weakened [118–120]. As a result, electrostatic repulsion is reduced, and hydrogen bonds and salt bridges form between DNA and the silica surface. At the same time, proteins are denatured, and their solubility decreases, thereby reducing their co-adsorption [121, 122].

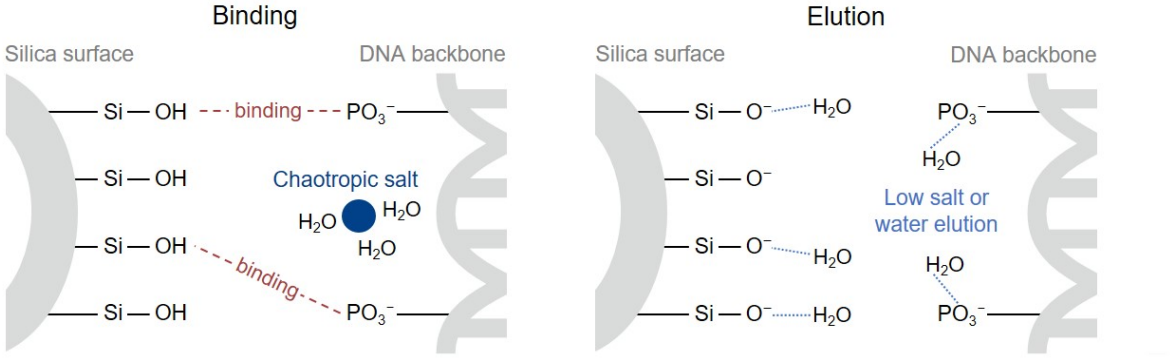


Figure 1.3 Schematic representation of DNA adsorption to silica surfaces. In the presence of chaotropic salts, hydration shells are disrupted, and electrostatic repulsion is reduced, enabling interactions between the DNA phosphate backbone and silanol (Si–OH) groups through hydrogen bonding and cation bridging. Elution with a low-salt buffer or water restores hydration and electrostatic repulsion, releasing DNA from the surface.

Although state-of-the-art cfDNA extraction kits differ in matrix format and composition, most rely on this chemistry in a multi-step bind-wash-elute workflow [12, 95]. First, plasma is incubated with a highly denaturing lysis buffer containing chaotropic salts, and often proteinase K to release nucleic acids associated with histones, protein complexes, or membrane-bound vesicles and to inactivate nucleases [123, 124]. After adjusting the binding conditions with buffers, the sample is loaded onto the solid phase, where cfDNA adsorbs to the silica surface. In contrast, most contaminants remain in solution or are removed during subsequent washes [125]. Alcohol-containing

wash buffers further reduce water activity, thereby stabilizing DNA adsorption while removing residual salts and impurities [13]. Elution is achieved by lowering ionic strength and increasing water activity, thereby restoring electrostatic repulsion and releasing DNA into solution [126]. In most kits, DNA is eluted in nuclease-free water or a low-salt buffer such as Tris-EDTA. Magnetic bead-based systems operate on the same physicochemical principle as kits using silica membranes, but replace centrifugation or vacuum steps with magnetic separation using paramagnetic particles coated with silica or related functional groups [127, 128]. Rapid separation is achieved by applying an external magnetic field, facilitating automation and parallel processing [129, 130].

Adsorption-based methods are robust and widely validated in clinical laboratories [131, 132]. However, their underlying mechanism has inherent limitations. Because cfDNA is extremely dilute, incomplete adsorption or inefficient elution directly reduces analytical sensitivity [126]. Fragment-size-dependent adsorption kinetics may introduce bias against very short DNA fragments [14, 15]. In addition, residual chaotropic salts or alcohol can inhibit polymerases and other enzymes if washing is incomplete. The multi-step bind-wash-elute workflow increases the risk of cross-contamination and adds process complexity and cost, particularly for large sample numbers [13]. Although silica-based workflows remain the current gold standard, their dependence on chaotropic reagents, solid-phase adsorption equilibria, and multiple handling steps can limit recovery efficiency, size selectivity, and process integration.

Another widely used nucleic acid isolation method is alcohol precipitation, often preceded by organic solvent extraction with phenol-chloroform [13, 133, 134]. Adding ethanol or isopropanol in the presence of monovalent cations reduces the dielectric constant of the solution, shielding the negative charges on the DNA phosphate backbone, thereby decreasing intermolecular electrostatic repulsion and promoting nucleic acid aggregation and precipitation [135]. Although conceptually simple, this method's efficiency depends strongly on nucleic acid concentration and fragment length. Given the low abundance and short size of cfDNA, yields may be reduced, and co-precipitation of salts and proteins can compromise downstream applications [135]. Consequently, alcohol precipitation is rarely used for routine cfDNA isolation. However, it has been implemented as a final concentration step in an innovative kit presented by Janku et al. [136].

These considerations have motivated the exploration of alternative separation principles. For example, Obino et al. reviewed microfluidic lab-on-a-chip approaches for nucleic acid extraction from raw human samples, although most still rely on silica-based adsorption [137]. One emerging strategy, which, to the best of our knowledge, has so far been exploited commercially only by PHASE Scientific International [138], is the use of ATPS, which may offer a gentler, potentially scalable liquid–liquid extraction approach for cfDNA isolation.

1.2 Aqueous two-phase systems

1.2.1 Fundamentals and phase separation

Aqueous two-phase systems (ATPS) form when two incompatible hydrophilic components are mixed in water above a critical concentration, leading to spontaneous phase separation into two immiscible aqueous phases [139, 140]. In contrast to conventional organic-aqueous extraction systems, both phases in an ATPS are water-rich, resulting in low interfacial tension and mild, near-physiological separation conditions [141]. Phase separation typically occurs either between two polymers, such as polyethylene glycol (PEG) and dextran, or between a polymer and a salt, commonly PEG and phosphate [142, 143]. Compared with organic solvent extraction, ATPS avoid volatile or denaturing

solvents, reducing biomolecule degradation and safety risks [144]. Unlike solid-phase methods, partitioning in ATPS occurs in a homogeneous liquid phase and does not rely on surface adsorption equilibria, potentially minimizing irreversible binding or incomplete elution [126, 145]. Due to their mild conditions, simple mixing, scalability, and relatively low cost, ATPS are widely used to separate and purify biomolecules, including proteins and nucleic acids [21, 146].

In polymer–salt systems, demixing is driven primarily by differences in water structure and preferential hydration [147]. Kosmotropic salts strongly interact with water molecules, effectively competing with polymers for hydration [118]. This competition dehydrates polymers and promotes polymer–polymer association, thereby driving phase separation [147]. The resulting top phase is typically enriched in polymer (PEG-rich phase), whereas the bottom phase contains higher salt concentrations (salt-rich phase). Each phase exhibits distinct physicochemical properties, including polarity, ionic strength, density, and osmotic pressure, which govern solute partitioning behavior [148]. The phase behavior of ATPS is represented in phase diagrams, which typically plot the concentrations of the two phase-forming components against each other at a specific temperature and pH (Figure 1.4) [149]. The binodal curve delineates the boundary between the monophasic and biphasic regions [150]. Compositions below the binodal form a single homogeneous phase, whereas those above the curve undergo spontaneous phase separation driven by thermodynamic incompatibility [151].

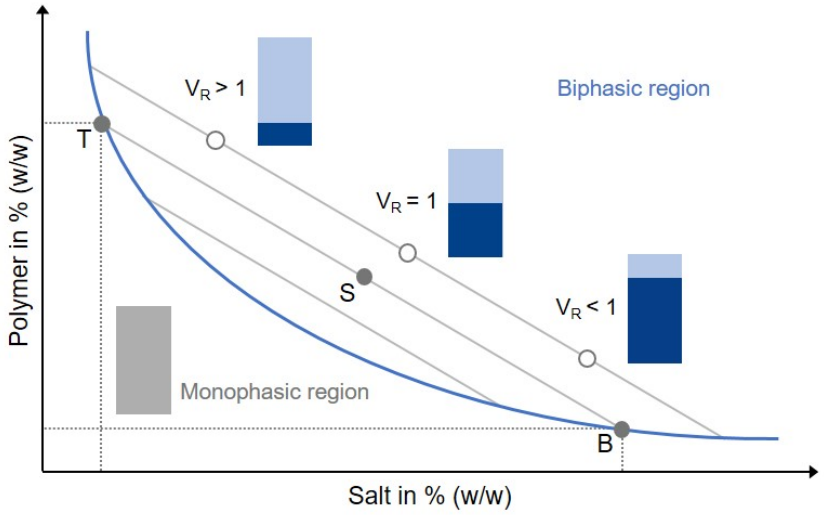


Figure 1.4 Schematic phase diagram of a polymer–salt ATPS. The binodal curve (blue) separates the monophasic and biphasic regions, while tie-lines (gray) connect the equilibrium compositions of coexisting phases. A system point S within the biphasic region separates into a polymer-rich top phase (T) and a salt-rich bottom phase (B), whose compositions are given by the intersections of the corresponding tie-line with the binodal. The phase volume ratio V_R varies along a given tie-line with the overall system composition; at $V_R = 1$, the top and bottom phases have equal volumes.

For any system within the biphasic region, the compositions of the coexisting phases are determined by thermodynamic equilibrium [144]. These equilibrium compositions lie on the binodal curve and are connected by tie-lines, which describe the compositional difference between the polymer-rich and salt-rich phases at a given set of system conditions [150]. The extent of compositional separation between these phases is quantified by the tie-line length (TLL), defined as the distance

between the phase compositions in the diagram [152]. It can be calculated from the equilibrium compositions of the top and bottom phases using

$$\mathrm{TLL} = \sqrt{(w_{\mathrm{PEG,T}} - w_{\mathrm{PEG,B}})^2 + (w_{\mathrm{salt,T}} - w_{\mathrm{salt,B}})^2} \tag{1.1}$$

where w is the mass fraction of the component in the top (T) and bottom (B) phases [153]. Experimentally, the compositions of the coexisting phases can be determined from phase equilibrium data, while the relative amounts of the phases are related by the lever rule

$$\frac{V_{\mathrm{T}}\rho_{\mathrm{T}}}{V_{\mathrm{B}}\rho_{\mathrm{B}}} = \frac{\overline{SB}}{\overline{ST}} \tag{1.2}$$

where V and ρ are the phase volume and density, respectively, and $\overline{SB}$ and $\overline{ST}$ denote the tie-line segment lengths. Together, these relationships allow determination of the tie-line position and length from experimental phase compositions. Increasing TLL generally amplifies differences in physicochemical properties, including polarity, ionic strength, and excluded volume, thereby influencing solute partitioning.

The phase volume ratio, defined as the ratio of top to bottom phase volumes after phase separation, is given by

$$V_R = \frac{V_{\mathrm{T}}}{V_{\mathrm{B}}} \tag{1.3}$$

and depends on the overall system composition and the phase densities [154]. Although biopartitioning in well-defined, ideal systems is independent of V_R, this independence does not necessarily hold for PEG/salt ATPS containing biomass, crude lysate, or other complex matrices such as plasma [23, 155]. Phase separation is influenced by several factors, including polymer molecular weight, salt type, initial system composition, pH, and temperature, all of which ultimately affect solute partitioning between the phases [143].

1.2.2 Biopartitioning mechanisms

Partitioning in polymer–salt ATPS is governed by differences in a solute's chemical potential between the coexisting phases [16]. At equilibrium, this distribution is described by the partition coefficient

$$K = \frac{c_{\mathrm{T}}}{c_{\mathrm{B}}} \tag{1.4}$$

where c_{T} and c_{B} denote the concentrations of a specific solute in the top and bottom phases, respectively [156]. In practice, several interdependent mechanisms contribute to this distribution.

Electrostatic interactions are central to polymer–salt systems. Differential ion partitioning generates differences in electrostatic potential [77, 157]. In PEG/phosphate systems, phosphate anions preferentially accumulate in the bottom phase, increasing ionic strength and creating a more negatively charged environment [158]. Because of the high charge density of the DNA phosphate backbone, partitioning is strongly influenced by ionic strength, charge screening, and possible Donnan-type effects [20, 159]. Changes in salt composition or the addition of neutral salts can therefore significantly alter DNA distribution [160, 161].

The molecular weight of the polymer further influences phase behavior and partitioning by affecting phase separation, hydrophobicity, and excluded volume [143, 162]. Higher-molecular-weight

PEG promotes phase separation at lower concentrations and increases steric constraints, thereby affecting TLL and size-dependent partitioning [139, 143, 163]. Excluded-volume effects arise from the high polymer concentration in the top phase, which reduces the free volume available to solutes and induces steric repulsion [164, 165]. Consequently, macromolecules preferentially partition into the phase with greater accessible volume [166]. For nucleic acids, this effect is size-dependent because the hydrodynamic radius increases with molecular length, leading to stronger exclusion of long DNA strands than of short DNA fragments [167, 168].

In addition, differences in solute-water interactions lead to unequal distributions of water mole fraction and solvent properties across the phases [147]. The polymer-rich phase is generally less polar and more hydrophobic than the salt-rich phase, reflecting differences in polymer concentration and ion distribution [139, 142, 168]. Hydrophobic interactions are generally more influential in protein partitioning within ATPS, whereas nucleic acid partitioning is predominantly governed by electrostatic interactions [140, 169].

1.2.3 Nucleic acid partitioning

Partitioning of nucleic acids in ATPS follows distinct trends that differ from those of many proteins. In polymer–salt systems such as PEG/phosphate, DNA mainly partitions into the salt-rich bottom phase, whereas proteins typically accumulate in the polymer-rich top phase [21, 141]. This behavior is attributed to the combined effects of electrostatic interactions, excluded volume, and phase-specific solvent properties, which favor the partitioning of highly charged polyanions into the salt-rich environment [139, 143]. Accordingly, ATPS have been successfully used to recover pDNA or remove gDNA, often yielding high recovery in optimized systems [161, 170, 171].

However, large nucleic acids differ substantially from cfDNA. Short DNA fragments (∼150–200 bp) have distinct physicochemical properties, including reduced hydrodynamic size and altered interaction behavior, that can influence their partitioning. Despite this relevance, systematic investigations of short DNA fragments in ATPS remain limited. In particular, quantitative studies on size-dependent partitioning under controlled tie-line conditions and phase compositions are scarce, and experiments using plasma-relevant matrices are rarely reported.

1.2.4 High-throughput screening and automation

The design of ATPS for biomolecule separation cannot be reliably predicted from theory alone. Phase formation and biomolecule partitioning depend on multiple coupled parameters, including polymer molecular weight, phase composition, TLL, pH, and ionic strength, which together determine the phases' physicochemical properties [140]. Consequently, empirical screening is typically required to identify suitable system compositions and operating conditions [172]. High-throughput experimentation provides an efficient strategy for exploring this multidimensional parameter space. By miniaturizing experiments and running them in parallel, large numbers of ATPS compositions can be prepared and evaluated in short time frames while consuming minimal sample volumes [140, 173]. Many downstream processing operations, including aqueous two-phase extraction, have therefore been adapted to microtiter-plate formats compatible with robotic liquid-handling stations [171, 174–176]. These systems enable automated dispensing, mixing, and sampling of microliter-scale volumes. They are often paired with statistical methods, such as the Design of Experiments, to systematically identify relevant process parameters [177–179].

Beyond accelerating process development, automation and parallelization are also highly relevant for routine clinical applications [13]. Liquid biopsy workflows often require processing large numbers of samples with high reproducibility and minimal manual intervention. Consequently, developing extraction strategies compatible with automated liquid-handling platforms is an important consideration for clinical implementation [180, 181].

While high-throughput screening enables rapid identification of suitable ATPS conditions for cfDNA extraction, the resulting polymer- and salt-rich phases can interfere with downstream analytical methods. Therefore, additional purification or desalting steps are often required before DNA quantification and molecular analysis.

1.3 Desalting and purification

Although ATPS enable efficient partitioning of nucleic acids from complex biological matrices, the resulting polymer- and salt-rich phases are often incompatible with downstream analytical methods. In PEG/phosphate ATPS, residual salts and polymers can interfere with spectroscopic measurements, inhibit enzymatic reactions such as PCR, and compromise fluorescence-based detection [182, 183]. Gel filtration, or size-exclusion desalting, is a widely used approach for removing low-molecular-weight contaminants (Figure 1.5). In this method, the sample passes through a porous resin. Small molecules, such as salts, enter the pores and are retained in the stationary phase, whereas larger macromolecules, such as DNA, are excluded and elute in the void volume, enabling gentle desalting without compromising DNA integrity [184, 185].

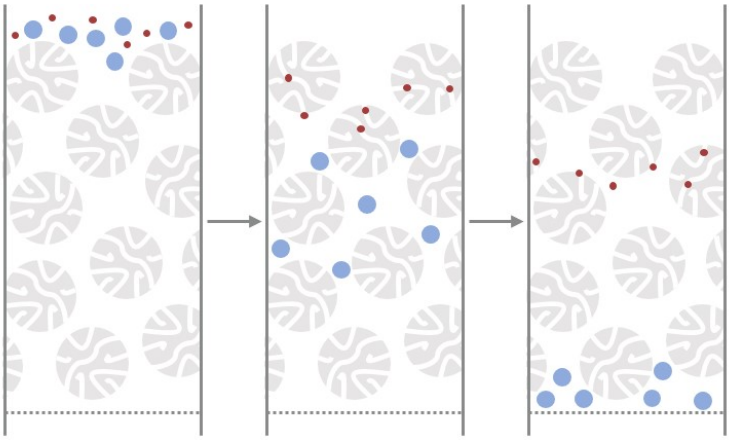


Figure 1.5 Schematic representation of gel filtration (size-exclusion desalting). Macromolecules (blue), such as DNA, are excluded from the pores of the stationary-phase particles (gray) and elute in the void volume. In contrast, smaller molecules (red), such as salts, enter the pores and are retained longer. This size-dependent separation enables efficient desalting and purification of samples.

The efficiency of gel filtration depends on resin properties and operating conditions. Pore size and resin chemistry determine the exclusion limit, which separates macromolecules from small solutes, and the ratio of sample volume to resin bed volume determines both desalting efficiency and DNA recovery [186, 187]. Overloading can lead to incomplete salt removal, whereas excessive matrix may reduce DNA yield. In addition, capillary forces and flow resistance within the porous structure may affect elution behavior during centrifugation-based operations [188]. Efficient desalting is

therefore essential for ATPS-derived samples because residual phase components can compromise analytical accuracy, and DNA losses must be minimized given the low concentrations of short cfDNA-like fragments. Consequently, purification conditions must balance the effective removal of low-molecular-weight components with maximal DNA recovery to ensure compatibility with downstream analysis.

1.4 DNA quantification

UV-Vis spectroscopy is a simple, widely used method for quantifying nucleic acids [189]. DNA exhibits a characteristic absorption maximum near 260 nm due to its aromatic nucleobases, and absorbance at this wavelength is proportional to concentration according to the Beer-Lambert law [190, 191]. However, the method lacks selectivity because proteins and other organic compounds may also absorb in this spectral region [192]. In addition, reliable quantification typically requires DNA concentrations in the nanogram-per-microliter range [192, 193]. These limitations, together with potential matrix effects from ATPS components, make UV-Vis spectroscopy unsuitable for dilute cfDNA samples.

Fluorescence-based assays provide significantly higher sensitivity for DNA detection. Dyes such as PicoGreen® exhibit a strong increase in fluorescence upon binding to dsDNA, with signal intensity proportional to DNA concentration over a broad dynamic range and detection limits in the picogram range [194, 195]. Because such dyes bind preferentially to dsDNA, fluorescence assays offer improved selectivity over UV-Vis absorbance, although residual matrix components may still affect measurements.

In addition to total DNA quantification using calibration curves, qPCR enables selective detection of amplifiable DNA [196]. The accumulation of PCR products is monitored in real time using fluorescent probes or intercalating dyes, and DNA concentration is determined from the cycle threshold (Ct) at which the fluorescence signal exceeds the background level [98]. Due to its high sensitivity and sequence specificity, qPCR is widely used in cfDNA analysis, but amplification efficiency can be affected by residual inhibitors [104, 197, 198].

Complementary to these methods, agarose gel electrophoresis and microfluidic capillary electrophoresis systems, such as the TapeStation, are used to assess DNA fragment size distribution and integrity [199, 200]. These techniques separate DNA fragments by size under an electric field, enabling visualization of characteristic fragment patterns and detection of high-molecular-weight contamination. Although not suitable for absolute quantification, they provide important qualitative and semi-quantitative information on fragment-length profiles, which is particularly relevant to cfDNA analysis [201, 202].

Together, fluorescence-based assays and qPCR enable sensitive quantification of total and amplifiable DNA, while electrophoretic methods provide complementary information on fragment-size distribution, allowing a comprehensive assessment of DNA recovery and quality after ATPS extraction.

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2

Thesis outline

2.1 Research proposal

Molecular diagnostics based on circulating biomarkers have become central to precision medicine, enabling minimally invasive disease characterization and longitudinal monitoring. In this context, liquid biopsy has emerged as a powerful approach for analyzing tumor-derived genetic material in body fluids. Among the different analytes, circulating cell-free DNA (cfDNA) holds particular promise, as it provides access to genetic and epigenetic tumor information without the need for invasive tissue biopsies. Its potential for applications such as early detection, therapy selection, and monitoring of treatment response has driven rapid advances in downstream analytical techniques, including droplet digital PCR and next-generation sequencing (NGS).

Despite these developments, the reliable extraction of cfDNA from plasma remains a critical bottleneck in the liquid biopsy workflow. Present at low concentrations and in a highly fragmented form, cfDNA coexists with high-molecular-weight genomic DNA (gDNA) and abundant plasma proteins, imposing strict requirements on extraction methods for recovery, selectivity, and compatibility with downstream analyses. Current standard approaches are predominantly based on solid-phase adsorption using silica membranes or magnetic beads under chaotropic conditions. While well established, these methods involve multi-step bind-wash-elute protocols that increase handling complexity and can lead to sample loss, contamination, and biased recovery of short DNA fragments. In addition, the use of chaotropic reagents and specialized consumables can limit scalability and integration into automated workflows.

These limitations motivate the exploration of alternative extraction strategies that better align with the physicochemical properties of short DNA fragments and the practical requirements of clinical diagnostics. Aqueous two-phase systems (ATPS) represent a promising liquid–liquid extraction approach that enables the partitioning of biomolecules between two immiscible aqueous phases under mild conditions. ATPS-based processes are rapid, scalable, and amenable to automation, as phase separation can be achieved by simple centrifugation. Against this background, the objective of

this thesis is to develop an ATPS-based extraction workflow for cfDNA-like fragments from plasma by integrating fundamental insights into partitioning behavior with process-oriented development. Although ATPS have been widely applied for the separation of proteins and long DNA molecules, their application to short DNA fragments resembling cfDNA remains insufficiently explored. In particular, the partitioning behavior of short DNA in polymer–salt systems is still poorly understood, and existing studies are largely limited to simplified model systems. Furthermore, the influence of complex biological matrices such as plasma and the integration of ATPS into a complete extraction and purification workflow compatible with downstream molecular analyses have not been systematically investigated. To address these gaps, this work progresses from studies in well-defined model systems to application in a complex biological matrix and, finally, to the development of an integrated workflow, with each study building on the previous one.

The partitioning behavior of nucleic acids has been extensively studied, but prior work has primarily focused on large DNA molecules, such as plasmid DNA or gDNA. Developing an ATPS-based extraction process requires a mechanistic understanding of short DNA partitioning and clear design rules for system selection. The first study (Chapter 3) addresses the lack of data on the behavior of short DNA fragments in polymer–salt ATPS. Given the limited predictability of solute partitioning and the strong interdependence among system parameters, a systematic, largely empirical approach is used. The effects of polyethylene glycol (PEG) molecular weight, salt type, tie-line length, phase volume ratio, and the addition of neutral salts on DNA partitioning and recovery are investigated in well-defined PEG/salt systems. High-throughput screening with automated liquid-handling routines enables efficient exploration of a broad design space, supported by automated binodal and tie-line determination. In addition, preliminary experiments in a synthetic plasma matrix are conducted to assess the transferability of the observed trends to more complex environments. This study provides the foundation for rational system selection in subsequent extraction experiments.

Most partitioning studies are limited to simplified systems containing only water, polymer, and salt. However, complex matrices such as blood plasma can significantly affect phase formation and solute distribution. Plasma contains a wide range of components, including proteins and electrolytes, which may interfere with DNA partitioning and recovery. Therefore, the second study (Chapter 4) investigates the extraction of short DNA fragments from human plasma using ATPS. Building on results from model systems, the study is extended to biologically relevant samples to assess how plasma components influence phase behavior and DNA distribution. The robustness of the ATPS-based capture step is evaluated using plasma samples from different donors and blood collection tubes. To enable higher plasma input volumes while minimizing dilution from pre-dissolved phase-forming components, an ATPS design based on solid PEG and salts is implemented. In parallel, downstream processing is addressed through the development of a reverse-elution strategy to desalt and concentrate the DNA-containing, salt-rich bottom phase. By combining extraction with a subsequent purification step, this study establishes key process elements required for practical implementation.

Sequencing-based analytical methods are central to modern molecular diagnostics and impose strict requirements on cfDNA extracts. In particular, sufficient DNA concentration must be achieved from limited input material, and potential inhibitors of enzymatic reactions must be effectively removed. Although ATPS enables simultaneous capture and concentration of target molecules, additional purification is required to remove residual polymer or salt from the phase of interest. The third study (Chapter 5) focuses on integrating and optimizing the complete ATPS-based extraction workflow and assessing its compatibility with sequencing-based downstream analyses. Building on

the established system understanding and process elements, different lysis and clearing strategies are evaluated to improve DNA accessibility and reduce matrix interference. Process conditions are further optimized to increase DNA concentration while minimizing losses, and a serial ATPS approach is explored for additional enrichment. The performance of the developed workflow is assessed with respect to recovery, fragment-size selectivity, and potential PCR inhibition, and is compared with an adsorption-based reference extraction method. Finally, the applicability of the process is demonstrated by extracting cfDNA reference material from plasma and successfully performing NGS analysis. This study provides proof of concept that ATPS-based extraction can yield sequencing-compatible DNA and be integrated into liquid biopsy workflows. While clinical validation is beyond the scope of this work, the results contribute to a deeper understanding of biomolecular partitioning in complex systems and support the further development of liquid–liquid extraction strategies for molecular diagnostics.

2.2 Manuscript overview

Within the scope of this thesis, three manuscripts were prepared and published. The publications have been slightly revised to ensure consistency in the format and layout of this thesis. In this section, each manuscript is briefly introduced with a concise summary and the corresponding citation. Detailed information on individual author contributions, including the respective signatures, is provided in the examination copy of this thesis.

The included research articles build on one another and aim to develop an ATPS-based extraction process for cfDNA-like fragments from plasma. Chapter 3 lays the groundwork by investigating the partitioning behavior of short DNA fragments in defined PEG/phosphate ATPS, addressing a previously unexplored aspect. Chapter 4 extends this work to human plasma, focusing on DNA distribution and separation from proteins, and introduces reverse elution as a desalting step for the DNA-containing, salt-rich bottom phase. Finally, Chapter 5 presents a proof of concept for the full extraction and purification workflow by demonstrating successful sequencing of cfDNA reference material isolated from plasma using NGS.

Chapter 3

Partitioning behavior of short DNA fragments in polymer/salt aqueous two-phase systems 29

Rafaela Meutelet, Lea J. Bisch, Benedikt C. Bürfent, Markus Müller, and Jürgen Hubbuch

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Chapter 3 investigates the partitioning behavior of short DNA fragments in polymer–salt ATPS. The effects of PEG molecular weight, tie-line length, phase volume ratio, salt type, and neutral salt additives on DNA partitioning and recovery are evaluated in clean, well-defined systems using high-throughput screening on a liquid-handling platform, with automated binodal and tie-line determination. In addition, preliminary separation experiments in a synthetic plasma matrix are conducted to assess the transferability of the observed trends. The results provide a basis for selecting suitable system conditions for subsequent extraction studies.

Chapter 4

Extraction and purification of short DNA fragments from plasma using aqueous two-phase systems for liquid biopsy 45

Rafaela Meutelet, Lea J. Bisch, Benedikt C. Bürfent, Johannes Oldenburg, Timo Hess, Heiko Rühl, and Jürgen Hubbuch

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Chapter 4 builds on these findings by investigating the extraction of short DNA fragments from human plasma using ATPS. The distribution of DNA in plasma-containing systems is analyzed, and the robustness of the extraction step is evaluated using samples from different donors and

blood collection tubes. To enable higher plasma input volumes while minimizing dilution effects, an ATPS design based on solid PEG and salts is implemented. Furthermore, an adapted reverse elution protocol is introduced as a desalting and concentration step for the DNA-containing bottom phase, enabling downstream processing.

Chapter 5

Proof of concept for aqueous two-phase system-based extraction of cell-free DNA from plasma for liquid biopsy applications 67

Rafaela Meutelet, Benedikt C. Bürfent, Timo Hess, Julia Teply-Szymanski, Paul Jank, Johannes Oldenburg, Heiko Rühl and Jürgen Hubbuch

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Chapter 5 presents a proof of concept for the complete ATPS-based extraction and purification workflow. The process is further optimized by evaluating lysis and clearing strategies, identifying system conditions that increase DNA concentration, and exploring serial ATPS for additional enrichment. Performance is assessed in terms of recovery, fragment-size selectivity, and potential PCR inhibition, and compared with a state-of-the-art silica-based extraction method. Finally, the optimized workflow is applied to extract cfDNA reference material from plasma, demonstrating compatibility with NGS and the ability to generate sequencing-quality DNA.

3

Partitioning behavior of short DNA fragments in polymer/salt aqueous two-phase systems

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Abstract

The development of liquid biopsy as a minimally invasive technique for tumor profiling has created a need for efficient biomarker extraction systems from body fluids. The analysis of circulating cell-free DNA (cfDNA) is especially promising, but the low amounts and high fragmentation of cfDNA found in plasma pose challenges to its isolation. While the potential of aqueous two-phase systems (ATPS) for the extraction and purification of various biomolecules has already been successfully established, there is limited literature on the applicability of these findings to short cfDNA-like fragments. This study presents the partitioning behavior of a 160 bp DNA fragment in polyethylene glycol (PEG)/salt ATPS at pH 7.4. The effect of PEG molecular weight, tie-line length, neutral salt additives, and phase volume ratio is evaluated to maximize DNA recovery. Selected ATPS containing a synthetic plasma solution spiked with human serum albumin and immunoglobulin G are tested to determine the separation of DNA fragments from the main plasma protein fraction. By adding 1.5% (w/w) NaCl to a 17.7% (w/w) PEG 400/17.3% (w/w) phosphate ATPS, 88% DNA recovery was achieved in the salt-rich bottom phase while over 99% of the protein was removed.

3.1 Introduction

In recent years, liquid biopsy has become a promising diagnostic tool in clinical and precision oncology, as it enables the molecular characterization of tumors without the need for invasive surgical tissue biopsies [203]. Its minimally invasive nature and easy repeatability offer potential benefits in cancer applications such as early cancer detection, real-time tumor monitoring, personalized treatment selection, patient response assessment, and detection of minimal residual disease after surgery [35]. Liquid biopsy is based on analyzing various tumor-derived entities found in body fluids, especially in the bloodstream. One of the most investigated biomarkers is circulating cell-free DNA (cfDNA), more specifically circulating tumor DNA (ctDNA), as its analysis provides not only a comprehensive profile of the tumor genome but also the assessment of intratumor heterogeneity and total tumor burden [51, 204].

The double-stranded cfDNA is released through apoptosis and necrosis, resulting in high fragmentation with a predominant fragment length of 167 bp [205]. This characteristic length comes from a 147 bp DNA strand wound around a histone octamer, forming a nucleosomal structure attached to a strand of linker DNA. Studies have shown that ctDNA fragments are even shorter than cfDNA, ranging from 132 to 143 bp, which can challenge efficient ctDNA extraction using conventional kits [206]. Additionally, the dilute ctDNA concentration of around 1–40 ng/mL plasma and the high risk of contamination from genomic DNA (gDNA) shed by lysed white blood cells make extraction even more challenging [10].

Commercial extraction kits primarily rely on DNA adsorption to solid phases like silica membranes or magnetic beads in the presence of chaotropic salts. If not removed, residual chaotropic salts can inhibit downstream amplification-based analyses. Therefore, standard kits use bind-wash-elute protocols with multiple steps, which can limit cfDNA yields [14]. Furthermore, a fragment length bias may be introduced due to uneven extraction efficiency of shorter fragments [15]. These limitations and the lack of standardization hinder the broader implementation of cfDNA analysis in clinical settings, highlighting the need for novel cfDNA extraction methods [11, 207].

An alternative approach for the integrated extraction and concentration of cfDNA from plasma samples can be achieved using aqueous two-phase systems (ATPS). With their high water content,

ATPS offer mild conditions for isolating biomolecules such as cells, viruses, proteins, and nucleic acids [21]. The extraction using ATPS is rapid, easily scalable, low-cost, and compatible with high-throughput processes [140, 208]. Purification processes using ATPS have already been successfully established for various biomolecules, including nucleic acids like gDNA and plasmid DNA (pDNA). Table 3.1 lists ATPS-based extraction and purification processes for DNA of different sizes described in the literature.

Table 3.1 ATPS-based extraction and purification processes for DNA of different sizes reported in the literature. bp, base pairs; Da, Dalton; ds, double stranded; EO-PO, ethylene oxide-propylene oxide; gDNA, genomic DNA; pDNA, plasmid DNA; PAA, polyacrylate; PEG, polyethylene glycol; ss, single stranded.

DNA size	ATPS	Isolation process	Reference
3×10^7 Da	PEG/Dextran	dsDNA separation from ssDNA	[209]
2×10^7 Da	PEG/Dextran	DNA isolation from microorganisms	[210]
15000 bp	Triton X-114/PBS	gDNA concentration as biomarker	[22]
-	PEG/phosphate	gDNA removal from protein product	[161]
8500 bp	PEG/phosphate	pDNA purification from lysate	[170]
7796 bp	PEG/phosphate	pDNA partitioning in clean systems/lysate	[23]
6700 bp	PEG/phosphate	pDNA partitioning in clean systems	[171]
-	PEG/phosphate	pDNA partitioning in clean systems/lysate	[211]
6050 bp	EO-PO/Dextran	pDNA purification from lysate	[160]
6050 bp	PEG/sulfate	pDNA purification from lysate	[212]
6050 bp	PEG/citrate/sulfate	pDNA purification from lysate	[162]
3000 bp	PEG/citrate	pDNA purification from lysate	[213]
2686 bp	PEG/PAA/salt	pDNA partitioning in clean systems/lysate	[214]
250–10000 bp	PEG/PAA/salt	PCR DNA fragment isolation	[215]
145 bp	not described	cfDNA purification from plasma	[136]
20 bp	PEG/sulfate	ss oligonucleotide partitioning	[216]

Several papers discuss pDNA partitioning in clean systems and pDNA extraction from crude cell lysate for large-scale production in gene therapy [170, 171, 211, 217]. The literature also covers gDNA isolation and its removal from complex mixtures for target protein purification using ATPS [161, 210, 218]. However, little is known about the partitioning behavior of DNA fragments shorter than 200 bp which resemble cfDNA.

As the partitioning behavior in ATPS depends on many factors, including ATPS composition, pH, additives, and target molecule characteristics [21], findings for pDNA or gDNA may not apply to shorter cfDNA or ctDNA fragments. Matos et al. [215] described isolating DNA fragments after polymerase chain reaction (PCR) using ATPS. Fragments up to 6000 bp are recovered in the polyethylene glycol (PEG)-rich top phase of a PEG/polyacrylate system before back-extraction into the salt-rich bottom phase of a PEG/Na_2SO_4 ATPS. However, the smallest fragments studied are 250 bp, too large to represent cfDNA or the even shorter ctDNA. Additionally, it is uncertain if this method applies to cfDNA extraction from a complex matrix like plasma. Janku et al. [136] reported using an ATPS-based kit, PHASIFY MAX, to purify cfDNA from blood for liquid biopsy, promising a 60% yield increase over the silica-based QIAamp Circulating Nucleic Acid kit. The DNA is recovered in the bottom phase, concentrated in the top phase of a second ATPS, and

precipitated with isopropanol. The exact ATPS compositions in the kit, commercialized by Phase Scientific (Hong Kong), are undisclosed.

High concentrations of polymer or salt, even if kosmotropic, necessitate a purification step to remove components that could affect downstream analysis. Different purification approaches can be employed depending on whether the DNA is enriched in the polymer- or salt-rich phase. For example, the PHASIFY MAX kit uses alcohol to precipitate the DNA from the top phase, while desalting resins are suitable for the salt-rich bottom phase.

Because the partitioning of molecules in ATPS is hard to predict and relies heavily on empirical experiments, this paper aims to provide comprehensive insights into the partitioning behavior of short DNA fragments in PEG/salt systems, comparing trends with other reported DNA types. The effects of PEG molecular weight, salt type, tie-line length (TLL), volume ratio (V_R), and neutral salt addition on DNA partitioning and recovery in clean PEG/salt ATPS are investigated. The applicability of the observed partitioning trends to systems containing a synthetic plasma solution is tested, providing a basis for developing an ATPS-based cfDNA extraction process from plasma samples for liquid biopsy.

3.2 Materials and methods

3.2.1 Chemicals and stock solutions

PEG 200, PEG 400, and PEG 600 of synthesis grade as well as 85% methyl orange dye, human serum albumin (HSA), and immunoglobulin G (IgG) were purchased from Sigma-Aldrich (St. Louis, MO). PEG 1000, $NaH_2PO_4 \cdot H_2O$, NaCl, KCl, LiCl, $MgCl_2$, and $CaCl_2$ were obtained from Merck KGaA (Darmstadt, Germany). K_2HPO_4 was purchased from VWR International GmbH (Radnor, PA). Stock solutions and buffers were prepared using ultra-pure water as follows: 70% (w/w) PEG, 40% (w/w) phosphate, and 25% (w/w) neutral salt solutions. Phosphate stock solution was prepared by combining 30.91% (w/w) NaH_2PO_4 and 9.09% (w/w) K_2HPO_4 in water to yield pH 7.4. A synthetic plasma electrolyte solution with physiological pH 7.4 was prepared by dissolving 0.9% (w/v) NaCl and 0.2% (w/v) $NaHCO_3$ in water.

3.2.2 DNA fragment preparation

Purified DNA fragments with a length of 160 bp were prepared using pKRrha_eGFP_JG 1163 plasmid isolated from E. coli as a template DNA. The desired fragment length was amplified by PCR using accordingly designed primer sequences obtained from Merck KGaA. One 50 µL reaction volume contained 50 ng of plasmid, 2 µL of both forward (TAAACGGCCACAAGTTCAG) and reverse (GGGTAGCGGCTGAAGCAC) primer, 10 µL of Hifi Buffer, and 0.5 µL of PCRBIO Hifi Polymerase (PCR Biosystems Ltd., London, UK) as well as DNase-free water. Twenty-four cycles consisting of heat denaturation at 95°C for 3 s, primer annealing at 60°C for 30 s, and elongation at 72°C for 10 s were carried out on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories GmbH, Hercules, CA). The PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega GmbH, Madison, WI) and diluted with DNase-free water to create a 10 ng/µL DNA fragment stock solution.

3.2.3 Liquid handling station

A Tecan Freedom EVO system (Tecan, Crailsheim, Germany) was used as a liquid handling platform for automated ATPS experiments. The system setup and liquid handling calibration procedure were described in earlier work [175]. The robotic workstation was controlled using Evoware 2.4 SP3. Importing pipetting volumes and exporting photometric data were done using Excel 2007 (Microsoft Corporation, Redmond, WA).

3.2.4 ATPS screening procedure

Automated routines adapted from previous methods developed by our group were used for binodal curve and tie-line determination [173, 175] as well as for DNA partitioning experiments in different ATPS [161]. Binodal points were identified using the cloud point method and fitted to the following equation given by Merchuk et al. [219]:

$$x_{PEG} = a * exp(b * x_{salt}^{0.5} + c * x_{salt}^{3}) \tag{3.1}$$

Tie-lines were determined as described by Diederich et al. [208], except that methyl orange dye was used instead of methyl violet for mass balance measurements and subsequent V_R calculations. System points with V_R near 1 were selected to investigate the DNA fragment partitioning behavior in clean ATPS. Various parameters including the molecular weight of PEG, TLL, and the type and concentration of neutral salt additives, were varied to evaluate the effects on DNA partitioning and recovery in the ATPS top and bottom phases. A total of 600 µL ATPS with a final concentration of 100 ng/mL 160 bp DNA were prepared in 1 mL 96-deep well polypropylene plates (Thermo Fisher Scientific Inc., Waltham, MA) by adding stock solutions in the following order: water, phosphate salt, additive salt, and PEG. This spike-in concentration was chosen to mimic the realistic concentration range of cfDNA in blood samples. ATPS were mixed for 1 min at 1100 rpm on an orbital shaker before spiking with DNA stock solution, mixing for incubation, and centrifuging at 4000 rpm for 5 min to achieve phase separation. One hundred microliters were sampled from top and bottom phases using an optimized sampling method [175] and desalted using proprietary purification plates (BioEcho Life Sciences GmbH, Cologne, Germany) to reduce interference by the phase-forming components for subsequent DNA quantification. Phase volumes and the DNA concentration in each phase were measured in triplicate. DNA partitioning trends were assessed using DNA recovery as a percentage instead of concentration, as the latter depends on phase volume, which varies across experiments. DNA recoveries in the top and bottom phases were calculated by multiplying the measured DNA concentrations with the respective phase volumes and dividing by the amount of input DNA. Similar partitioning experiments were performed by spiking selected ATPS with a final concentration of 2.5 mg/mL of either HSA or IgG instead of DNA to investigate the partitioning behavior of the main plasma protein fractions.

3.2.5 Separation experiments with synthetic plasma

Separation experiments with synthetic plasma were conducted manually in 1.5 mL reaction tubes for selected ATPS. Six hundred microliters ATPS with a final concentration of 100 ng/mL 160 bp DNA were prepared by adding stock solutions in the following order: plasma electrolyte solution, phosphate salt, additive salt, and PEG. After brief vortexing of the phase-forming components, a protein mix consisting of 60 mg/mL HSA and 15 mg/mL IgG was added to achieve a final protein

concentration of 10 mg/mL in the ATPS. Lastly, ATPS were spiked with 160 bp DNA fragments, vortexed for incubation, and centrifuged in a microcentrifuge at 4000 rpm for 5 min for phase separation. Phase volumes were determined visually. Phase samples were not desalted but diluted tenfold in water to reduce interference of the phase-forming components with DNA and protein quantification analyses. Adequate blank phases were used for background correction, and the same blank phases were spiked with a defined amount of DNA as references for recovery calculations.

3.2.6 Analytical methods

3.2.6.1 DNA quantification

The DNA concentration in the ATPS top and bottom phases was measured using the Quant-iT PicoGreen dsDNA HS assay kit (Thermo Fisher Scientific Inc.). Black 96-well half-area polystyrene microplates (Greiner Bio-One, Kremsmünster, Austria) (50 µL per well) and black 384-well microplates (10 µL per well) were used for the automated screening and the manually prepared ATPS, respectively. Samples were analyzed against blanks with similar phase composition but without DNA to account for interference. Four-point calibration curves were constructed by spiking 160 bp DNA fragments into reference top and bottom phases. For the automated screening, the PEG and salt concentrations in these reference phases were set to half the average concentrations in the top and bottom phases of the screened system points. This was done to ensure the reference phases remained in the one-phase region and to prevent DNA precipitation upon spiking. ATPS samples were diluted 1:2 with water after desalting to match the reference phase composition. Fluorescence measurements were performed according to the manufacturer's instructions after a 2-minute incubation.

3.2.6.2 Agarose gel electrophoresis

The DNA fragment partitioning into the top and bottom phases was visualized on 2% agarose gels stained with 1 µL/mL Midori Green (Nippon Genetics Europe, Düren, Germany). Electrophoresis was carried out at 120 V for 40 min using TAE as a running buffer. The gels were visualized and photographed using the GelDoc Go Gel Imaging System by Bio-Rad (Hercules, CA).

3.2.6.3 Protein quantification

Protein concentrations in the ATPS top and bottom phases from the automated screening were determined by UV-Vis absorbance at 280 nm in Greiner Bio-One 350 µL-polystyrene UV Star plates (300 µL per well). The samples from the manually prepared separation experiments were measured using the NanoDrop 2000/2000c. ATPS samples were diluted tenfold in water rather than desalted to reduce polymer and salt interference while avoiding protein loss.

3.3 Results and discussion

3.3.1 ATPS characterization

Phase diagrams were determined for several PEG/salt combinations using automatic routines on the liquid handling station to select appropriate system points for partitioning experiments (Figure A3.1). The binodal curves match those of Effio et al. [161], confirming the robustness of the

automated method developed by Oelmeier et al. [175]. As PEG molecular weight increases, the curves shift toward the origin, indicating that fewer phase-forming components are needed for phase separation. Larger PEG chains have a more hydrophobic character and lower solubility in water, correlating with a higher excluded volume and increased salting-out ability. This leads to greater incompatibility between the polymer and salt components, resulting in a larger two-phase region and steeper tie-line slopes [143, 220]. Adding NaCl also causes a slight shift of the binodal curves toward the origin. This shift is consistent with the literature and can be explained by the increased salting-out effect upon NaCl addition [143].

3.3.2 Effect of PEG molecular weight on DNA fragment partitioning

The partitioning behavior of a pure 160 bp DNA fragment in clean ATPS was investigated by varying PEG molecular weight and TLL. Systems points with V_R near 1 were chosen following the characterization experiments and spiked with 100 ng/mL DNA. Figure 3.1 shows the DNA distribution in PEG 200, PEG 400, PEG 600, and PEG 1000/phosphate systems at pH 7.4. The colored dots along the binodal curve represent the DNA recovery in the top and bottom phases of the system points marked with a cross on the corresponding tie-line. The axes show the concentrations of the phase-forming components plotted against each other in weight percent. For exact recovery values, ATPS compositions, and TLL, see Table A3.1.

A trend of top-phase DNA fragment partitioning was observed for PEG 200 and PEG 400 ATPS, while bottom-phase partitioning was favored for PEG 600 and PEG 1000 ATPS. As PEG molecular weight increases, the exclusion volume decreases, reducing space in the top phase for DNA fragments [162]. This effect seems consistent across different DNA sizes, with studies reporting that a PEG molecular weight of 600 g/mol reliably excludes pDNA ranging from 2700 to 8500 bp from the top phase of PEG/phosphate ATPS [170, 212]. Additionally, higher molecular weight polymers increase the hydrophobicity of the top phase, making it less suitable for hydrophilic DNA fragments [221].

The partitioning behavior of nucleic acids is determined not only by hydrophobic forces but also by electrostatic ones. The strong one-sided partitioning observed is partly due to the high net charge of DNA, causing electrostatic repulsion from the ion-rich bottom phase [222]. Except for one PEG 600 system point near the binodal, where DNA was partitioned evenly, all systems showed one-sided distribution. This indicates that the interplay between hydrophobic and electrostatic forces governs the partitioning behavior of short DNA fragments, as it does for longer DNA forms [149]. When there is enough “space” in the top phase to accommodate the DNA, it will partition away from the salt-rich bottom phase. Beyond a certain exclusion limit, here at PEG 600, DNA distributes into the bottom phase, despite the electrostatic repulsion with the phosphate anions. When top and bottom phase compositions are too similar, the partitioning effects balance each other out, resulting in a more even DNA distribution.

3.3.3 Effect of TLL on DNA fragment recovery

DNA fragment recovery reached 90%–100% in the top phase of the tested PEG 200 ATPS (Figure 3.1A). A trend of decreasing total recovery with increasing TLL was observed for the tested PEG molecular weights. The longer the tie-line, the higher the phosphate concentration in the bottom phase and PEG concentration in the top phase, implying an intrinsic reduction of water content [168]. High salt concentrations lead to a strong salting-out effect which lowers the solubility of additional solutes in the bottom phase, potentially causing precipitation even at DNA input

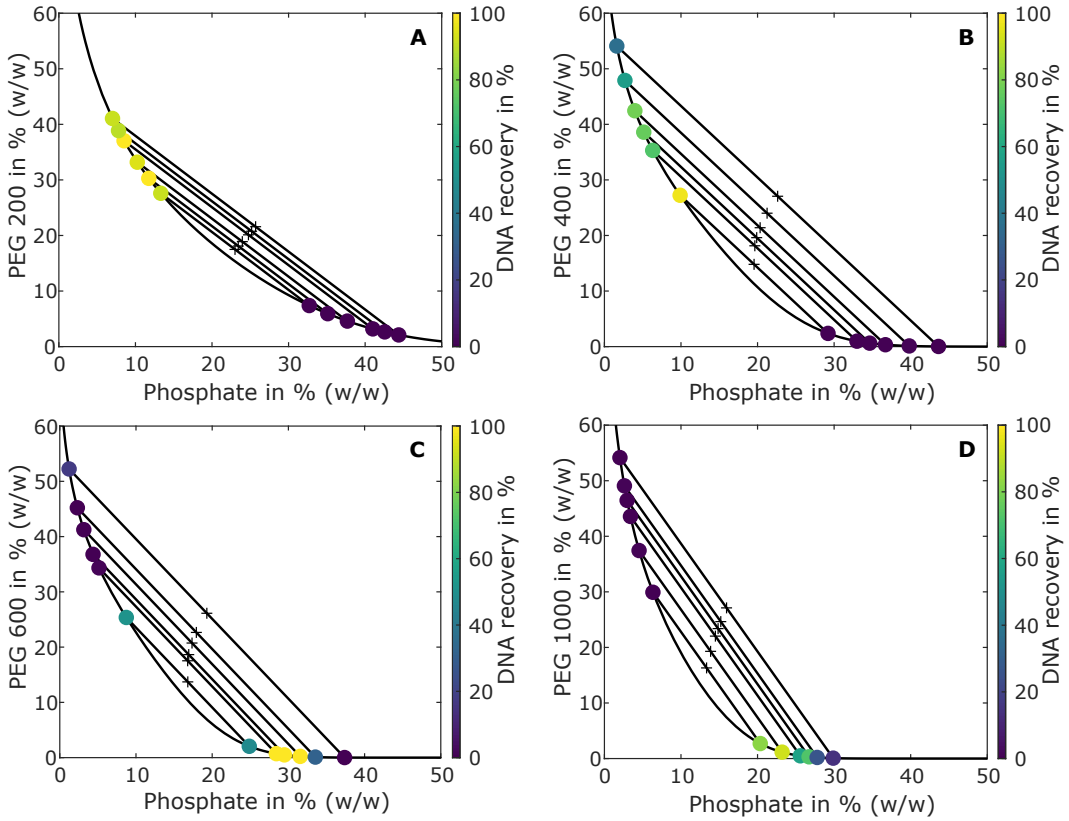


Figure 3.1 Partitioning of a 160 bp DNA fragment in selected PEG/phosphate ATPS at pH 7.4. Binodal curves and tie-lines are shown, with "x" marking the system compositions of the screened ATPS spiked with 100 ng/mL DNA. Color-coded dots along the binodal curve illustrate the DNA recovery in the respective top and bottom phases. Each partitioning experiment was carried out in triplicate.

concentrations as low as 100 ng/mL. PEG is known to precipitate nucleic acids [223], an effect that increases with PEG molecular weight [167]. This explains the decreased DNA recovery with increasing TLL and why this trend was not observed for PEG 200, since its exclusion limit allows the partitioning of the 160 bp fragment into the top phase without precipitation. Despite no visible interphase, it is presumed that the DNA missing from the mass balance accumulated at the interface between the top and bottom phases when both the exclusion effect of the polymer phase and the salting-out effect of the salt phase were too high.

A system point on a shorter tie-line should be selected to avoid precipitation due to the phase-forming components. However, points too close to the binodal curve, with similar top and bottom phase compositions, may lead to even target distribution, longer phase separation times, and reduced process robustness. This should be considered when working with biological samples like plasma that can vary in composition. Similar findings for pDNA in PEG/polyacrylate/salt ATPS were reported by Johansson et al. [214]. Thus, an optimal TLL must be identified for one-sided DNA recovery while minimizing DNA loss due to precipitation. This is especially important for cfDNA extraction for early cancer detection, as cfDNA levels in plasma are typically minimal, and losing DNA during the preanalytical steps could compromise detection [11].

3.3.4 Effect of neutral salt additives on DNA phase transition

Both gDNA and pDNA undergo a drastic phase transition from top to bottom phase upon adding neutral salt to PEG/salt ATPS for PEG with a molecular weight of less than 600 g/mol. This is likely due to uneven ion partitioning in ATPS, altering the physicochemical properties of the phases [161, 167]. The applicability of this directed partitioning behavior to cfDNA-like fragments was investigated using different neutral salts. Figure 3.2A–C shows the recovery of 100 ng/mL spiked 160 bp DNA fragment in the top and bottom phases of a 17.7% (w/w) PEG 400/17.3% (w/w) phosphate ATPS with the addition of various neutral salts up to 1.5% (w/w). This system composition was selected based on the high top phase recovery of 98% achieved in previous partitioning experiments without neutral salts. The slight shift of the binodal curve due to the salting-out ability of the added salts was not considered here due to the low salt amounts added, but changes in phase volume ratios were taken into account [143, 167].

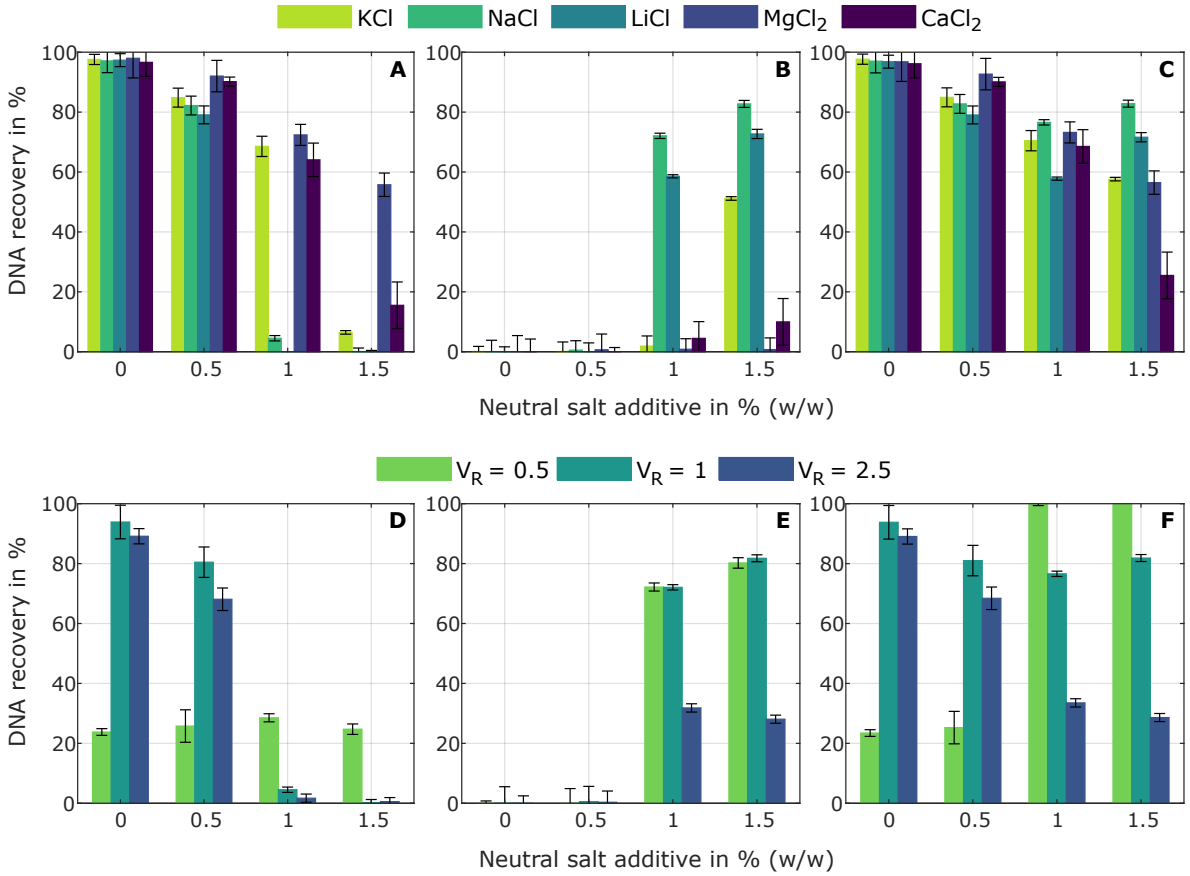


Figure 3.2 Partitioning of 160 bp DNA fragment in a 17.7% (w/w) PEG 400/17.3% (w/w) phosphate ATPS with varying neutral salt additives: (A) top phase, (B) bottom phase, and (C) total recovery. Partitioning of 160 bp DNA fragment in ATPS along the same tie-line with varying phase volume ratio (VR) for different NaCl concentrations: (D) top phase, (E) bottom phase, and (F) total recovery. The respective PEG 400/phosphate compositions for VR = 0.5, 1, and 2.5 are 12.2% (w/w)/21.0% (w/w), 17.7% (w/w)/17.3% (w/w), and 24.0% (w/w)/13.0% (w/w). Systems were spiked with 100 ng/mL DNA in duplicate.

A clear phase transition from top to bottom was observed with 1.5% (w/w) KCl, but total DNA recovery was only 58%, suggesting DNA may have accumulated at the interface between the top and bottom phases rather than fully partitioning into the bottom phase. The lack of a visible interphase made sampling and DNA quantification infeasible, though other studies confirm interphase accumulation at higher pDNA concentrations [211]. The phase transition was more pronounced for systems containing NaCl or LiCl, occurring at 1% (w/w) added salt. For 1.5% (w/w) NaCl and LiCl, bottom phase recoveries were 83% and 73% respectively, while top phase recoveries fell below the detection limit. This may be attributed to the chloride anions' preferential partitioning into the PEG-rich top phase, leading to DNA exclusion from the top phase into the interphase. As more neutral salt is added, the exclusion effect of the chloride anions becomes stronger and more co-cations facilitate the transition of the DNA from the interphase into the bottom phase [157]. However, adding even more salt may not necessarily increase bottom-phase recovery due to solubility limitations and DNA precipitation.

Systems containing $MgCl_2$ or $CaCl_2$ did not exhibit a phase transition at tested concentrations. Instead, total DNA recovery decreased, with only 56% and 25% recovered at 1.5% (w/w) $MgCl_2$ or $CaCl_2$, respectively. Precipitation was visible even in blanks without DNA containing $MgCl_2$ or $CaCl_2$, and to a lesser extent LiCl, suggesting the solubility limit was exceeded. The precipitating salts may lead to DNA co-precipitation, explaining the low recoveries. The observed precipitation correlates with the position of the chloride counter-cations in the Hofmeister series. The Mg^{2+} and Ca^{2+} cations are more chaotropic than the other tested cations, exhibiting higher incompatibility with the PEG and phosphate phases.

The NaCl-induced phase transition was visualized using agarose gel electrophoresis to qualitatively confirm the fluorescent dye measurements (Figure A3.2). No band was visible in the bottom phase samples with 0% and 0.5% (w/w) added salt, whereas a band appeared only in the bottom phase for samples with over 1% (w/w) NaCl. The low resolution is attributed to the low DNA concentration in the ATPS.

These results demonstrate that short DNA fragments behave similarly to other negatively charged molecules when neutral salt is added to PEG/salt ATPS. Among the salts tested, NaCl was the most effective in directing DNA partitioning from the top to the bottom phase. NaCl did not cause visible precipitation, and a bottom phase recovery of 83% was achieved with just 1.5% (w/w) added salt, corresponding to 0.3 M.

3.3.5 Effect of phase volume ratio on DNA concentration

In addition to capturing target molecules from complex mixtures, ATPS offer the advantage of integrating a volume reduction step by adjusting the phase volume ratio. Theoretically, all system points along the same tie-line result in the same target distribution regardless of the volume ratio, as the top and bottom phases share the same equilibrium composition [139]. This means a target molecule can be concentrated by reducing the volume of the phase where it accumulates. Three ATPS along the same tie-line with PEG 400/phosphate compositions of 12.2% (w/w)/21.0% (w/w), 17.7% (w/w)/17.3% (w/w), and 24.0% (w/w)/13.0% (w/w) and respective top to bottom phase V_R of 0.5, 1, and 2.5 were spiked with 100 ng/mL DNA and up to 1.5% (w/w) NaCl. Figure 3.2D–F shows the recovery in the top and bottom phases and the total DNA recovery for these system points.

Contrary to expectations, the partitioning behavior of pure DNA fragments in clean ATPS was not independent of V_R. Previous results showed a phase transition upon NaCl addition for $V_R = 1$,

with DNA recovery shifting from 94% in the top phase to 82% in the bottom phase after adding 1.5% (w/w) NaCl. However, partitioning and total recovery differed for the other two V_R values tested. Luechau et al. [23] also observed varying partition behavior of pDNA in PEG 300/phosphate ATPS with different V_R, attributing it to changes in the PEG/salt ATPS properties induced by the solute. This explanation is unlikely to apply here, as the systems investigated contain only PEG 400 and salt, with a negligible amount of pure spiked DNA.

Instead of a DNA concentration in the top phase of the $V_R = 0.5$ system point without NaCl, recovery dropped to 24%. Systems with added salt exhibited a nearly constant top phase recovery, rather than the drastic phase switch observed for $V_R = 1$ and 2.5. This suggests that reducing the volume of the phase where DNA is expected to accumulate might lead to DNA loss rather than concentration. It appears that the solubility limit is reached as the phase volume decreases, making the partitioning coefficient dependent on DNA concentration. However, it remains unclear whether DNA exceeding the solubility limit precipitates or accumulates at the interphase, where it is difficult to sample and hence not detectable. The observation that DNA recovery in the bottom phase of the $V_R = 0.5$ system with over 1% (w/w) added salt reaches up to 80%, while top phase recovery remains constant at around 24%, suggests DNA may have migrated from the interphase to the bottom phase. Again, this is likely due to the preferential ion distribution upon NaCl addition, making bottom phase distribution more favorable for DNA fragments.

DNA was undetectable in the bottom phase of all system points containing less than 1% (w/w) added NaCl, regardless of the V_R. At 1.5% (w/w) NaCl, bottom phase recovery was about 80% for the $V_R = 1$ and 0.5 ATPS, but only 28% for $V_R = 2.5$, despite expectations of DNA concentration in the bottom phase. This discrepancy may result from the higher PEG input concentration required for ATPS with a higher V_R. High polymer concentrations with adequate salt concentrations can favor DNA condensation, leading to precipitation [77]. Since the top and bottom phases of system points along one tie-line should have the same compositions, DNA may precipitate before phase separation.

3.3.6 Selective DNA phase transition by NaCl addition

Further partitioning experiments with plasma proteins assessed the selectivity of the NaCl-induced DNA phase switch. To avoid potential interactions between the components, DNA, HSA, and IgG were spiked separately into 17.7% (w/w) PEG 400/17.3% (w/w) phosphate ATPS containing NaCl concentrations up to 1.5% (w/w). The recoveries in the top and bottom phases and total recoveries are shown in Figure 3.3. The x-axis indicates the NaCl concentrations in weight percent.

Protein concentrations remained below the detection limit in the bottom phases of the investigated systems, whereas 82% of spiked DNA was recovered in the bottom phase with 1.5% (w/w) NaCl. HSA recovery in the top phase ranged between 84% and 92%, while IgG top phase recovery was around 81% across the investigated NaCl range. This suggests hydrophobic interactions play a larger role in protein distribution than electrostatic effects, at least at the investigated salt concentrations [163]. In previous studies, where gDNA was seen as a contaminant to protein targets, adding NaCl to the ATPS helped improve purification efficiency [161]. Here, a DNA-selective phase transition could be utilized to purify cfDNA from protein contaminants in a plasma sample by enriching the DNA in the protein-depleted bottom phase while retaining an acceptable yield.

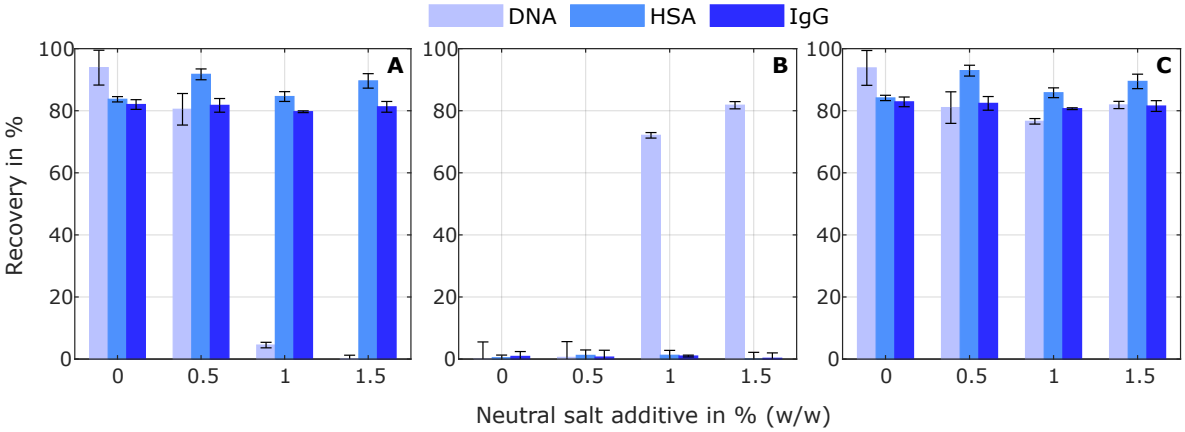


Figure 3.3 Partitioning of 160 bp DNA fragment, HSA, and IgG in a 17.7% (w/w) PEG 400/17.3% (w/w) phosphate ATPS with varying NaCl concentrations: (A) top phase, (B) bottom phase, and (C) total recovery. Systems were made in triplicate and spiked with either 100 ng/mL DNA, 2.5 mg/mL HSA, or 2.5 mg/mL IgG.

3.3.7 DNA fragment separation from synthetic plasma

Promising system points from pure component experiments were tested with a synthetic plasma matrix to evaluate whether potential interactions between plasma proteins and DNA could alter the observed partitioning behaviors. The matrix, composed of a plasma-like electrolyte solution, was used to substitute water in the selected ATPS. The systems were spiked with the two main plasma protein fractions HSA and IgG, and with DNA, only after premixing the phase-forming components to rule out the possibility of initial precipitation due to the high PEG and salt concentrations used. The top and bottom phase recoveries are listed in Table 3.2 along with the exact ATPS compositions. Values below the detection limit are reported as zero.

Table 3.2 160 bp DNA fragment and total protein recoveries in the top and bottom phases of selected PEG/phosphate ATPS made with a synthetic plasma matrix. [a] HSA:IgG spike-in ratio of 4:1 for a final protein concentration of 10 mg/mL; [b] PEG 400; [c] PEG 1000.

ATPS composition in % (w/w)			**Top phase recovery** in %		**Bottom phase recovery** in %	
PEG	**Phosphate**	**NaCl**	**DNA**	**Protein**[a]	**DNA**	**Protein**[a]
17.7[b]	17.3	0	96.2 ± 10.3	72.0 ± 0.5	0	1.0 ± 0.1
17.7[b]	17.3	1.5	0	73.7 ± 0.4	87.7 ± 8.8	1.0 ± 0.1
19.5[c]	13.8	0	0	32.6 ± 0.3	86.6 ± 4.0	9.6 ± 0.3

The DNA and protein partitioning behavior in PEG 400 systems with synthetic plasma matched those of clean systems with separate components. DNA partitioning remained unchanged, indicating that interactions with proteins or electrolytes did not play a significant role at the tested concentrations. Adding 1.5% (w/w) NaCl drastically changed DNA distribution without affecting protein distribution, confirming the selectivity of the NaCl-induced phase switch for systems with mixed components. This enabled a DNA recovery of up to 88% in the virtually protein-free bottom phase of the PEG 400/phosphate system. Another way to separate the DNA fragments from the protein fraction is to use the precipitating ability of higher molecular weight PEG to an advantage.

Because of the reduction of excluded volume and increasing upper phase hydrophobicity [224] as well as the solubility limitation in the bottom phase, 42.2% of the total protein precipitated and formed a visible interphase in the tested PEG 1000 system. Only 9.6% of the protein partitioned into the bottom phase, where 87% of the DNA was recovered.

These partitioning experiments with synthetic plasma offer promising tools for cfDNA separation from plasma samples, such as selectively switching DNA distribution or increasing protein precipitation. However, more research is needed to confirm their applicability to complex plasma samples, where protein concentration is 6–8 times higher than the protein input tested here [225]. Also, cfDNA concentrations in plasma vary widely based on cancer type and stage, exceeding 100 ng/mL only in some types of advanced cancer cases. Additionally, the nucleosomal nature of cfDNA might affect partitioning, as histone-bound DNA could behave differently if no lysis step is performed. However, a lysis step could alter protein distribution and introduce new components to the ATPS that must be accounted for.

3.4 Conclusions

This work presents the basics of short DNA fragment partitioning in PEG/phosphate ATPS, laying the groundwork for developing an ATPS-based cfDNA extraction system from plasma for liquid biopsy. Distribution experiments with pure components were carried out on a high-throughput screening platform to assess the effect of PEG molecular weight, TLL, neutral salt additive, and phase volume ratio on DNA partitioning and recovery. The partitioning behavior aligns with previous pDNA studies. Short DNA fragments distribute into the bottom phase for higher molecular weight PEG and into the top phase for PEG smaller than 600 g/mol. Minimizing DNA loss from precipitation can be managed by selecting system points on shorter tie-lines and considering solubility limits when varying the phase volume ratio. Adding neutral salts like NaCl can selectively direct DNA fragments into the protein-depleted bottom phase. Separation experiments with a synthetic plasma matrix containing HSA and IgG resulted in 88% DNA recovery and nearly 100% protein removal in the salt-rich bottom phase, demonstrating the potential of ATPS for cfDNA extraction from plasma.

Acknowledgments

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Supporting information

Appendix **A3** contains the Supporting Figures associated with this chapter.

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4

Extraction and purification of short DNA fragments from plasma using aqueous two-phase systems for liquid biopsy

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Abstract

Liquid biopsy is a minimally invasive method for tumor profiling, relying on detecting and analyzing tumor-derived biomarkers present in bodily fluids. Key biomarkers, such as circulating cell-free DNA (cfDNA), are often present at very low concentrations in plasma. Efficient extraction and purification of cfDNA are critical, as impurities such as proteins and salts can interfere with downstream applications like next-generation sequencing. This study demonstrates the potential of aqueous two-phase systems (ATPS) as a capture step for short DNA fragments from human plasma, building on prior findings with synthetic systems. DNA partitioning, recovery, and separation from plasma proteins were evaluated in selected polyethylene glycol (PEG)/phosphate/plasma ATPS. PEG 1000 systems proved to be more effective than PEG 400 systems, achieving up to 90% DNA recovery in the bottom phase. Total protein concentration was reduced to 3 mg/mL, while most of the protein was removed as reversible aggregates forming an interphase. System robustness was confirmed using plasma from various donors and blood collection tube types, showing consistent DNA recovery and phase separation behavior. To address dilution caused by liquid phase-forming components, a modified ATPS design using solid PEG and phosphate was implemented. This approach increased plasma input from 37.7 to 66.7% (w/w) without altering DNA or protein partitioning, effectively maximizing the plasma load for extraction. Lastly, a reverse elution protocol using purification plates containing a desalting matrix was introduced to concentrate the DNA-containing phase while removing salts and residual proteins, improving its suitability for downstream sequencing-based applications.

4.1 Introduction

Cancer remains one of the leading causes of death worldwide, with the global cancer burden projected to rise by 77% by 2050, according to the International Agency for Research on Cancer [226]. This underscores the need for precise genetic profiling to improve cancer diagnosis and treatment. Tissue biopsy, the gold standard for tumor characterization, is limited by its invasive nature and dependence on single, localized tumor samples.

Liquid biopsy has emerged as a minimally invasive alternative to overcome these limitations, particularly for tumors inaccessible through surgery [227]. Liquid biopsy analyzes cancer-derived biomarkers found in the bloodstream and other bodily fluids, enabling tumor profiling from a simple blood draw. Its ease of repetition and ability to capture tumor heterogeneity make liquid biopsy a versatile tool for various applications across disease progression, including early cancer detection, monitoring treatment response, tracking residual disease post-surgery, and identifying emerging tumor clones [5].

Among the various biomarkers accessible through liquid biopsy, circulating cell-free DNA (cfDNA) holds particular promise. This double-stranded DNA is released primarily during apoptosis, resulting in highly fragmented DNA with a predominant length of 167 bp [205]. A small fraction of cfDNA originates from tumor cells as circulating tumor DNA, which is often even shorter, typically ranging from 132 to 143 bp [206].

The cfDNA-based liquid biopsy workflow typically begins with blood collection in specialized stabilizing tubes to prevent contamination by genomic DNA (gDNA) from lysed blood cells [11]. Plasma is then fractionated using a double centrifugation protocol to maximize recovery and remove residual blood cells [81]. cfDNA is extracted from the plasma fraction, purified, and concentrated for

sequencing-based analysis. Techniques such as droplet digital PCR or next-generation sequencing (NGS) are employed based on the required sensitivity and coverage [228]. Although sequencing yields actionable insights into tumor genetics, cfDNA extraction remains a critical bottleneck, requiring high recovery and purity from minimal starting material, typically only 1–40 ng/mL of plasma [10].

Commercial cfDNA extraction kits primarily rely on DNA adsorption to solid phases, such as silica membranes or magnetic beads, in the presence of chaotropic salts [95]. While effective, these methods can lead to residual chaotropic salt contamination, which inhibits amplification-based assays. To address this, standard kits use bind-wash-elute protocols with multiple wash steps. However, this can reduce overall recovery and introduce a fragment length bias due to uneven extraction efficiency, particularly of short fragments [14][15]. These limitations highlight the need for novel approaches to cfDNA extraction that improve efficiency, scalability, and standardization, facilitating the broader implementation of liquid biopsy analyses in clinical settings.

Aqueous two-phase systems (ATPS) provide a promising, low-cost, and rapid alternative for initial DNA capture and concentration, particularly in biochemical and analytical diagnostic applications such as liquid biopsy, where scalability and automation are critical [229]. By adding phase-forming components, such as polymers and salts, directly to plasma, ATPS can enable the selective partitioning of cfDNA into one phase while enriching other plasma components, such as proteins, in the opposite phase. To ensure compatibility with downstream analysis, an additional purification step is required to remove residual phase-forming components and contaminants that may interfere with sensitive detection techniques. For DNA collected in the polyethylene glycol (PEG)-rich top phase, alcohol-induced precipitation is a viable approach [136]. When DNA is found in the salt-rich bottom phase, desalting resins or reverse elution techniques can be utilized. These methods allow the purified DNA to be recovered in the flow-through, while salt and other impurities are effectively retained [185].

Studies on ATPS-based DNA extraction have predominantly focused on gDNA [210][22] or plasmid DNA [23][211], which differ significantly from cfDNA in both size and conformation. Detailed insights into the partitioning behavior of short DNA fragments that mimic cfDNA [136] remain limited. Additionally, most existing studies investigate partitioning effects in clean ATPS or through single-solute experiments [230]. However, it has been reported that biomass in ATPS can influence phase formation, often causing shifts in the binodal curve [25].

Available studies exploring ATPS loaded with whole blood [218] or plasma [24] typically focus on separating blood proteins, such as albumin, immunoglobulin G (IgG), or hemoglobin, with little or no mention of DNA. Selvakumar et al. [230] describe an approach to increase biomass loading by introducing solid phase-forming components, such as undissolved PEG and salts, instead of relying on pre-dissolved solutions. This allows the substitution of the liquid typically used to dissolve the phase-forming chemicals, such as water, with biological samples like plasma containing a high amount of water.

In a previous study, we investigated the partitioning behavior of a 160 bp DNA fragment in clean PEG/salt ATPS using automated routines on a liquid handling station [231]. Optimal conditions for short DNA fragment recovery were identified through high-throughput screening, and experiments with selected system points were carried out using a synthetic plasma solution. The results demonstrated that a PEG 1000/phosphate system led to selective DNA partitioning into the bottom phase, with proteins mainly remaining in the top phase. Furthermore, the addition of neutral salts, such as NaCl, to a PEG 400/phosphate system significantly shifted DNA partitioning from the top to the bottom phase without affecting protein distribution. Both systems achieved high DNA recoveries of around 87% with substantial protein removal, meeting the criteria for selective

cfDNA recovery. However, it remains uncertain whether the partitioning behavior and separation efficiency observed in synthetic systems can be replicated when using a complex biological matrix like human plasma in ATPS.

Based on the insights described above, we present data on the distribution of short DNA fragments in ATPS containing human plasma. The robustness of the proposed ATPS capture step is evaluated using blood collected from individual donors and stored in various tube types. To increase plasma input volume and minimize the dilution of target DNA by phase-forming components, an ATPS design incorporating solid PEG and salts is introduced. In addition, further purification and simultaneous concentration of the DNA-containing phase are explored using an adapted reverse elution protocol. This study establishes a foundation for developing an ATPS-based cfDNA extraction process tailored for clinical liquid biopsy applications.

4.2 Materials and methods

4.2.1 Chemicals and stock solutions

PEG 400 (Sigma-Aldrich, St. Louis, MO, USA) and PEG 1000 (Merck KGaA, Darmstadt, Germany) were used in synthesis grade. $NaH_2PO_4 \cdot H_2O$ and NaCl were obtained from Merck, while K_2HPO_4 was purchased from VWR International (Radnor, PA, USA). PEG and salt stock solutions, along with purified 160 bp DNA fragments for spiking experiments, were prepared as described by Meutelet et al. [231].

4.2.2 Acquisition and processing of blood samples

Blood samples were collected from healthy donors at the Institute of Experimental Hematology and Transfusion Medicine at the University Hospital Bonn (Bonn, Germany). The study procedures were performed in compliance with the approval granted by the Ethics Committee of the Medical Faculty at the University of Bonn (protocol code 070/05) and adhered to the Declaration of Helsinki. Written informed consent was obtained from all participants, and the procedures were carried out in accordance with institutional guidelines. After giving consent, approximately 10 mL of whole blood was collected into one of the following stabilizing blood collection tubes: EDTA, PAXgene® Blood ccfDNA Tube (PreAnalytiX GmbH, Hombrechtikom, Switzerland), or Cell-Free DNA BCT® (Streck, La Vista, NE, USA). Blood samples were shipped at room temperature and processed within 24 hours using a double centrifugation protocol to maximize plasma recovery. Whole blood was centrifuged in the collection tubes at 1600 × g for 10 min at room temperature. The plasma layer was then collected and centrifuged again at 16000 × g for 10 min to remove any remaining blood cells. Plasma aliquots of 1 mL were stored at -80°C until further use.

4.2.3 ATPS preparation and sampling

All ATPS partitioning experiments were performed at room temperature in an air-conditioned laboratory to minimize temperature-related variability in phase behavior. Systems were prepared with a physiological pH of 7.4 by adjusting the salt ratio in the phosphate buffer stock solution. A schematic overview of the experimental workflow is provided in Figure A4.1 in the supplementary material, and includes the following main steps: ATPS preparation, sampling of the individual phases, sample dilution, and subsequent analysis. Key system characteristics, including binodal

coefficients and tie-line slopes, are summarized in Table A4.1. The methods used for automated binodal and tie-line determination are described in detail in Meutelet et al. [231], along with the corresponding phase diagrams.

Plasma aliquots were thawed at room temperature on the lab bench for 45 min. Any sedimented material was left undisturbed to avoid resuspension. ATPS with a total volume of 600 µL were manually prepared in 1.5 mL Eppendorf tubes by sequentially adding plasma, 160 bp DNA fragments (final concentration of 200 ng/mL per ATPS), 40% (w/w) phosphate stock solution, and 70% (w/w) PEG stock solution. The specific volumes of components used to prepare each system point are listed in Table A4.2. For each ATPS composition, a blank control was prepared by spiking plasma with water instead of DNA stock to allow background correction. After thorough mixing by vortexing, phase separation was accelerated by centrifugation at 8000 × g for 5 min. Phase sampling and volume determination were performed with care using a pipette. The top phase was first collected and transferred to a new tube without disturbing the interphase or bottom phase. The bottom phase was then recovered by gently pressing the interphase layer against the tube wall. Remaining liquid trapped in the aggregated interphase was removed by brief centrifugation before the interphase was resuspended in 100 µL water. All samples were then diluted tenfold in water to reduce interference of phase-forming components during quantification analyses.

4.2.4 ATPS preparation with solid components

To increase the plasma input volume without altering the amount of phase-forming components in the ATPS, PEG and salts were added directly to the plasma as solids rather than pre-dissolved stock solutions. Waxy PEG cylinders of the appropriate weight were prepared by melting PEG 1000, solid at room temperature, in a microwave and allowing it to harden in a cylindrical mold for a few minutes. The PEG cylinders and phosphate salts were then added to 1.5 mL Eppendorf tubes containing plasma spiked with 160 bp DNA fragments. To ensure complete solubilization, the tubes were placed on a thermo-shaker at 25°C for 5 min, briefly vortexed, and mixed for an additional minute. After a final vortexing step, the tubes were centrifuged at 8000 × g for 5 min to achieve phase separation. Sampling and sample preparation for analysis followed the same protocol used for ATPS prepared with pre-dissolved components. The amount of phase-forming components and plasma used to prepare the investigated system points is specified in Table A4.3.

4.2.5 Combined DNA purification and concentration

Reverse elution plates from the EchoCLEAN Organic Solvent DNA CleanUp Kit (BioEcho, Cologne, Germany) were tested as a combined DNA purification and concentration step from the salt-rich phase. This reverse elution method uses a purification matrix that retains impurities and salts, allowing DNA fragments to be recovered in the flow-through. The plates were used according to the manufacturer's instructions with slight modifications to the commercially available product. Specifically, a custom matrix filling volume of 500 µL was used, and various centrifugation speeds were tested during conditioning and elution to optimize DNA recovery and concentration. Each well was loaded with 100 µL of a 200 ng/mL 160 bp DNA solution, and each combination of conditioning and elution speed was tested in triplicate. To assess the desalting capacity of the matrix, conductivity measurements were performed using the conductivity sensor of an ÄKTApurifier system (Cytiva, Uppsala, Sweden).

4.2.6 Analytical methods

4.2.6.1 DNA quantification

DNA was quantified using the Quant-iT™ PicoGreen™ dsDNA HS assay kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Black 384-well microplates with a total volume of 20 µL per well were used for the top, bottom, and interphase samples. Each sample was analyzed alongside blanks with similar composition but without DNA to account for any interference from phase-forming or plasma components with the assay. These blank phases were also spiked with a known amount of 160 bp DNA fragments to generate a reference signal for recovery calculations. Fluorescence measurements were conducted according to the manufacturer's instructions after an incubation period of 2 min. Each sample, blank, and reference was measured in triplicate. DNA recoveries in each phase were calculated by multiplying the measured DNA concentrations by the respective phase volumes and dividing by the amount of input DNA.

4.2.6.2 Protein quantification

Total protein concentrations in the ATPS phases were measured in triplicate using the NanoDrop™ 2000/2000c UV/Vis spectrometer (Thermo Fisher Scientific Inc.). Water was used as a blank, and plasma used in the ATPS was diluted tenfold and measured to serve as a reference for recovery calculations. An average extinction coefficient of 10 was assumed for the plasma protein absorbance measurements at 280 nm.

4.2.6.3 SDS-PAGE analysis

Protein content in the phases was visualized using reducing SDS-PAGE with 50 mM DTT. Phase samples were diluted tenfold in water or purified using reverse elution plates (BioEcho) before mixing with NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) and denaturation at 70°C for 10 min. Proteins were separated on NuPAGE 4–12% BisTris Mini Protein Gel using an Xcell SureLock Mini-Cell (Invitrogen) at 200 V, stained with InstantBlue Coomassie protein stain (US Biological, Salem, MA, USA), and de-stained in water. A Novex Mark12 protein standard (Invitrogen) was used as a molecular weight marker, and gels were imaged with a GelDoc Go system by Bio-Rad (Hercules, CA, USA).

4.3 Results and discussion

4.3.1 DNA capture from plasma using ATPS

4.3.1.1 ATPS formation

The applicability of previous findings from synthetic plasma experiments was assessed for ATPS prepared with human plasma. In preliminary experiments, phase formation of an exemplary PEG/phosphate/plasma ATPS was investigated, as shown in Figure 4.1.

Upon adding PEG and salt solutions to plasma, initial turbidity indicated the formation of protein aggregates [232]. After centrifugation, the resulting ATPS exhibited three distinct regions: a yellowish top phase, a visibly defined interphase, and a clear bottom phase. These findings suggest a three-phase partitioning system with a solid interphase rather than the conventional aqueous two-phase partitioning typically observed in synthetic systems. However, it remains unclear whether

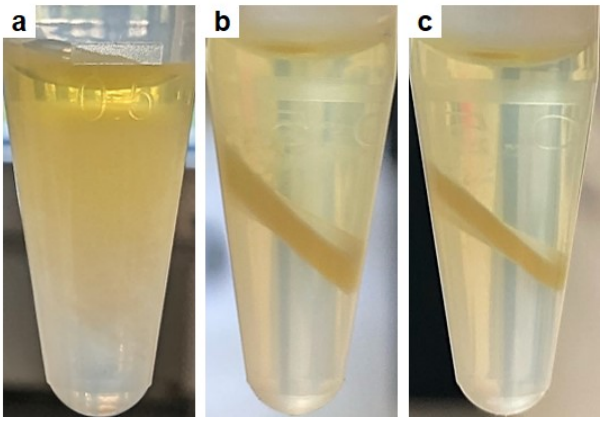


Figure 4.1 PEG 1000/phosphate/plasma ATPS: (a) before phase separation, (b) after centrifugation at 2000 × g, (c) after centrifugation at 8000 × g.

DNA detected in the interphase actively partitions into this region or if it co-precipitates or becomes physically trapped, thereby altering its initial partitioning behavior. The thickness and appearance of the interphase varied with the specific ATPS composition. For example, PEG 1000 systems formed thicker and cloudier interphases compared to PEG 400 systems, where the interphase appeared as a thin, compact disk. This observation aligns with the higher precipitating power of PEG with increased molecular weight [167].

Similar to synthetic PEG/phosphate systems, the top phase of the plasma-containing ATPS was primarily composed of polymer, while the bottom phase consisted predominantly of phosphate salts. After removing all liquid constituting the top and bottom phases, the interphase was redissolved in water, indicating it consisted predominantly of reversibly aggregated proteins. This enabled the analysis of DNA and protein content in the interphase, facilitating the closure of mass balances which was previously impossible for clear, synthetic systems lacking a visible interphase.

Interestingly, aside from the formation of an interphase, the observed phase volume ratios were comparable to those measured in control systems using water as the matrix instead of plasma. This indicates that replacing water with plasma does not significantly alter the phase equilibrium of the selected formulations, aligning with the findings of Selvakumar et al. for ATPS PEG 1000/phosphate ATPS loaded with bovine plasma [25]. Manually determined phase volumes for each system point are provided in Table A4.4.

Additional experiments revealed that centrifuging the mixed systems at a higher centrifugation speed of 8000 × g rather than the 1500 × g used for synthetic ATPS produced a slightly more compact and manageable interphase (Figure 4.1b and c). This adjustment did not affect overall DNA and protein distribution but improved the removal of residual liquid from the interphase. Consequently, all plasma ATPS experiments were performed with phase separation at 8000 × g for better handling and consistency.

Although the DNA input concentration of 200 ng/mL used in this study exceeds typical cfDNA levels in plasma [10], it was chosen to ensure reliable detection with fluorescence-based assays. Since partitioning behavior in ATPS remains independent of concentration as long as solubility limits are not exceeded [168], the observed DNA distribution is expected to remain consistent at lower, clinically relevant DNA concentrations.

4.3.1.2 PEG 400/phosphate/plasma ATPS

In previous synthetic plasma experiments, DNA and protein were predominantly recovered in the top phase of a PEG 400/phosphate ATPS. However, the addition of NaCl resulted in DNA migrating almost entirely into the bottom phase, separating it from the main protein fraction. As explained in more detail in Meutelet et al. [231], this directed switch is mainly due to uneven ion distribution in the ATPS, which changes the physico-chemical properties of the phases [167]. The partitioning behavior of a 160 bp DNA fragment in a 17.7% (w/w) PEG 400/17.3% (w/w) phosphate ATPS containing 24.0% (w/w) human plasma with and without NaCl was investigated for comparison. Figure 4.2 shows the respective DNA and plasma protein recoveries in the top phase (TP), bottom phase (BP), and interphase (IP).

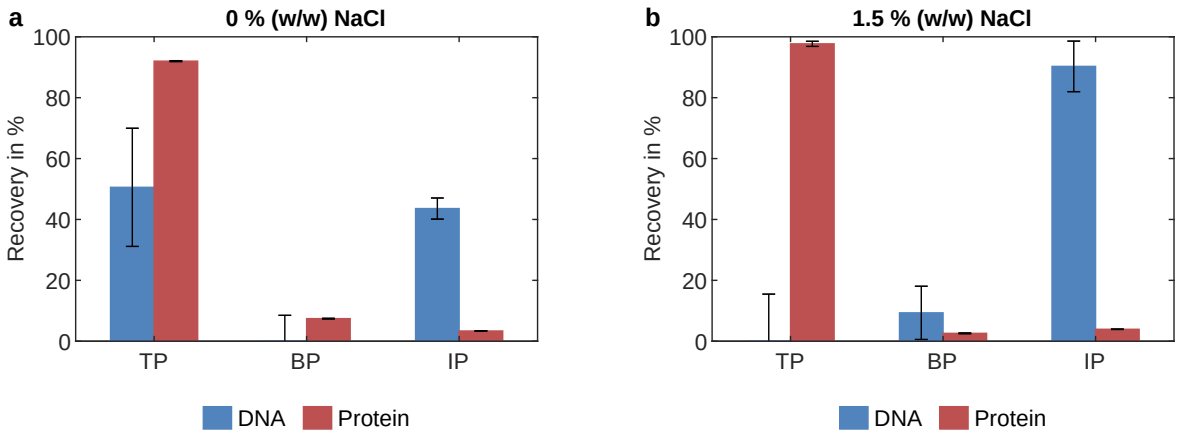


Figure 4.2 160 bp DNA and plasma protein recoveries in the top phase (TP), bottom phase (BP), and interphase (IP) of a 17.7% (w/w) PEG 400/17.3% (w/w) phosphate/24.0% (w/w) plasma ATPS (a) without (b) and with 1.5% (w/w) NaCl.

Unlike the synthetic plasma system, where DNA was fully recovered in the top phase, DNA was almost evenly distributed between the top phase and the interphase. Protein distribution, in contrast, closely mirrored prior findings, even though the total protein concentration in the plasma sample was approximately 6 times higher than the 10 mg/mL human serum albumin (HSA) and IgG mix spiked into the synthetic solution. Upon adding NaCl to the plasma-based ATPS, DNA did not transition to the bottom phase as observed in synthetic plasma but was instead primarily retained in the interphase. These results suggest that the interphase interferes with DNA partitioning and phase transition, likely because of the physical barrier formed by aggregated proteins and potential DNA–protein interactions [233].

Interestingly, while the formation of an interphase may appear disadvantageous, it presents a potential opportunity for selective DNA capture. In this PEG 400 system, the interphase contains only a fraction of the total protein and is virtually free of PEG and salt, reducing the risk of contaminants that could interfere with downstream analyses [103]. DNA concentration can be significantly increased by redissolving the interphase in a small volume of water or buffer, making this approach a promising initial capture and concentration step for cfDNA from plasma.

Additional experiments demonstrated that DNA collected in the interphase could be concentrated 25-fold, from an initial concentration of 200 ng/mL in plasma to over 5000 ng/mL, by redissolving the interphase in just 10 µL of water (data not shown). However, despite the low protein recovery in the redissolved interphase (3% of the input amount), the resulting protein concentration was

21.3 mg/mL, far exceeding the tolerance thresholds for sequencing-based downstream analyses [234]. Performing a lysis step on the DNA-containing interphase, followed by an additional purification step, could be a viable option to reduce residual protein levels.

4.3.1.3 PEG 1000/phosphate/plasma ATPS

Building on the insights from earlier PEG 1000 ATPS investigations, where DNA partitioned into the bottom phase with minimal protein recovery, the distribution of DNA and protein in a 19.5% (w/w) PEG 1000/13.8% (w/w) phosphate/37.7% (w/w) plasma system was analyzed (Figure 4.3). For a detailed description of the observed partitioning behavior, see Meutelet et al. [231]. The higher molecular weight of PEG 1000 requires fewer phase-forming components compared to PEG 400 systems, allowing for a greater plasma input volume. This effect arises from the binodal shift towards the origin, attributed to the increased hydrophobicity and exclusion effects of higher molecular weight PEG [221]. Processing larger plasma volumes offers a distinct advantage, as the probability of detecting potential rare cfDNA variants present at very low concentrations is increased [235][236].

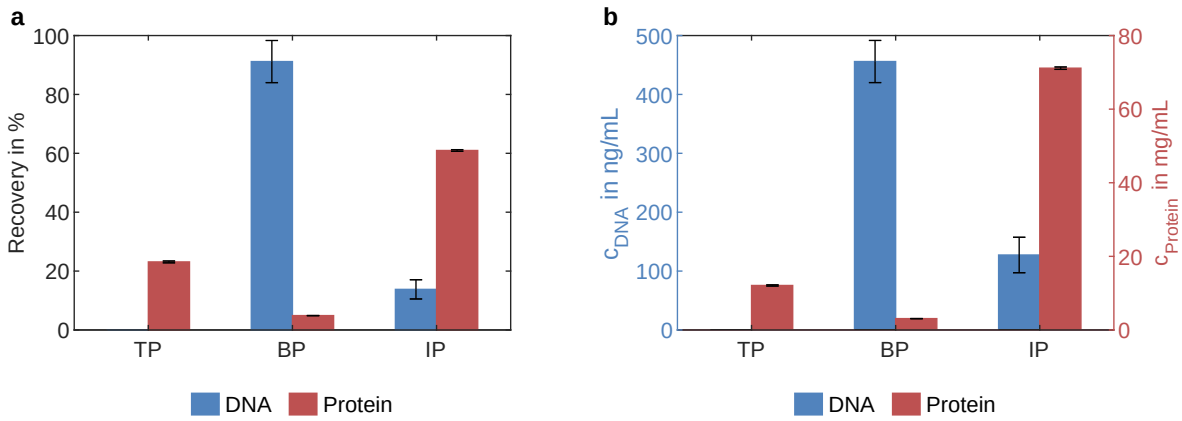


Figure 4.3 160 bp DNA and plasma protein (a) recoveries and (b) concentrations in the top phase (TP), bottom phase (BP), and interphase (IP) of a 19.5% (w/w) PEG 1000/13.8% (w/w) phosphate/37.7% (w/w) plasma ATPS.

With the ability to sample the redissolved interphase, we confirmed that the discrepancies in DNA and protein mass balances in clean systems without plasma were due to accumulation at the interface between the top and bottom phases. As expected from the synthetic plasma system experiments, up to 90% of the spiked DNA was recovered in the bottom phase, while only 5% of the total plasma protein, corresponding to a concentration of 3.1 mg/mL, was detected in the same phase. Reducing SDS-PAGE analysis (Figure A4.2) was used to qualitatively assess the protein content in the phases of the PEG 1000 and PEG 400 systems. The results indicated that protein distribution varies with the polymer molecular weight and confirmed that protein recovery in the bottom phase is the lowest. The lower protein concentration left in the bottom phase than the interphase, along with the capability for higher plasma input, suggests that PEG 1000 systems are better suited than PEG 400 systems for efficient cfDNA extraction from plasma. This is why all following experiments were performed using PEG 1000 systems.

However, although the DNA concentration in the bottom phase can reach several hundred ng/mL, the protein concentration remains substantially higher. This poses a significant challenge

for downstream sequencing techniques requiring PCR, as blood proteins like IgG, lactoferrin, and hemoglobin are known to be potent PCR inhibitors [237]. These findings highlight the inherent limitations of relying on an entirely ATPS-based system for extraction, as achieving sufficient DNA concentration and purity in a single step is not feasible. Nevertheless, as an initial capture step, ATPS proves to be an efficient and rapid method, removing over 95% of plasma proteins while retaining high DNA recovery. Following this primary capture step, addressing the remaining challenges, namely removing salts and residual protein from the DNA-containing bottom phase, is critical for achieving the purity required for sensitive downstream analyses.

To the best of our knowledge, only one other study has described an ATPS-based extraction kit for cfDNA purification from plasma [136]. In that approach, cfDNA is first captured in the bottom phase of an initial ATPS, then transferred and concentrated in the top phase of a second ATPS, followed by isopropanol precipitation, washing, and redissolution. Other recent studies on nucleic acid extraction rely on different capture mechanisms, most commonly the adsorption of cfDNA onto silica matrices [127]. Examples include the use of silica-coated magnetic beads [181][238] or column-based kits [239][202]. A comprehensive overview of microfluidic systems for nucleic acid extraction from human samples is provided by Obino et al. [137]. While these methods generally report high cfDNA recovery and purity, ATPS-based extraction may offer distinct advantages in terms of simplicity, cost-effectiveness, speed, and scalability. A direct comparison of yield, purity, and overall handling will be performed once the extraction process incorporating the investigated ATPS as a capture step has been fully established.

4.3.2 System robustness

4.3.2.1 Blood collection tube variability

The performance of cfDNA analysis in liquid biopsy is affected not only by the low initial DNA concentrations and the challenge of sample purity but also by various preanalytical factors, including blood collection, processing, and storage conditions [92]. Several studies have investigated cfDNA quantity and integrity when stored in different types of blood collection tubes [240][83]. While K_2/K_3EDTA-containing tubes remain the most commonly used devices, specialized blood collection tubes developed by companies like Streck or PreAnalytiX have been commercialized specifically for cfDNA storage [79]. These tubes contain anticoagulants and distinct stabilizing agents [11], which may impact DNA distribution and recovery in the ATPS capture step employed in this study. To evaluate the robustness of the selected ATPS against variability introduced by different blood collection tube types, the distribution of 160 bp DNA and plasma proteins was analyzed in 19.5% (w/w) PEG 1000/13.8% (w/w) phosphate/37.7% (w/w) plasma systems. Pooled plasma samples from EDTA (E), Streck (S), or PAXgene (P) tubes were compared, as shown in Figure 4.4.

Most DNA was consistently recovered in the bottom phase across all three tube types, with tubes S and E achieving similar recoveries over 75%, while tube P recovered 66%. As expected, DNA recovery in the top phase was minimal, with values below the detection limit for tubes S and E. Tube P showed the highest recovery in the interphase with 36%, compared to tubes S (23%) and E (17%). However, the interphase measurements exhibited the highest standard deviations for all tube types, likely due to protein interference that could have affected fluorescence readings [183]. Protein concentration measurements confirm that DNA capture in the bottom phase is a viable option, as protein concentration remains below 3 mg/mL for all tube types. The most significant

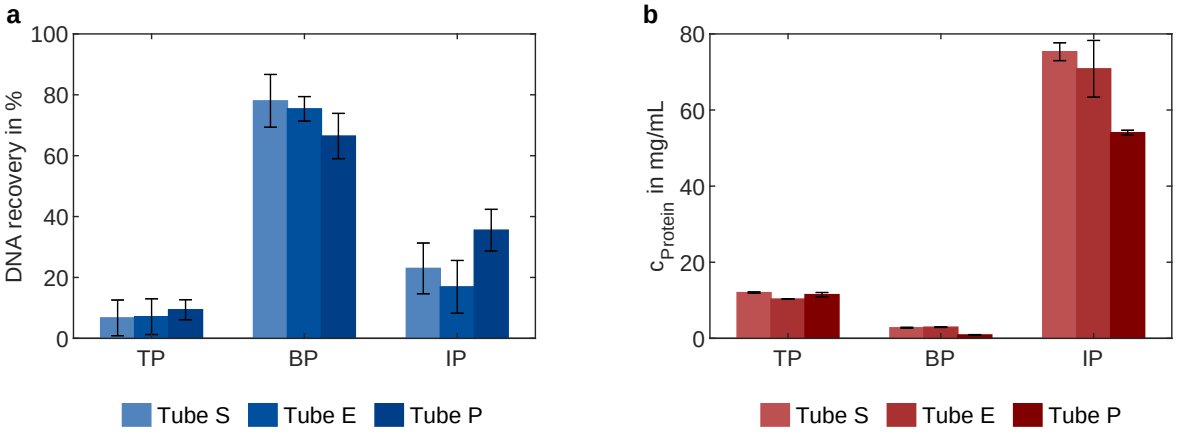


Figure 4.4 (a) 160 bp DNA recovery and (b) plasma protein concentration in the top phase (TP), bottom phase (BP), and interphase (IP) of 19.5% (w/w) PEG 1000/13.8% (w/w) phosphate/37.7% (w/w) plasma ATPS with pooled plasma from EDTA (E), Streck (S) or PAXgene (P) blood collection tubes. Partitioning experiments were performed in duplicate, and samples were measured in triplicate.

difference was observed in the interphase, where protein accumulation was lowest for tube P (54 mg/mL) compared to tubes S and E (over 70 mg/mL).

Interestingly, tube P exhibited higher DNA recovery in the interphase despite lower protein concentrations in the same phase. This could be due to the specific stabilizing agents in PAXgene tubes, which may promote DNA partitioning to the interphase by altering its interactions with plasma proteins or phase-forming components. Although the stabilization reagent is not disclosed, the manufacturer claims that the tube is free of cross-linking substances and does not chemically modify cfDNA [11]. Another notable observation was that pooled plasma from tube P had a reddish hue, possibly indicating hemolysis [241]. This was also reflected in the final ATPS, where the interphase appeared more red than yellow like in the other tubes. This suggests that cell lysis was not entirely prevented [240], with released hemoglobin likely aggregating in the interphase. This may explain the divergent DNA and protein measurements observed in the interphase of tube P, either due to interference with the analytical assays or altered distribution behavior.

Overall, the ATPS proved robustness across different tube types, achieving high DNA recovery in the bottom phase and a controlled distribution of plasma proteins. PAXgene tubes exhibited distinct behavior, warranting further investigation. For consistency, Streck or EDTA tubes may be preferred going forward.

4.3.2.2 Plasma variability

Another factor potentially affecting phase behavior and recovery outcomes in an ATPS composed of plasma is the plasma variability across individuals. Differences in plasma composition can arise due to variable water, protein, and metabolite contents resulting from donor-specific characteristics such as genetics, environment, lifestyle, and health status [242]. To investigate this, the same PEG 1000 system was examined using plasma from individual anonymous donors (A-F), each collected in at least two of the tube types S, E, or P. Figure 4.5 displays the resulting DNA recovery and protein concentration across the top phase (TP), bottom phase (BP), and interphase (IP).

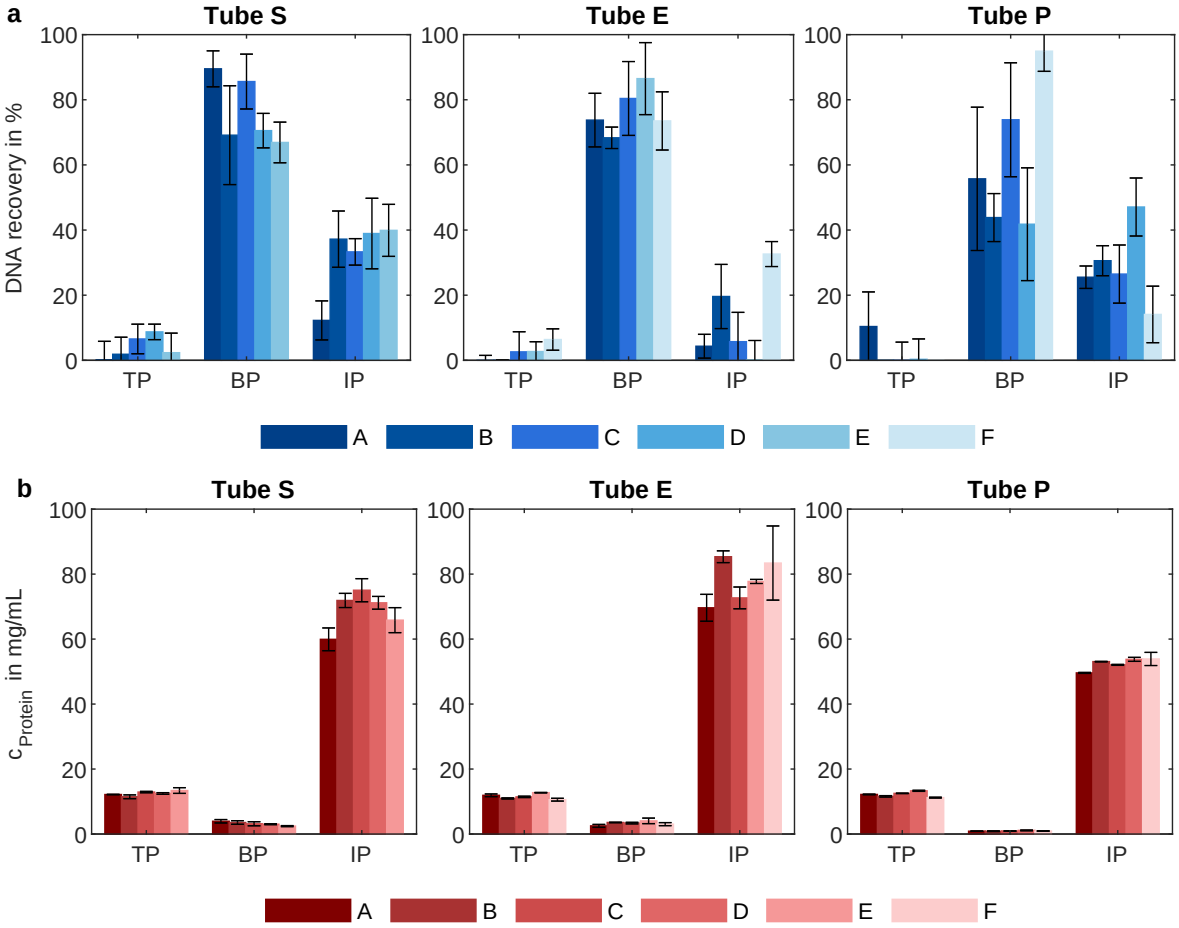


Figure 4.5 (a) 160 bp DNA recovery and (b) plasma protein concentration in the top phase (TP), bottom phase (BP), and interphase (IP) of 19.5% (w/w) PEG 1000/13.8% (w/w) phosphate/37.7% (w/w) plasma ATPS with plasma from individual donors (A-F) from EDTA (E), Streck (S) or PAXgene (P) blood collection tubes. Partitioning experiments were performed in duplicate and samples were measured in triplicate.

DNA recovery in the top phase was negligible across all plasmas and tube types. Most DNA partitioned into the bottom phase while the plasma protein bulk primarily accumulated at the interphase, consistent with pooled plasma experiments. This trend was evident across all plasmas tested for tube types S and E.

For tube S systems, DNA recovery in the bottom phase ranged from 67% (donor E) to 90% (donor A). Interphase recoveries were similar across plasmas, reaching up to 40%, except for donor A, where it was only 12%. This resulted in total recoveries exceeding 100% for most plasmas except for donor A. This discrepancy may be due to interference from the stabilizing reagent, as it has been suggested that Streck tubes contain a formaldehyde-releasing substance that can chemically modify DNA through crosslinking [11], potentially leading to an overestimation of DNA quantities. This effect may be more pronounced in the interphase due to the high protein content, which could exacerbate interference with the fluorescence measurements. Warton et al. [83] reported contamination with high molecular weight DNA in Streck tubes, which may have accumulated in the interphase in this study. However, these explanations seem unlikely, as all samples were

background-corrected using appropriate blank ATPS phase samples, and the effect was not observed consistently across all experiments with individual or pooled plasma. Another explanation is the trapping of bottom-phase liquid in the aggregated protein interphase, which could lead to an overestimation of the DNA content in the interphase.

The DNA and protein distribution in tube type E systems was similar to that of type S, with DNA recoveries ranging from 67% (donor B) to 86% (donor E). Interphase recoveries were lower overall, with some plasmas (donors A, C, and E) exhibiting values below the detection limit.

In contrast, tube P systems exhibited less reproducible results, with higher standard deviations and lower protein concentrations overall. DNA recovery in the bottom phase ranged from 42% (donor D) to 95% (donor F). Notably, the protein concentration in tube P systems was also lower in the bottom phase (around 1 mg/mL compared to 3 mg/mL for tubes S and E) and in the interphase (up to 53 mg/mL, compared to 69–85 mg/mL for tube E and 60–75 mg/mL for tube S). This suggests potential interference of either the stabilization reagent or partial hemolysis of the tube P samples, which showed a reddish hue, affecting protein absorbance readings [241].

Protein concentrations in the top phase were constant at approximately 12 mg/mL across all plasmas and tube types. In the bottom phase, concentrations plateaued at around 3 mg/mL for tubes S and E. This threshold effect is likely due to solubility limits in the PEG- and salt-rich phases [168] and could explain why the variations within the different plasmas were highest in the interphase, where all supplementary proteins excluded from the top and bottom phases tended to aggregate. This effect underscores a key advantage of the ATPS-based capture, as plasma composition variability does not significantly affect protein distribution in the bottom phase, ensuring reproducible outcomes.

Although some variability in DNA recovery was observed, particularly with tube type P samples, tubes S and E demonstrated consistent trends across donor samples. With plasma from these tubes, acceptable DNA recovery and low protein contamination in the bottom phase were achieved, highlighting the reproducibility and reliability of the ATPS-based capture step. Nevertheless, further optimization may be needed to address the variability introduced by specific tube formulations and improve performance with hemolyzed samples.

4.3.3 DNA concentration

4.3.3.1 ATPS formation with solid components

One of the major challenges in liquid biopsy is the inherently low concentration of cfDNA, especially during early-stage cancer detection when cfDNA levels are at their lowest [203]. To increase the likelihood of detecting rare cfDNA variants, a logical approach is to increase the plasma volume to be processed.

The plasma input volume can be increased without altering the relative concentrations of PEG and salt by adding the phase-forming components as solids directly into the plasma. This approach eliminates the need for pre-dissolving of the components, and the water content of the stock solutions is replaced with plasma [230]. This method also offers the advantage of preventing transiently high PEG and salt concentrations that could lead to DNA loss through precipitation [223], as the components gradually dissolve in plasma. Additionally, using solid components is more convenient for ready-to-use extraction kits, as the reduced water content decreases weight during logistics, reduces the risk of contamination, and extends shelf life.

To assess the effectiveness of this strategy, DNA and protein recoveries were compared between ATPS prepared with pre-dissolved components (Figure 4.5) and those prepared with solid phase-forming components (Figure 4.6), using individual plasma samples (donors A-E) collected in Streck tubes.

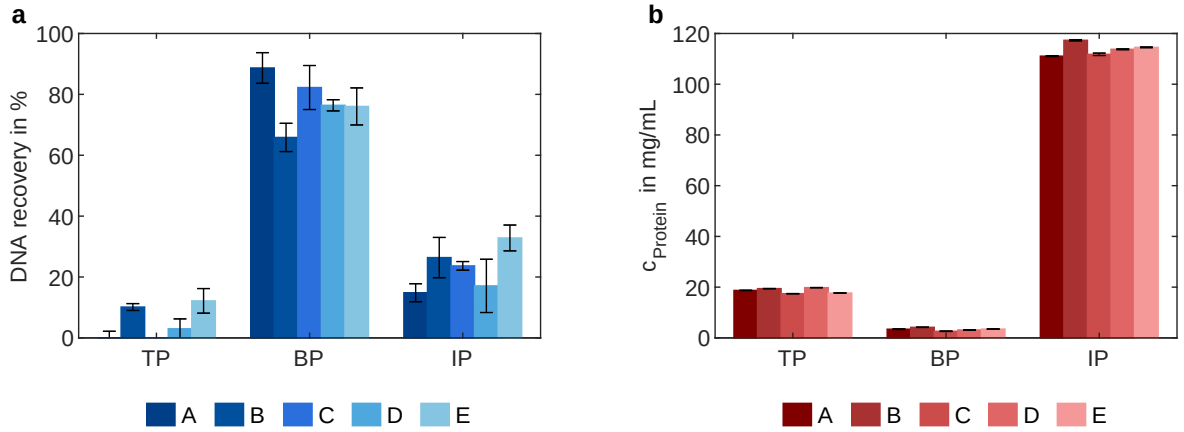


Figure 4.6 (a) 160 bp DNA recovery and (b) plasma protein concentration in the top phase (TP), bottom phase (BP), and interphase (IP) of 19.5% (w/w) PEG 1000/13.8% (w/w) phosphate/66.7% (w/w) plasma ATPS with solid components for different plasma donors (A-E) from Streck tubes. Partitioning experiments were performed in duplicate and samples were measured in triplicate.

By adding the components as solids, 29.0% (w/w) of the water from the pre-dissolved stock solutions could be replaced with plasma without altering the amount of PEG and phosphate, resulting in 66.7% (w/w) plasma in the ATPS. This approach not only increases the amount of extractable cfDNA but also minimizes the dilution associated with the addition of phase-forming components. DNA recoveries between the two preparation methods were comparable across all plasma samples, confirming consistent performance. The DNA recovery in the interphase was slightly lower when solid components were used, possibly due to reduced DNA precipitation attributed to the slower dissolution of PEG. The most notable difference was an increased protein concentration in the interphase when solid components were used (over 110 mg/mL compared to approximately 70 mg/mL). This was expected due to the higher plasma input volume and was visibly reflected in the thicker interphase. Despite this, phase formation and volume ratios were similar between preparation methods. Importantly, protein concentrations in the bottom phase remained unchanged despite the increased plasma volume, underscoring the ability of the ATPS capture step for controlled protein removal. This control could be particularly valuable for a subsequent lysis step, allowing precise adjustment of lysing agent quantities based on the known protein concentration in the bottom phase.

To evaluate the scalability of the solid-components plasma ATPS, the PEG 1000 system was prepared using 4 mL of pooled plasma. The phase volume ratio and the DNA and protein partitioning behavior remained consistent (Figure A4.3). DNA recovery in the bottom phase was 80%, with a protein concentration of 4.5 mg/mL.

These findings highlight the feasibility of dissolving solid phase-forming components directly into plasma. This method simplifies the preparation process, maximizes the cfDNA concentration available during extraction, and presents a scalable solution for processing larger plasma volumes.

4.3.3.2 Phase volume ratio manipulation

Another advantage of ATPS is the potential to concentrate target molecules by adjusting the phase volume ratio (V_R). Five systems were prepared along the same tie-line but with different V_R to investigate if the DNA could be concentrated in the bottom phase by reducing its volume without changing phase equilibrium composition [139]. To ensure comparability, all systems contained the same amount of plasma, with water added as needed to maintain the total volume. The exact compositions of the investigated system points are found in Table A4.3 in the supplementary material. In the first experiment, systems were spiked with 200 ng/mL of a 160 bp DNA fragment, while in the second experiment, a concentration of 100 ng/mL was used. DNA recovery and concentration, as well as protein concentration in the phases, are shown in Figure 4.7.

A plateau in DNA concentration was observed in the bottom phase instead of the expected increase with decreasing phase volume. The plateau occurred at approximately 500 ng/mL for a 200 ng/mL input and at 200 ng/mL for a 100 ng/mL input across all tested V_R, except at $V_R = 5.3$, where the DNA concentration in the bottom phase was only half the plateau value. One hypothesis was that the plateau reflects the solubility limit of DNA in the bottom phase. However, this hypothesis is unlikely because the plateau was observed at 200 ng/mL when the input concentration was lowered to 100 ng/mL, even though up to 500 ng/mL DNA could theoretically partition into the bottom phase. Instead, the limiting factor appears to be the DNA concentration in the interphase, as comparable values were observed for both input concentrations. This data suggests that any DNA unable to accumulate in the interphase partitions into the bottom phase, explaining the observed plateau behavior.

Consequently, DNA recovery in the bottom phase and overall DNA recovery decreased with increasing V_R. The most likely explanation for the incomplete mass balances observed at $V_R \geq 3.1$ is the high amount of PEG 1000 required for phase formation, exceeding 27% (w/w) for this specific tie-line. This elevated PEG concentration possibly caused irreversible DNA precipitation [77], contributing to the observed losses.

Protein distribution exhibited comparable trends across the two experiments, regardless of the DNA input amount. Protein concentration in the top phase plateaued at 10 mg/mL, while protein concentration in the bottom phase decreased as the bottom phase volume decreased. This result is counterintuitive, as one would expect both DNA and protein concentrations in the bottom phase to increase. Notably, protein concentration in the interphase peaked at $V_R = 2.1$ but decreased for $V_R = 5.3$, potentially due to the precipitating effects of the high PEG 1000 concentration [243]. This denaturation could prevent proper protein detection, mirroring the challenges observed with DNA, as total recoveries declined at higher V_R.

The same experiment was repeated using solid phase-forming components added directly to plasma to investigate whether the slower solubilization of PEG would improve DNA recovery. The plasma content in the solid component systems was higher than in the liquid component systems and was kept constant across all V_R values. All systems were spiked with 100 ng/mL DNA for comparability.

Like the liquid component systems, DNA recoveries in the bottom phase decreased with increasing V_R when using solid components. However, bottom phase recoveries were approximately 19% higher across all V_R, while interphase recoveries were lower, confirming that adding the components as solids reduces DNA precipitation. Despite this notable improvement, bottom phase recovery at $V_R = 5.3$ remained low (32%), indicating that the PEG concentration was still too high to achieve an adequate DNA concentration. An additional experiment was conducted using a system point on a

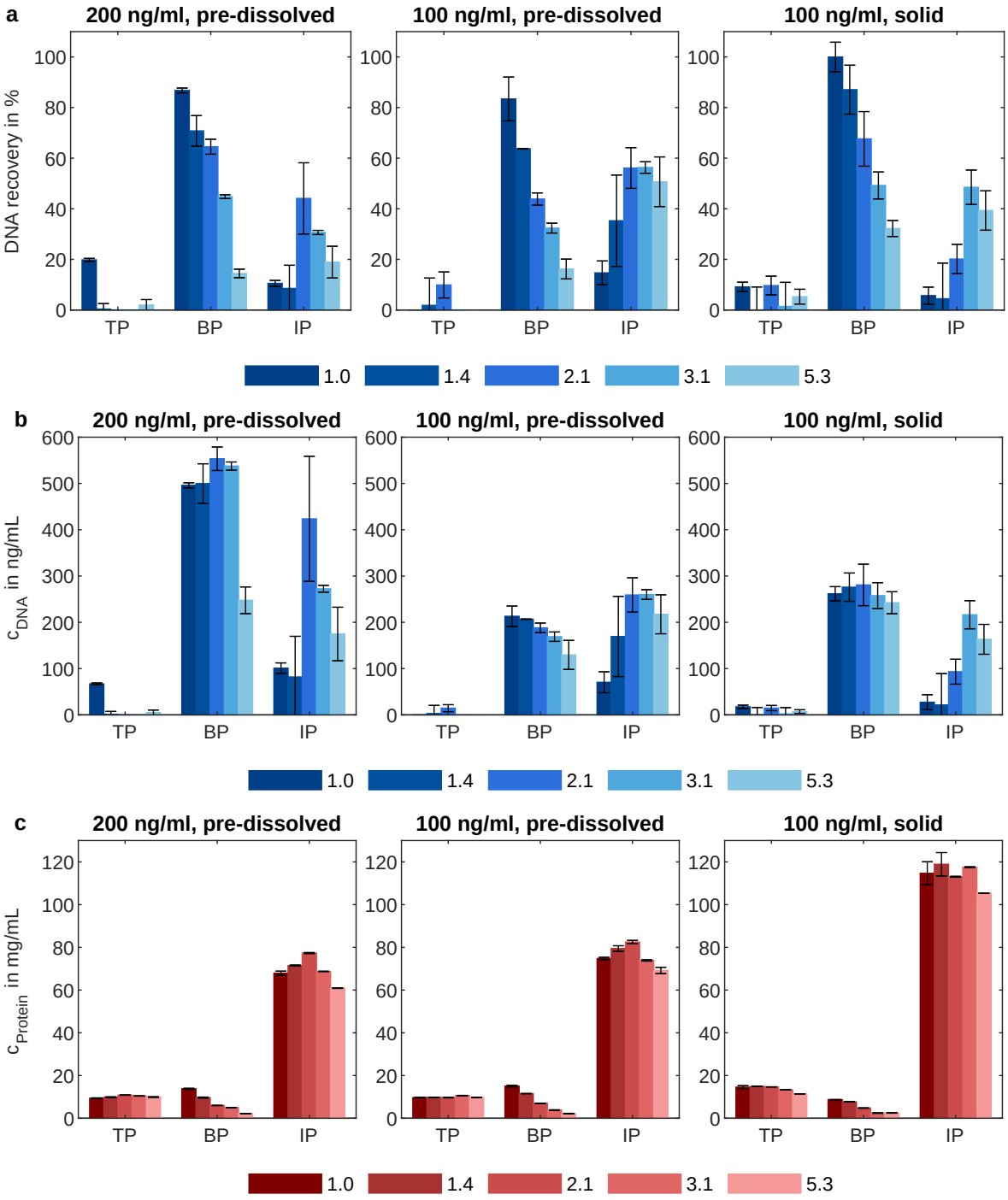


Figure 4.7 (a) 160 bp DNA recovery, (b) concentration, and (c) plasma protein concentration in the top phase (TP), bottom phase (BP), and interphase (IP) of five PEG 1000/phosphate/plasma ATPS with phase volume ratios ranging from 1.0 to 5.3. ATPS were spiked with 200 ng/mL or 100 ng/mL DNA and were prepared with pre-dissolved or solid components. Partitioning experiments were performed in duplicate and samples were measured in triplicate.

shorter tie-line, which required less PEG 1000 and salt, to improve DNA recovery and concentration while minimizing losses. While this adjustment reduced DNA precipitation, the lower PEG levels also decreased protein precipitation, shifting protein distribution from the interphase to the bottom phase (data not shown). These findings suggest that an optimal PEG concentration is essential to effectively precipitate plasma proteins while preventing DNA co-precipitation or entrapment, ensuring efficient DNA extraction and concentration.

4.3.4 Combined purification and concentration

Another approach to increase the DNA concentration involved removing liquid from the DNA-containing phase after the ATPS capture step. For this purpose, EchoLUTION purification plates with a custom matrix filling volume of 500 µL provided by BioEcho were used. The purification plates follow a reverse elution principle, where target DNA flows through while contaminants such as salt and residual proteins are retained [185]. A so-called conditioning step is required to remove the storage solution before the target solution is applied to the matrix. The standard protocol, according to the manufacturer's instructions, specifies a conditioning and elution centrifugation rate of 1000 × g for 1 min. In the following experiment, the conditioning and elution rates were modified to achieve simultaneous removal of contaminants and liquid, effectively concentrating the DNA in the flow-through. The flow-through volume, DNA concentration, and recovery for different combinations of conditioning and elution rates are presented in Figure 4.8.

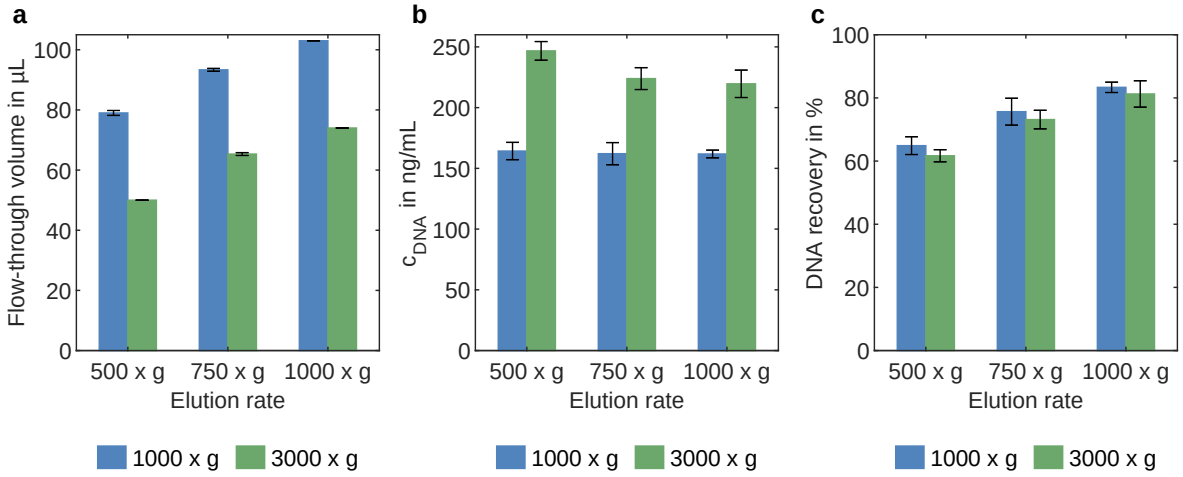


Figure 4.8 (a) Flow-through volume, (b) concentration, and (c) recovery of a 160 bp DNA fragment after reverse elution. An input volume of 100 µL of 200 ng/mL DNA in water was applied per 500 µL matrix-filled well in triplicate. Conditioning centrifugation rates of 1000 × g and 3000 × g were tested in combination with elution rates of 500 × g, 750 × g, and 1000 × g.

The flow-through volume decreased with decreasing elution rates, but increasing the conditioning speed achieved an even more significant reduction. At a conditioning rate of 3000 × g, a higher removal of storage solution effectively "dried out" the matrix, enabling it to retain more input liquid within the pores and between particles [185]. Combined with a lower elution rate, which increases the residence time of the liquid in the matrix, an efficient volume reduction of the flow-through was achieved. By combining conditioning at 3000 × g with elution at 750 × g, the input volume

was reduced by 35%, while DNA concentration was enhanced by 12% compared to the standard protocol.

Interestingly, DNA recovery was not significantly affected by the increased conditioning rate. However, lower elution centrifugation rates led to a decrease in DNA recovery. This could be attributed to insufficient centrifugal force to overcome capillary forces and prolonged residence time within the matrix, which may cause DNA retention between particles or diffusion into pores [184]. Supplementary experiments demonstrated that unspecific adsorption to plate walls and frit material accounted for no more than 5% of DNA loss (data not shown). These findings suggest that the remaining DNA loss likely stems from electrostatic or hydrophobic interactions with the matrix or entrapment within its structure [244].

The desalting capacity of the purification plate was evaluated under the adapted conditions (conditioning 3000 × g, elution 750 × g). Conductivity measurements with increasing phosphate stock concentrations (Figure 4.9) demonstrated that salt removal remained as effective as the standard protocol for up to 30% (w/w) input salt. Less than 5% of the salt remained in the flow-through, corresponding to conductivity values below 2 mS/cm.

Additionally, purification experiments of blank bottom phases revealed a reduction in plasma protein concentrations from 4 mg/mL to below 0.5 mg/mL, with successful protein removal confirmed via SDS-PAGE (Figure A4.4). However, when plasma was purified without a prior ATPS capture step, only 37.5% of total protein was removed, leaving a substantial amount of protein exceeding 30 mg/mL. These results highlight the necessity of the ATPS capture step for significant protein content reduction, as the plasma protein concentration is too high for the purification plates alone. Conversely, the purification step is essential for removing residual protein and reducing the high salt concentration introduced by ATPS, which can inhibit downstream applications such as PCR. Indeed, preliminary quantitative PCR runs confirmed that amplification was only possible for desalted samples (data not shown). While some DNA loss remains inevitable, the modified reverse elution protocol demonstrates strong potential for achieving combined DNA purification and concentration, towards meeting the requirements for amplification-based downstream applications.

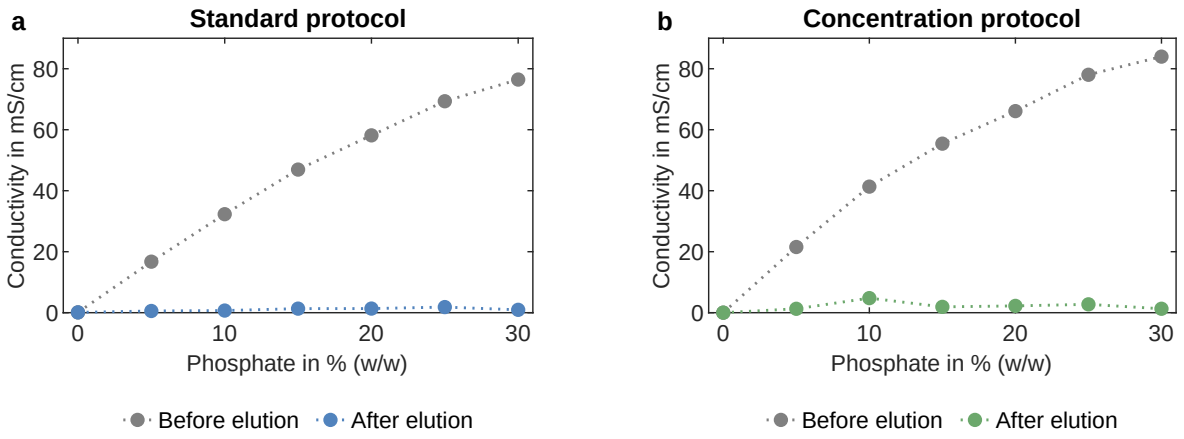


Figure 4.9 Conductivity measurements before and after reverse elution using: (a) standard protocol (conditioning and elution at 1000 × g), (b) concentration protocol (conditioning at 3000 × g, elution at 750 × g). Increasing amounts of phosphate salt were used as input (100 µL per well filled with 500 µL matrix).

4.4 Conclusions

This study demonstrates the potential of ATPS as a robust and efficient approach for cfDNA capture from plasma. The presented PEG/phosphate systems achieved DNA purification and concentration by selectively partitioning DNA into the bottom phase while effectively separating it from the plasma protein bulk. Despite variability in plasma and blood collection tube types, consistent DNA recovery and controlled protein removal were observed, underscoring the robustness of the ATPS capture step. The addition of solid phase-forming components enabled increased plasma input without compromising DNA recovery and protein distribution, highlighting this method's scalability and practicality. Additionally, an adapted reverse elution protocol demonstrated promise as a tool for streamlined downstream processing of DNA-containing, salt-rich solution after ATPS-based capture. Nonetheless, further improvements are necessary to remove residual proteins and phase-forming components that could interfere with downstream analyses such as PCR or NGS. Ongoing efforts focus on implementing a lysis step post-capture, which, when combined with reverse elution, could streamline the simultaneous removal of salts and proteins without relying on adsorption-based processes. These findings establish a solid foundation for developing a scalable and reliable ATPS-based workflow for efficient cfDNA extraction in liquid biopsy applications.

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Supplementary data

Appendix **A4** contains the Supporting Figures associated with this chapter.

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5

Proof of concept for aqueous two-phase system-based extraction of cell-free DNA from plasma for liquid biopsy applications

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Abstract

Efficient extraction of circulating cell-free DNA (cfDNA) from plasma is critical for liquid biopsy applications, but remains challenging due to cfDNA's short fragment size, low concentration, and the presence of interfering proteins and genomic DNA. This study presents a simple extraction process based on aqueous two-phase system (ATPS) capture and reverse elution purification as a potentially lysis-free alternative to adsorption-based methods. Short DNA fragments selectively partitioned into the salt-rich bottom phase, achieving up to 65% recovery after purification while removing 99.7% of plasma proteins. Reverse elution enabled efficient desalting and up to fourfold DNA concentration under mild, aqueous conditions. The extracts showed no qPCR inhibition and displayed a cfDNA-like size profile with a cutoff around 750 bp, indicating selective enrichment of short fragments. Although the total yield was slightly lower than that of the QIAamp Circulating Nucleic Acid Kit (QIAGEN), the ATPS workflow substantially reduced processing time, equipment needs, and cost. Next-generation sequencing of cfDNA reference material extracted from DNA-free plasma confirmed compatibility with library preparation and sequencing workflows, providing proof of concept for a simplified cfDNA extraction strategy for amplification-based liquid biopsy applications.

5.1 Introduction

Molecular diagnostics based on nucleic acids have become central to precision medicine, enabling individualized disease characterization and treatment [245, 246]. Circulating cell-free DNA (cfDNA), referring to extracellular DNA fragments released into the bloodstream primarily through apoptosis and necrosis of cells, circulates in blood and other body fluids and has emerged as a key biomarker for liquid biopsy, offering a minimally invasive alternative to tissue biopsy [203, 227]. Its analysis serves applications ranging from cancer management to prenatal testing and transplant monitoring [41, 247–249]. In oncology, a small fraction of cfDNA originates from tumor cells and is referred to as circulating tumor DNA (ctDNA), which carries tumor-specific genetic alterations [51].

However, the analysis of cfDNA remains challenging, mainly due to its low abundance in plasma [10]. Typically present at concentrations below 40 ng/mL, cfDNA consists of short, nucleosome-associated fragments around 167 bp that are rapidly degraded and easily lost during processing [205, 206]. Because ctDNA represents only a minute fraction of total cfDNA, contamination with high-molecular-weight genomic DNA (gDNA) released from lysed blood cells during sample handling can compromise mutation detection and quantification [11]. Consequently, cfDNA extraction remains a critical bottleneck for sensitive and reproducible downstream molecular analyses, such as next-generation sequencing (NGS), as extraction efficiency and preservation of fragment size can significantly influence analytical outcomes [15, 207, 250]. These challenges are further amplified in clinical diagnostics, where rapid turnaround times and high throughput are required with limited sample volumes.

Commercial cfDNA extraction kits predominantly rely on solid-phase adsorption, most commonly using silica membranes or silica-coated magnetic beads under high-salt conditions [95]. In these systems, nucleic acids bind to the silica surface in the presence of chaotropic salts and are subsequently recovered through sequential washing and elution steps [12]. While these approaches provide high purity and are widely used as reference methods, such as the QIAamp Circulating Nucleic Acid Kit (QIAGEN), they present several practical and analytical limitations. The multi-step bind-wash-elute

workflow is time-consuming, requires repeated manual handling and vacuum or centrifugation steps, and may increase the risk of cross-contamination [13]. Automated magnetic-bead-based systems can mitigate this risk and improve workflow consistency, but other limitations remain. For instance, incomplete removal of chaotropic salts may inhibit downstream PCR. In addition, because cfDNA is typically present at very low concentrations, incomplete adsorption or inefficient elution can directly reduce analytical sensitivity [126]. From an analytical perspective, the recovery of short DNA fragments may be less efficient, potentially introducing fragment-size bias and affecting the representation of cfDNA populations [14, 15]. Furthermore, contamination with high-molecular-weight gDNA released during sample handling can compromise the detection of rare tumor-derived variants. Together with high reagent costs, limited throughput, and the need for specialized equipment, these factors highlight the need for simpler, scalable, and cost-effective extraction strategies. Consistent with this, comparative studies have reported substantial variability in cfDNA yield, purity, and fragment size distribution across manual and automated extraction workflows, underscoring that cfDNA isolation remains a critical and not yet fully standardized pre-analytical step in liquid biopsy analysis [95, 131, 201, 251].

Given these challenges, liquid–liquid extraction using aqueous two-phase systems (ATPS) offers a promising alternative to conventional solid-phase approaches. ATPS enable biomolecule partitioning between two immiscible aqueous phases without chaotropic reagents or specialized consumables, providing a fast, affordable, and easily scalable method that requires only simple centrifugation for phase separation [229].

In our previous work [252], we demonstrated efficient and robust capture of short DNA fragments from plasma using polyethylene glycol (PEG)/phosphate ATPS and introduced a solid-component design to increase plasma input while minimizing dilution. Coupling the extraction with a reverse elution step further improved DNA concentration and purity. However, the resulting concentrations remained below the input requirements of standard NGS library preparation kits, and the influence of a lysis step had not yet been investigated.

Building on this foundation, the present study optimizes the ATPS-based extraction process to address these limitations. We assess the effects of different lysis conditions, identify alternative system points for higher DNA concentration with minimal loss, and explore additional strategies, such as serial ATPS, for further enrichment. The optimized workflow is evaluated for PCR inhibition and compared to the QIAamp Circulating Nucleic Acid Kit (QIAGEN) using varying DNA inputs and fragment lengths. Finally, the complete ATPS-based process is applied to a spiked cfDNA reference set for NGS analysis, demonstrating that the method can yield sequencing-quality DNA suitable for downstream molecular analyses.

5.2 Materials and methods

5.2.1 Blood collection and plasma processing

Blood samples were collected from healthy donors at the Institute for Experimental Hematology and Transfusion Medicine at the University Hospital Bonn (Bonn, Germany). The study procedures were performed in compliance with the approval granted by the Ethics Committee of the Medical Faculty at the University of Bonn (protocol code 070/05) and adhered to the Declaration of Helsinki. Written informed consent was obtained from all participants, and the procedures were carried out in accordance with institutional guidelines. After giving consent, approximately 10 mL of whole blood was collected into EDTA K3E S-Monovette collection tubes (Sarstedt, Nümbrecht,

Germany) or Cell-Free DNA BCT® (Streck, La Vista, NE, USA). Blood samples were shipped at room temperature and processed within 24 hours using a double centrifugation protocol to maximize plasma recovery. Whole blood was first centrifuged at 1600 × g for 10 min at room temperature. The plasma layer was then transferred and centrifuged at 16000 × g for 10 min to remove residual blood cells. Plasma aliquots of 1 mL were stored at -80°C until further use.

5.2.2 Preparation of ATPS components

Starting points for system optimization were selected based on our previous work, where the experimental workflow and phase sampling procedure are described in detail [252]. To maximize the plasma input volume per ATPS, PEG 1000 (Merck KGaA, Darmstadt, Germany) and the phosphate salts $NaH_2PO_4 \cdot H_2O$ (Merck) and K_2HPO_4 (VWR International, Radnor, PA, USA) were added as solids rather than as pre-dissolved stock solutions. Cylindrical PEG pieces of up to 150 mg were prepared by melting the waxy polymer in a microwave and allowing it to solidify in a cylindrical mold for a few minutes. The salts were placed into Eppendorf tubes, mixed with spiked plasma, and finally combined with the PEG. Plasma aliquots were thawed at room temperature on the lab bench for 45 min, leaving any sedimented material undisturbed to avoid resuspension. Purified 160 bp DNA fragments were prepared as described by Meutelet et al. [231] and gently mixed with plasma to the desired concentration before addition to the salts. For the NGS experiments, the 5-Gene-Multiplex Set cfDNA by SensID GmbH (Rostock, Germany) containing defined variants (AKT1/BRAF/ERBB2/KRAS/PIK3CA) at 0% and 5% VAF was spiked into human-tech DNA-free plasma (SensID GmbH). The amounts of phase-forming components and plasma varied with the investigated system point and experimental conditions. A summary of the relevant system points is provided in Table A5.1.

5.2.3 ATPS-based DNA extraction protocol

A schematic overview of the developed extraction process is shown in Figure A5.6a and comprises three main steps: ATPS capture, an optional second ATPS concentration step, and purification by reverse elution. Plasma was added to the tube containing phosphate salts, pulse-vortexed five times, followed by the addition of the PEG cylinders and another five pulse-vortexes. The tube was then mixed on a thermo-shaker at 1400 rpm and 25°C for 5 min, followed by vortexing to ensure complete solubilization. Phase separation was achieved by centrifugation at 8000 × g for 1 min. The top phase was carefully removed without disturbing the interphase or bottom phase. The bottom phase was collected using a pipette by gently puncturing the interphase and transferring the liquid to a new tube. Any recoverable liquid adhering to the tube walls or trapped within the interphase was added to the bottom phase, and the final volume was measured manually with a pipette. The remaining interphase was discarded. For the optional serial ATPS step, fresh plasma and PEG 1000 were added to the recovered bottom phase. Mixing, centrifugation, and phase extraction were performed as described for the first ATPS.

The resulting salt-rich bottom phase was purified using the reverse elution plates from the EchoCLEAN Organic Solvent DNA CleanUp Kit (BioEcho, Cologne, Germany). As described by Meutelet et al. [252], plates containing 500 µL matrix filling volume per well were centrifuged at 3000 × g for 1 min for conditioning, and elution was performed at 750 × g for 1 min. Each well was loaded with 100 µL or 50 µL, and the final purified DNA volume was measured manually using a pipette.

5.2.4 Lysis conditions

Three lysis methods were evaluated and compared with a non-lysed sample: Proteinase K with lysis buffer from the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) (Q), an enzyme-free lysis buffer with clearing solution (BioEcho) (B), and a reducing agent in water (T). Lysis was performed either on spiked plasma pre-ATPS capture or on bottom-phase samples before reverse elution. Reagent concentrations and volumes were adapted from the respective manufacturers' protocols to match the plasma or bottom-phase volumes of the non-lysed sample.

For method Q, 100 µL Proteinase K, 1 mL plasma, and 0.8 mL buffer ACL were combined, vortexed for 30 s, and incubated at 60°C for 30 min. After cooling, the lysed plasma supernatant was used for ATPS formation. For methods B and T, 0.5 mL lysis buffer or 50 mM reducing agent solution was added to 1 mL plasma, incubated at 80°C for 2 min, and cooled to room temperature. For lysis B, 43 µL clearing solution was then added, and only the supernatant was used. The same procedures were applied to bottom-phase samples in post-capture lysis experiments. Conductivity of purified lysates was measured using an ÄKTApurifier conductivity sensor (Cytiva, Uppsala, Sweden).

5.2.5 Reference adsorption-based extraction protocol

The adsorption-based QIAamp Circulating Nucleic Acid Kit (QIAGEN) was used as a reference extraction method. The protocol was performed according to the manufacturer's instructions, except that carrier RNA was omitted. In brief, the workflow included sample lysis, DNA binding to silica columns, sequential washing with buffer and ethanol using a vacuum manifold, drying, and elution in water with a microcentrifuge. A schematic overview of the workflow, including detailed process parameters, is provided in Figure A5.6b.

5.2.6 Analytical methods

5.2.6.1 Protein quantification

Protein concentration in purified samples was measured in triplicate using a NanoDrop™ 2000/2000c UV/Vis spectrometer (Thermo Fisher Scientific). Water served as a blank, and tenfold-diluted plasma served as a reference for removal-efficiency calculations. An average extinction coefficient of 10 was assumed for plasma protein absorbance at 280 nm. Reducing SDS-PAGE analysis was performed on selected samples following the protocol and equipment described by Meutelet et al. [252] to assess protein removal and composition after lysis and extraction.

5.2.6.2 DNA quantification

DNA was quantified using the Quant-iT™ PicoGreen™ dsDNA HS assay kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Unpurified samples were diluted tenfold prior to analysis to reduce interference from phase-forming components. Analysis was performed as described by Meutelet et al. [252], with blanks to account for background signal and references for recovery calculations. DNA recovery was calculated by multiplying the measured DNA concentration by the extract volume and dividing by the amount of input DNA.

5.2.6.3 Assessment of sample purity

Quantitative PCR (qPCR) was performed on selected purified samples to validate DNA quantification and assess sample purity after extraction, verifying the absence of amplification inhibitors. The assay targeted the spiked 160 bp DNA fragment. Reactions were performed in a total volume of 20 µL, containing 5 µL of sample or standard, 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Feldkirchen, Germany), and 0.25 µM each of forward (CTACGGCAAGCTGACCCT) and reverse (GAAGCACTGCACGCCGTAG) primers (Sigma-Aldrich, St. Louis, MO, USA). All reactions were performed in triplicate on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific). Thermal cycling conditions comprised an initial denaturation at 95°C for 7 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 20 s. Quantification was performed using a standard curve generated from serial dilutions of purified 160 bp DNA fragment (50 ng/mL to 0.005 ng/mL) and calculated by the Quant Studio™ Design & Analysis Software v1.5.1 (Thermo Fisher Scientific) based on cycle threshold (Ct) values. Amplification efficiency and linearity (R^2) of the standard curve were verified for each run. No-template controls and blank extraction controls were included on each plate to confirm assay specificity and exclude contamination.

5.2.6.4 Evaluation of DNA integrity

DNA integrity was assessed using an ALU-based qPCR assay adapted from Umetani et al. [253]. Two primer sets targeting repetitive ALU sequences were used to amplify 115 bp and 247 bp fragments. Each 20 µL reaction contained 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH), 0.15 µM each of forward and reverse primer (either ALU115 or ALU247) (Sigma-Aldrich), and 5 µL of sample or standard. Amplification was performed on the system described above with the following cycling conditions: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and annealing at 64°C for 1 min. DNA concentrations were determined from standard curves generated using serial dilutions of purified human genomic DNA (200–0.02 ng/mL), and the DNA integrity index (DI) was expressed as the ratio of ALU247 to ALU115 concentrations.

5.2.6.5 DNA fragment size analysis

DNA fragment size distribution was analyzed using the Agilent TapeStation 4150 system (Agilent Technologies, Santa Clara, CA, USA) with the D1000 ScreenTape assay, following the manufacturer's instructions. Samples (1 µL) were loaded together with the corresponding ladder, and electropherograms were evaluated using TapeStation Analysis Software. To investigate fragment size distribution in the extracts, the 160 bp PCR fragment was replaced with GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific).

5.2.6.6 Next-generation sequencing

NGS libraries for the customized LIQUIDPlex CSTM Solid Tumor panel (Archer, Inc./IDT, Boulder, CO, USA) were prepared according to the manufacturer's instructions, using 35 ng cfDNA as input material and targeting 35 genes. After quantification with the NEBNext Library Quant Kit for Illumina (New England Biolabs, Ipswich, MA, USA) on a QuantStudio5 DX qPCR cycler (Thermo Fisher Scientific), libraries were paired-end sequenced for 300 cycles on a NovaSeq6000 sequencer

(Illumina, San Diego, CA, USA). FASTQ files were generated on the Illumina DRAGEN v4 server and analyzed on the Archer analysis platform (version 7.3.1; Archer) with default settings.

5.2.6.7 Statistical analysis

Due to the exploratory nature of this proof-of-concept study and the limited availability of plasma samples, the number of biological replicates varied between experiments. Where possible, independent replicates were included to assess reproducibility. Measurements were generally performed in technical triplicate. Data are reported as mean ± standard deviation (SD). Error bars in figures represent SD of the indicated replicates. No formal statistical hypothesis testing was conducted, as the study was designed for exploratory validation rather than quantitative comparison.

5.3 Results

5.3.1 System optimization

In our previous work [252], a promising ATPS composition (19.5% (w/w) PEG/13.9% (w/w) salt; tie-line length 41%) was identified, which enabled efficient DNA capture in the bottom phase while separating it from the main plasma protein fraction. However, the DNA concentration in the final extract remained too low for reliable downstream analysis. Here, DNA concentration is expressed as a concentration factor, defined as the ratio of the DNA concentration in the final extract to that in the plasma input.

5.3.1.1 Effect of tie-line length and phase volume ratio

To increase DNA concentration without altering phase distribution, system compositions along different tie-lines were investigated. Previously, increasing PEG concentration to reduce bottom-phase volume led to DNA loss. To prevent this while maintaining a high phase volume ratio (V_R), a system point on a shorter tie-line, requiring lower PEG and salt concentrations to induce phase separation, was tested. Preliminary screening experiments identified a composition with a theoretical V_R of five and tie-line length of 34% (27.1% (w/w) PEG/8.1% (w/w) salt), which doubled the DNA concentration in the bottom phase without affecting recovery (70%) (Fig. A5.1a). Protein distribution remained comparable to the reference system, confirming that selectivity was maintained.

5.3.1.2 DNA concentration by reverse elution

To further enhance DNA concentration, the optimized system point was combined with the previously described reverse elution protocol [252]. Figure 5.1 shows DNA recoveries and concentration factors obtained with the standard and concentration purification protocols. The optimized ATPS composition with a higher PEG content doubled the DNA concentration factor relative to the reference system, without affecting recovery significantly (57–66%). Applying the optimized reverse elution protocol (3000 × g conditioning, 750 × g elution) nearly doubled the factor again (from 2.3 to 4.1), yielding an overall fourfold increase compared to the initial system. Moreover, conductivity measurements confirmed efficient salt removal for all conditions (Fig. A5.1b). Combining the optimized ATPS composition with the adapted reverse elution protocol substantially improved DNA concentration without compromising recovery or protein removal efficiency.

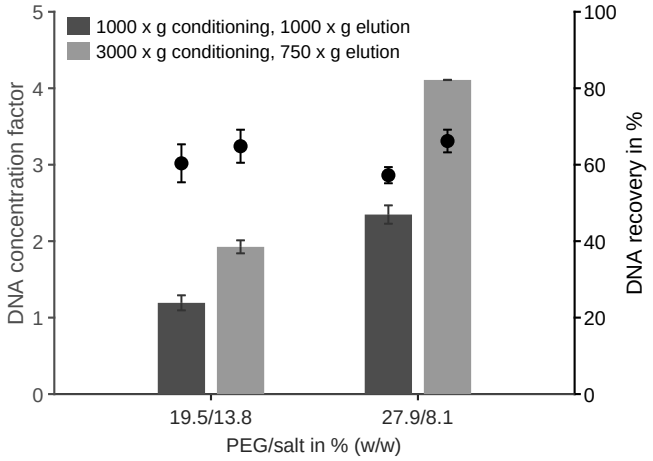


Figure 5.1 Effect of system composition and purification protocol on DNA recovery and concentration factor. DNA concentration factor (bars, left axis) and recovery (dots, right axis) are shown for two ATPS compositions with different PEG/salt contents (% (w/w)). Bottom-phase samples were purified using either the optimized (3000 × g conditioning, 750 × g elution) or the standard reverse elution protocol (1000 × g for both steps). Data represent mean ± SD of biological duplicates, each measured in technical triplicates.

5.3.2 Effect of lysis on extraction efficiency

To assess the effects of a lysis step in the ATPS-based extraction workflow, three different lysis methods were evaluated and compared with a non-lysed reference (No). The tested methods were Proteinase K digestion with QIAGEN lysis buffer ACL (Q), BioEcho lysis buffer with clearing solution (B), and lysis solution containing a reducing agent (T). For comparability, all systems contained identical concentrations of phase-forming components and were purified using the optimized reverse elution protocol. Figure 5.2 shows the resulting DNA concentration factors and recoveries, as well as protein concentrations and removal efficiency for the tested lysis conditions pre- and post-ATPS capture.

5.3.2.1 Plasma lysis pre-ATPS capture

When plasma was lysed before ATPS formation, phase behavior differed from the non-lysed reference, showing a thinner or more flocculent interphase depending on the lysis method (Fig. A5.2a). Despite these visual differences, protein removal remained high (99.4–99.9%) across all conditions. DNA recovery was highest in the non-lysed system (75%±2%) and comparable for lysis Q, while recoveries for lysis B and T were below 13%.

5.3.2.2 Bottom phase lysis post-ATPS capture

Applying lysis to the DNA-containing bottom phase after capture with the reference ATPS (19.5% (w/w) PEG/13.9% (w/w) salt) yielded comparable DNA concentration factors (1.0–1.2) across all methods. After accounting for dilution, recoveries reached approximately 75% for lysis B, 60% for lysis Q, and 53%±6% for the non-lysed reference. Protein removal again exceeded 99.0% for all methods, but SDS-PAGE analysis revealed differences in residual protein bands (Fig. A5.2b). In the non-lysed bottom phase, distinct bands were observed at 66 kDa (human serum albumin (HSA)),

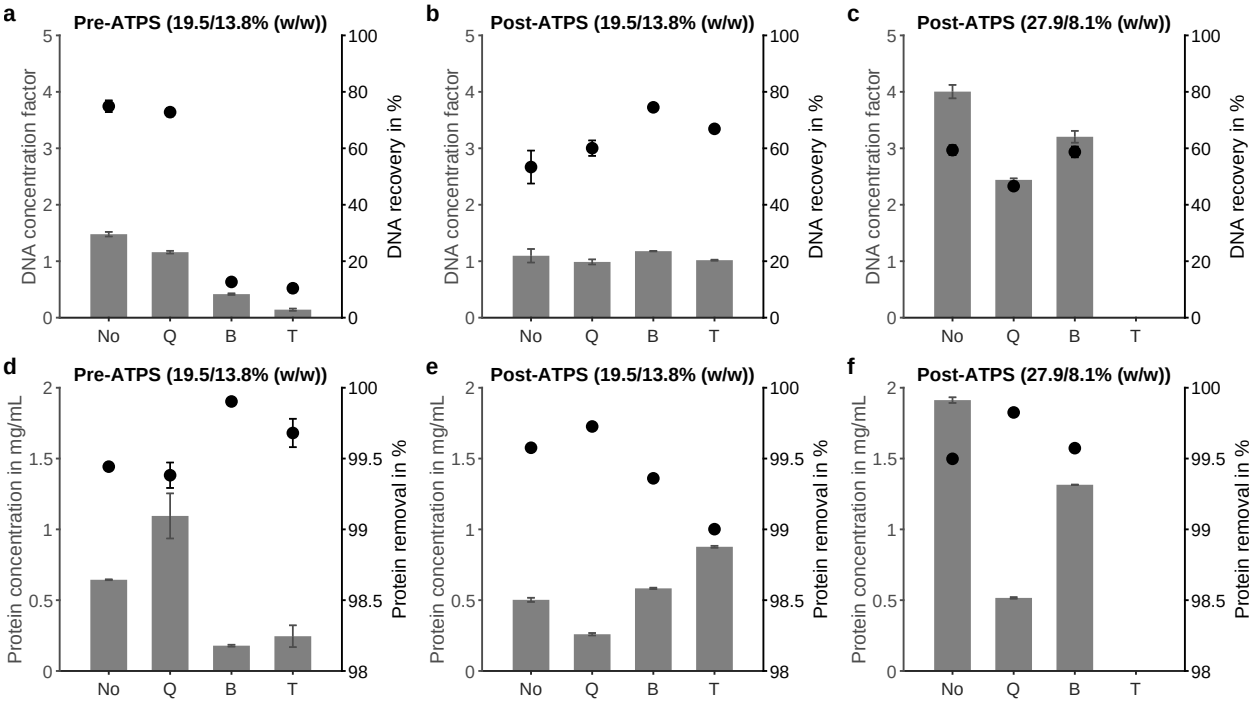


Figure 5.2 Impact of pre- and post-ATPS capture lysis methods on DNA and protein recovery in the bottom phase. (a-c) DNA concentration factor (bars, left axis) and recovery (dots, right axis) for two ATPS compositions with different PEG/salt contents. Lysis was performed on plasma pre-capture (a) or on the bottom phase post-capture (b-c) using enzymatic (Q), buffer-based (B), or reducing (T) methods, compared to a non-lysed reference (No). (d-f) Corresponding protein concentration (bars, left axis) and removal efficiency (dots, right axis) under identical conditions. Data represent mean ± SD of technical triplicates.

55 kDa (transferrin), ∼45 kDa (α1-antitrypsin), and 23 kDa (apolipoprotein A1). These bands appeared fainter after purification, confirming protein removal. Following lysis, the intensity and number of visible bands decreased: lysis T showed bands near 55 kDa, 45 kDa and 23 kDa, lysis B retained the 23 kDa band and a faint one around 45 kDa, and lysis Q displayed the ∼45 kDa band as well as a smear around 6 kDa. Conductivity values were slightly lower for lysed samples (0.5 mS/cm) than for the non-lysed bottom phase (0.9 mS/cm), suggesting minor salt precipitation effects (Fig. A5.2c).

For the optimized ATPS composition (27.1% (w/w) PEG/8.1% (w/w) salt), protein removal efficiency remained high (99.5%), and lysis showed no considerable benefit. The non-lysed sample achieved the highest DNA recovery (59%) and concentration factor (4.0). Lysis B produced a similar recovery but a lower concentration factor of 3.2 due to dilution with lysis buffer, while lysis Q performed less effectively with a recovery of 47%.

Overall, the reduction-based lysis method B achieved recoveries comparable to or higher than those of the enzymatic method Q, despite its shorter incubation time (2 min vs. 30 min) and the absence of enzymes. However, depending on the system composition, omitting lysis completely yielded equally efficient, if not higher, DNA extraction.

5.3.3 Performance comparison with silica-based extraction

The performance of the optimized, lysis-free ATPS-based extraction and purification process was evaluated and compared to the QIAamp Circulating Nucleic Acid kit (QIAGEN) across a range of DNA input concentrations and fragment lengths. Figure 5.3 shows the resulting DNA concentration factors and recoveries for a 160 bp DNA fragment as well as the fragment size distribution of a 1 kbp DNA ladder after extraction.

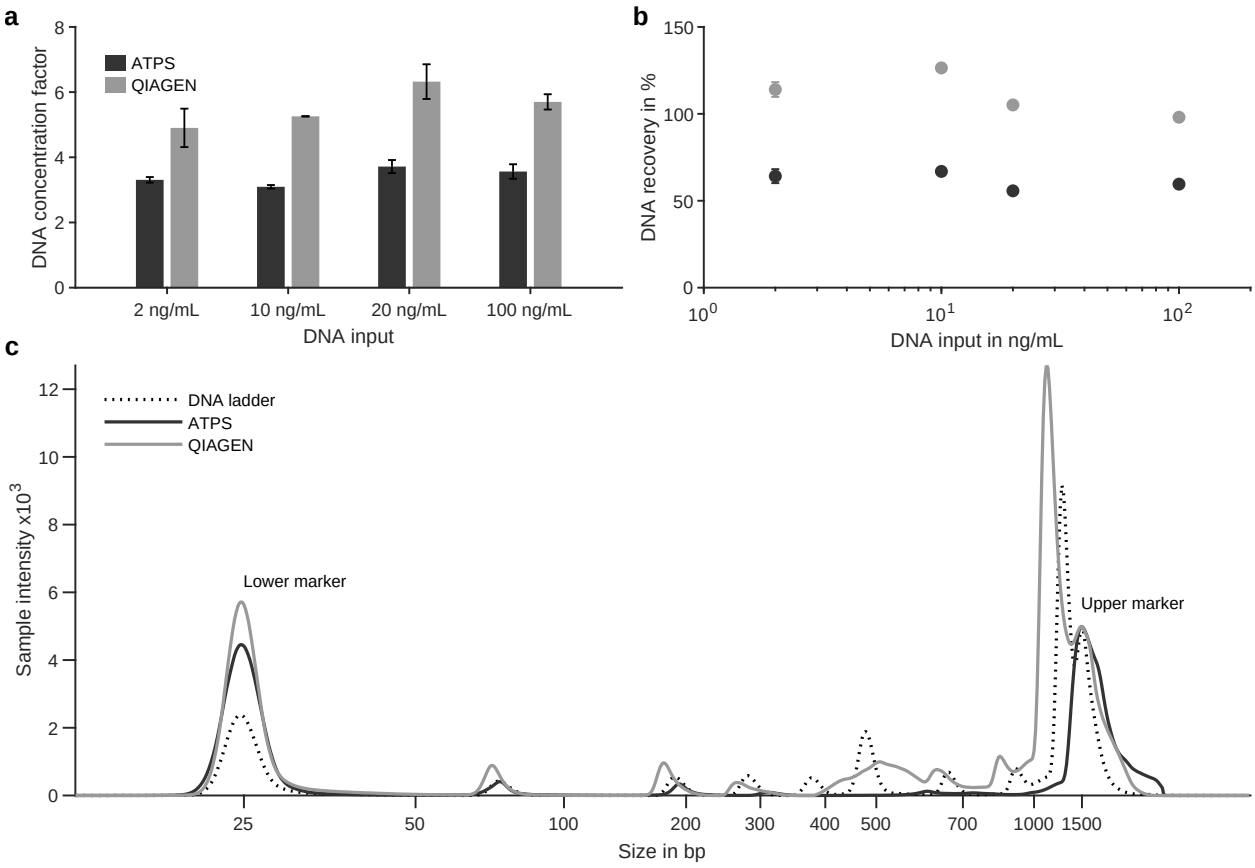


Figure 5.3 Comparative performance of ATPS- and adsorption-based QIAGEN extraction. (a) DNA concentration factor as a function of input concentration (2–100 ng/mL) for ATPS and QIAGEN extractions. (b) DNA recovery as a function of input concentration. (c) Fragment size distribution of a 1 kbp DNA ladder before and after extraction with both methods, showing relative fluorescence intensity over fragment length (bp). Input analyses were performed in technical triplicate. Fragment-length analysis was performed on a representative sample.

5.3.3.1 Effect of DNA input concentration

Plasma samples were spiked with a 160 bp DNA fragment at concentrations from 2 ng/mL to 100 ng/mL and extracted in parallel using the ATPS-based process and the QIAamp kit (QIAGEN). Due to elution in a small final volume (<100 µL), the QIAamp kit achieved higher concentration factors (4.9–6.3) across the tested input range. Nevertheless, the ATPS process yielded consistent recoveries above 60% even at low DNA inputs (e.g., 1 ng DNA in 500 µL plasma), indicating robust extraction efficiency. The recoveries calculated for QIAamp exceeded 100%, likely due to differences

in the blank/reference (water) and sample (elution buffer) compositions. Reported recoveries for this kit typically range between 70–80% [254]. While the QIAamp kit achieved higher apparent recoveries, its workflow was approximately six times longer (∼60 min) due to multiple incubation, vacuum, and centrifugation steps (Fig. A5.6). In contrast, the ATPS-based process required only a mixing step and centrifugation for phase separation and purification, offering reduced processing time (∼10 min turnaround, ∼2 min hands-on) and straightforward scalability, particularly for larger plasma volumes.

5.3.3.2 DNA fragment size distribution and integrity

Microelectrophoresis confirmed that the spiked 160 bp PCR fragment was successfully recovered using both extraction methods (Fig. A5.3a,b). The analysis also showed that desalting is essential for accurate fragment detection, as DNA bands and size markers did not migrate correctly in unpurified samples. To evaluate DNA integrity and size distribution, additional experiments were performed by spiking plasma with a 1 kbp DNA ladder. The ATPS-based extraction efficiently recovered fragments within the cfDNA size range, while fragments larger than 750 bp were largely absent from the extract. In contrast, the QIAamp extraction yielded higher overall DNA concentration for all fragment sizes but displayed broader or poorly resolved peaks for fragments above 270 bp, suggesting possible co-extraction of aggregated or high-molecular-weight DNA.

To further confirm the size selectivity of ATPS, plasma spiked with lambda DNA (48.5 kbp) was analyzed. More than 70% of the lambda DNA was recovered in the interphase, while none was detected in the bottom phase, confirming effective exclusion of long DNA fragments such as contaminating gDNA. The thicker interphase observed for lambda DNA samples visually corroborated these findings (Fig. A5.3c,d).

ALU-qPCR was performed on extracts from unspiked, healthy plasma to detect endogenous cfDNA and determine DNA integrity index (DI) following the method of Umetani et al. [253]. The DI of the QIAamp extract was 0.5, consistent with the presence of DNA fragments longer than 247 bp. In contrast, DI values for the ATPS extracts ranged from 0.1 to 0.2, indicating that nearly all DNA was truncated into fragments smaller than 247 bp. These results align with the electrophoresis data, confirming the size-selective recovery of short cfDNA-like fragments in ATPS.

5.3.4 Assessment of qPCR compatibility

To assess potential inhibition of amplification-based techniques by matrix components in the purified ATPS extracts, spike-in experiments were evaluated using qPCR. Figure 5.4a shows the amplification curves for a dilution series of blank extracts spiked with 5 ng/mL DNA, displayed for one representative replicate per condition for clarity (all curves shown in Fig. A5.4a). The curves exhibit the expected sigmoidal shape with minimal variation between triplicates, indicating the absence of inhibitory effects. The quantified DNA concentrations (4.9–5.5 ng/mL) closely matched the expected values, further confirming the lack of inhibition. A qualitative comparison with QIAamp extracts likewise showed comparable amplification performance, indicating that the ATPS workflow does not introduce detectable inhibitors unique to the extraction (Fig. A5.4b). Differences in Ct values between ATPS and QIAamp extracts corresponded to the respective DNA concentrations obtained after extraction. Similarly, lysed samples showed efficient amplification, suggesting that purification via reverse elution effectively removed potentially interfering components from the lysis step. Consistent amplification behavior across diluted samples from the DNA input experiments further supports the high purity and qPCR compatibility of ATPS extracts (Fig. 5.4b).

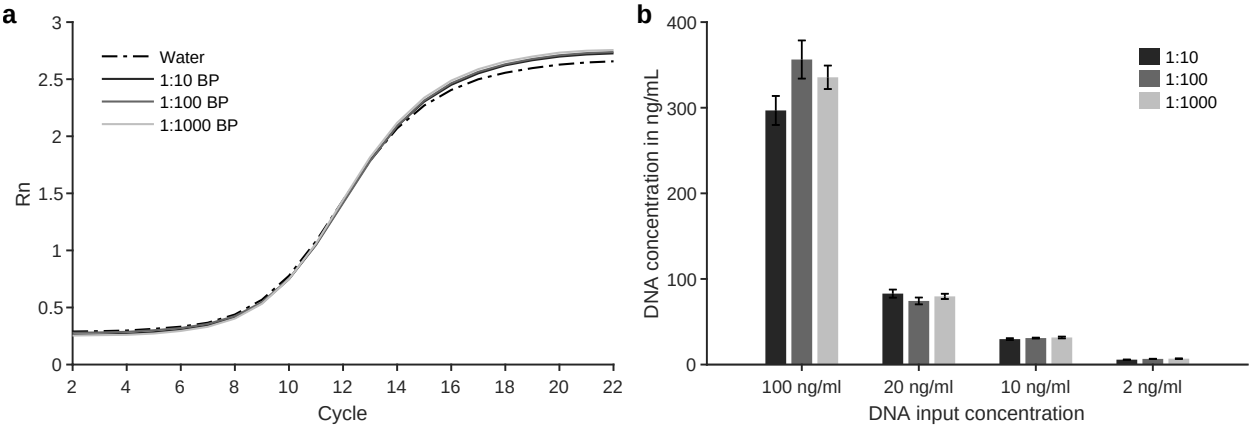


Figure 5.4 Quantitative PCR performance for spike-in experiments and dilution series to assess matrix inhibition. (a) Amplification curves for 160 bp DNA spiked at 5 ng/mL into water and into a dilution series of the ATPS bottom phase extracts (BP) to evaluate potential matrix inhibition of PCR amplification. Fluorescence intensity is plotted against cycle number. Each condition was measured in technical triplicate; one representative amplification curve per condition is shown for clarity. (b) Quantified DNA concentrations for input concentrations of 2–100 ng/mL measured in three dilutions (1:10, 1:100, 1:1000). Data represent mean ± SD of technical triplicates.

5.3.5 Serial ATPS extraction for enhanced DNA concentration

To further increase DNA concentration, the efficiency of serial ATPS steps was evaluated. In this setup, fresh PEG was added to the bottom phase of a first ATPS to induce a second phase separation, aiming to reduce the bottom-phase volume and remove residual protein. Due to mass balance constraints, adding PEG alone did not reproduce the composition of the first ATPS, so fresh plasma was added to dilute the rest of the phase-forming components. Although this increased total volume, it enabled reaching the desired PEG/salt content while introducing additional DNA into the system. This is a beneficial feature for liquid biopsy applications where larger plasma volumes increase the chance of recovering rare cfDNA fragments [235].

DNA recovery and concentration factors were analyzed across three consecutive ATPS steps (Tab. 5.1). Samples were diluted tenfold before analysis but were not purified by reverse elution due to sample volume constraints, each bottom phase being used to create the subsequent ATPS. Recovery for each step was calculated relative to the total DNA present before phase separation, including DNA added through fresh plasma and carried over from the preceding step. The total recovery was based on the total DNA input across all steps.

Serial ATPS formation was successfully achieved, though bottom-phase volume reduction for systems 2 and 3 was limited to a factor of 3, compared to 4.7 for system 1. Total protein removal reached 99.8% before reverse elution, confirming efficient protein depletion across all steps. Individual step recoveries were consistent (70–79%), yet cumulative recovery decreased to 53±4% after the second step and 46±3% after the third. Thus, while serial extraction increases plasma input and DNA concentration, it does so at the expense of overall yield. This trade-off requires careful optimization.

Table 5.1 Parameters and analytical outcomes for serial ATPS steps. Overview of system parameters and analytical results for consecutive ATPS steps, including plasma input, bottom phase (BP) volume, concentration factors (CF), DNA recovery, protein concentration, and protein removal efficiency before reverse elution. Data is reported for a representative experiment.

ATPS	Plasma input	BP volume	BP CF	DNA CF	DNA recovery	Protein concentration	Protein removal
1	1000 µL	215 µL	4.7	3.3±0.4	71±8%	3.0 mg/mL	99.0%
2	229 µL	145 µL	3.1	4.5±0.3	70±5%	1.5 mg/mL	98.6%
3	154 µL	103 µL	2.9	6.2±0.3	79±4%	1.3 mg/mL	98.7%
Total	1383 µL	103 µL	13.4	6.2±0.3	46±3%	1.3 mg/mL	99.8%

5.3.6 Process optimization

5.3.6.1 Selection of optimal system composition

Following these findings, a second screening was conducted to identify the optimal system-point composition that maximized bottom-phase volume reduction without compromising DNA recovery. Based on previously established phase diagrams [231], four system points with increasing PEG and decreasing salt concentrations were tested. The first composition was chosen to be similar to the previously optimized composition (27.9% (w/w) PEG/8.1% (w/w) salt). The samples were diluted tenfold prior to analysis, but were not purified by reverse elution. As expected, the plasma input to bottom-phase volume ratio increased from 4.2 to 6.2 with higher PEG content, but DNA recovery declined by more than 10% at a system composition of 31.0/6.5% (w/w) (Fig. 5.5a). Interestingly, protein concentration in the bottom phase decreased at PEG concentrations above 30% (w/w), indicating improved protein removal (Fig. 5.5b). The ATPS with 30.0% (w/w) PEG and 7.0% (w/w) salt provided the best compromise between DNA concentration, recovery, protein removal, and volume reduction, and was selected for subsequent experiments.

5.3.6.2 Influence of practical handling parameters

To streamline the ATPS-based extraction process, several practical parameters were evaluated, including PEG dissolution, tube filling ratio, component addition order, incubation temperature, and centrifugation time.

To accelerate PEG dissolution, thin shavings obtained from solidified PEG films were tested instead of cylindrical PEG pieces. Although initial dissolution appeared faster, total dissolution time was not reduced, and DNA recovery decreased to 36–53% compared to 45–74% with cylindrical PEG for the same tube-to-plasma volume ratio of 2.5 (Fig. 5.5c).

Process performance was also affected by the total ATPS volume relative to tube volume. Systems with a tube-to-plasma volume ratio below two showed large variability in DNA recovery (10–90%), likely due to insufficient mixing and incomplete dissolution of PEG and salt. A ratio of at least two is therefore recommended to ensure reproducible results.

The order of component addition had a minor but reproducible influence. Optimal performance was achieved when plasma was first mixed with the salt before adding PEG, using cylindrical pieces smaller than 150 mg rather than shavings.

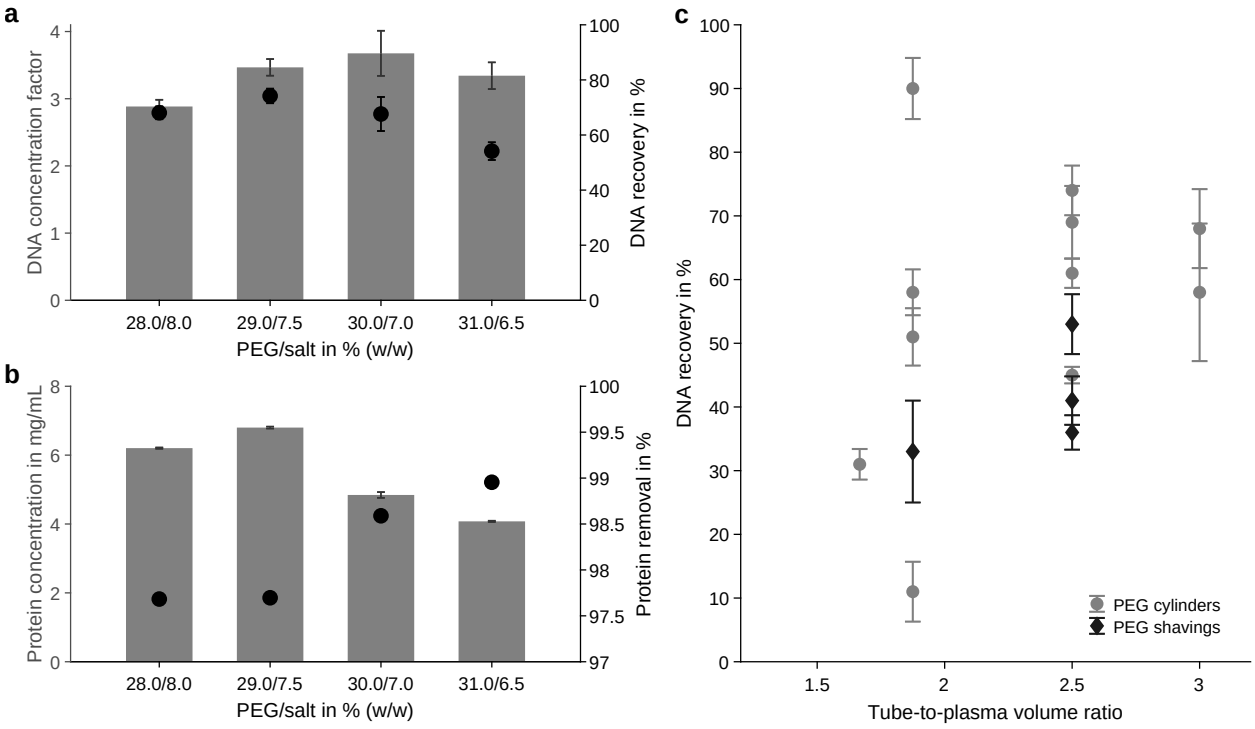


Figure 5.5 Effects of system composition and tube-to-plasma volume ratio on DNA recovery. (a) DNA concentration factor (bars, left axis) and DNA recovery (dots, right axis) before reverse elution for ATPS compositions with different PEG/salt contents (% (w/w)). (b) Corresponding protein concentration (bars, left axis) and removal efficiency (dots, right axis) under the same conditions. (c) DNA recovery of unpurified bottom phase samples as a function of tube-to-plasma volume ratio for PEG cylinders (dots) and PEG shavings (diamonds). Data represent mean ± SD of technical triplicates.

Incubation temperature during mixing was evaluated by increasing it between 30°C and 40°C compared to room temperature. Contrary to expectations based on PEG 1000's melting point (35–40°C), higher temperature did not accelerate dissolution, and led to increased protein partitioning into the bottom phase, indicating reduced selectivity (Fig. A5.5a). Room-temperature incubation was therefore selected for the final protocol, which also simplifies processing by eliminating the need for heating during mixing.

Finally, centrifugation time for phase separation was reduced from 5 min to 1 min without affecting phase formation (Fig. A5.5b), DNA recovery, or protein distribution. The resulting optimized workflow is summarized in Figure A5.6a.

5.3.6.3 Optimization of reverse elution conditions

Lastly, modifications to the reverse elution protocol were tested to improve DNA recovery and concentration. Specifically, it was observed that reusing purification plates that had undergone repeated high-speed centrifugation (3000 × g) led to substantial DNA loss, suggesting that DNA may become trapped within the damaged matrix. To verify this, the eluate was reapplied to the same well and eluted again at 750 × g for 1 min. The second elution yielded the same final volume as the first, but approximately twice the DNA amount was recovered, confirming that a large portion

of DNA remained in the matrix after the first elution. While this double-elution step can effectively recover retained DNA, it also increases the risk of co-eluting residual proteins or salts. Therefore, using fresh plates that have not been centrifuged above 1000 × g more than once is recommended to minimize DNA retention.

Reducing the input volume per well proved more directly beneficial for DNA concentration. In the standard protocol, 100 µL of the bottom phase were applied per well, containing 500 µL of the purification matrix. Experiments showed that the retained volume after conditioning at 3000 × g and elution at 750 × g remained approximately 30 µL, regardless of the input volume. Consequently, decreasing the input from 100 µL to 50 µL increased the relative volume reduction from 30.8% to 60.1% (Fig. 5.6a). When 100 µL of bottom phase were split into two wells (50 µL each, eluates pooled), the DNA concentration factor increased from 2.4 to 3.9 without affecting recovery (Fig. 5.6b). These results demonstrate that DNA concentration can be improved by lowering the input volume per well while maintaining recovery and desalting efficiency.

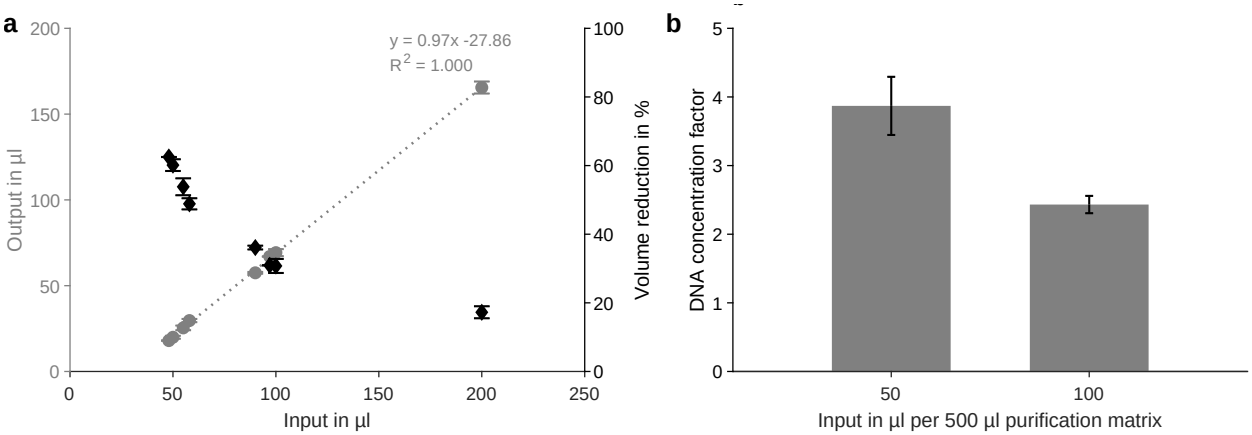


Figure 5.6 Correlation between input and output volumes during reverse elution and their effect on DNA concentration. (a) Output volume (dots, left axis) and relative volume reduction (diamonds, right axis) as a function of input volume during reverse elution using the optimized protocol (3000 × g conditioning, 750 × g elution), including a linear trend line. (b) DNA concentration factor for 100 µL and 50 µL input extract per well containing 500 µL purification matrix. Data in (a) represent mean ± SD from at least two independent experiments for each input volume (n ≥ 12 for 50 µL and 100 µL input). Data in (b) represent mean ± SD of technical triplicates.

5.3.7 Extraction of cfDNA reference material for sequencing analysis

As a proof-of-concept demonstration of sequencing compatibility, a cfDNA reference set containing defined variants was spiked into DNA-free plasma and processed using the developed ATPS-based extraction workflow. Sequencing analysis performed in a routine molecular pathology laboratory at a university clinic demonstrated that the resulting extracts enabled reliable NGS library preparation, with expected quality-control metrics, including TapeStation profiles and library concentrations. In addition, on-target deduplication ratio and median fragment lengths obtained on a NovaSeq 6000 sequencer (Illumina) were also within the expected range (Tab. A5.2). We further compared a single-step ATPS extraction with a serial two-step ATPS process. In both cases, all variants spiked at 5% variant allele frequency (VAF) were reliably detected, whereas no variants were observed in

the negative controls spiked with 0% VAF cfDNA (Tab. 5.2). These results demonstrate that DNA obtained via the ATPS extraction workflow is compatible with downstream library preparation and NGS-based variant detection.

Table 5.2 Next-generation sequencing results for cfDNA reference material with defined mutations extracted using the ATPS-based process. DNA-free plasma was spiked at 5% variant allele frequency (VAF). Detected variants, total sequencing depth, and measured VAF are reported for extracts obtained using a single ATPS step (ATPS 1) or two consecutive ATPS steps (ATPS 2). Data is reported for a representative experiment.

	Variant		ATPS 1		ATPS 2	
Gene	**cDNA**	**Protein**	**Depth**	**VAF**	**Depth**	**VAF**
AKT1	c.49G>A	p.E17K	4702	0.070	6789	0.068
BRAF	c.1799T>A	p.V600E	3431	0.055	4851	0.059
ERBB2	c.2313_2324dup	p.Y772_A775dup	7473	0.059	10915	0.060
KRAS	c.35G>A	p.G12D	4433	0.067	6471	0.075
KRAS	c.181C>A	p.Q61K	5151	0.086	7569	0.088
KRAS	c.436G>A	p.A146T	2964	0.054	4324	0.045
PIK3CA	c.1633G>A	p.E545K	2885	0.063	4169	0.052
PIK3CA	c.3140A>G	p.H1047R	4320	0.104	6488	0.102

5.4 Discussion

In this study, we developed and optimized an ATPS-based workflow to extract and purify short DNA fragments from plasma, demonstrating its potential for liquid biopsy applications. Although DNA recovery has not yet reached the efficiency of state-of-the-art adsorption-based methods such as the QIAamp kit (QIAGEN) [14], the presented method offers a rapid, simple, and cost-effective alternative that requires minimal equipment and handling. Furthermore, the ATPS-based workflow enabled reproducible DNA recovery from minute input amounts, efficient protein removal, compatibility with amplification-based downstream analyses, and robust performance under optimized parameters. This approach could facilitate the clinical implementation of cfDNA-based liquid biopsy assays, advancing individualized disease monitoring and treatment [41].

DNA concentration is critical for NGS library preparation, as most kits require defined input amounts of DNA in small volumes (e.g., 10 ng in 50 µL), and cfDNA concentrations in plasma typically range from 1 to 40 ng/mL [10]. The initially selected ATPS composition provided high recovery, but limited volume reduction (V_R=1). Shifting along the same tie-line to increase V_R caused substantial DNA loss [252], likely due to PEG-induced DNA precipitation [167, 223]. A shorter tie-line was identified where DNA and protein partitioning remained consistent, yet phase formation required less PEG and salt, enabling an effective reduction in bottom-phase volume while maintaining ∼60% recovery after purification. These findings confirm that ATPS composition can be tuned to balance recovery and selectivity, though approaching the binodal too closely reduces robustness and alters partitioning behavior [214], as observed before [252]. The second system optimization highlights the influence of PEG exclusion effects and solubility limits on DNA recovery [168, 221], as well as the inherent trade-off between concentration and yield in ATPS-based extraction.

Serial extraction experiments, inspired by back-extraction approaches found in the literature [136, 215], demonstrated that repeated ATPS steps can effectively increase DNA concentration and total plasma input. However, each step introduced incremental DNA loss, ultimately reducing total yield to about half after three ATPS. Despite the consistent formation of biphasic systems and enhanced protein removal, deviations in phase volume reduction in later cycles suggest compositional shifts. While PEG and salt ratios were theoretically identical across all systems, the lower plasma and protein content in systems 2 and 3 likely influenced phase formation and shifted the binodal, as previously observed for biomass-dependent phase behavior [25]. Thus, while serial extraction can improve concentration and purity, its advantages must be weighed against the cumulative DNA losses it entails.

The influence of lysis on extraction performance was evaluated both pre- and post-ATPS capture to determine whether additional disruption of protein-DNA complexes could enhance recovery. The results indicate that plasma lysis prior to ATPS formation is not beneficial, as it disrupts phase behavior due to altered protein composition and plasma dilution. Because each lysis method relies on different mechanisms (enzymatic digestion, reduction, precipitation), the resulting interphase appearance and composition vary accordingly. This was confirmed by SDS-PAGE analysis of lysed bottom-phase samples, which revealed method-specific protein band patterns reflecting distinct degradation pathways. Proteinase K is a serine protease that cleaves peptide bonds [255] and produces a broad smear of low-molecular-weight fragments, whereas the strong reduction agent used in lysis T disrupts disulfide bonds, yielding smaller protein subunits [256]. However, without a precipitating agent such as the clearing solution in lysis B, these subunits remain in solution.

In contrast, applying lysis post-ATPS, when most proteins have already been removed from the bottom phase, increased DNA recovery by up to 20%. This suggests that disrupting residual DNA–protein interactions within the bottom phase facilitates DNA release during purification. However, the benefit of post-capture lysis was not consistent across all systems. In the ATPS containing 7.6% (w/w) more PEG and 5.7% (w/w) less salt, the non-lysed sample performed best. This likely reflects unfavorable interactions between lysis reagents and phase-forming components, most likely the high amount of PEG, which could lead to increased PEG–salt precipitation [257].

Among the tested lysis methods, the rapid, enzyme-free lysis B achieved comparable recovery to the enzymatic standard while drastically reducing incubation time. Method Q was optimized for proprietary buffer systems and plasma lysis to increase DNA adsorption onto silica, which explains its reduced effectiveness in lysing high-salt bottom-phase samples. The results highlight that post-capture lysis can improve DNA yield, but is not strictly required, as the salt-rich bottom phase itself likely promotes histone dissociation and cfDNA release [258]. Because cfDNA is naturally associated with histones, conventional extraction workflows typically rely on lysis using strong chaotropes such as guanidine thiocyanate to improve DNA adsorption to silica membranes or beads [125, 259]. In ATPS systems, omitting this step could streamline the workflow, reduce reagent consumption, and facilitate automation, supporting potential clinical implementation.

Process parameter optimization revealed that seemingly minor handling conditions can impact the reproducibility and efficiency of ATPS-based DNA extraction. Attempts to accelerate PEG dissolution by using thin shavings unexpectedly reduced DNA recovery, likely due to transient local PEG concentration peaks that promote DNA precipitation, as previously observed for pre-dissolved PEG systems [252]. In contrast, slower, more gradual dissolution of larger PEG pieces improved DNA recovery, highlighting the importance of homogeneous component mixing and complete equilibration before phase separation [141, 260]. Similarly, systems processed in overfilled tubes (tube-to-plasma ratios <2) showed high variability, indicating insufficient mixing and impaired mass transfer. These

conditions could lead to increased DNA entrapment in the interphase rather than transfer to the protein-poor bottom phase. This hypothesis is supported by recent studies showing that mixing time and agitation speed influence partitioning and mass transfer in ATPS [261]. In addition, vessel geometry, particularly the height-to-diameter ratio, has been shown to affect phase separation kinetics and equilibrium in ATPS, further emphasizing the role of physical process parameters in system performance [262].

Further tests showed that elevated temperatures during mixing did not accelerate PEG dissolution but instead led to undesired protein partitioning into the bottom phase. Such temperature-induced binodal shifts have been reported previously for PEG–salt ATPS [263], and elevated temperature can also alter protein stability. Thus, room-temperature operation preserves selectivity while simplifying handling. Although the equilibration time could not be shortened without compromising performance, the centrifugation time was reduced from 5 min to 1 min without affecting phase formation or analyte distribution, thereby improving process throughput. Together, these findings demonstrate that control of practical handling parameters such as mixing, temperature, and tube volume is essential for reliable ATPS performance. Maintaining a simple, room-temperature workflow supports scalability and facilitates integration into automated extraction platforms. High-throughput processing could be achieved using a deep-well plate format and a liquid-handling station equipped with an integrated centrifuge and a rotational shaker, as in the setup described in our previous work [231].

Purification by reverse elution was optimized to increase DNA concentration and purity. As shown previously for 160 bp DNA fragments in water [252], higher conditioning speeds effectively "dried" the purification matrix, thereby allowing greater retention of the input liquid within pores and interparticle spaces [185]. Combined with a lower elution rate, which prolongs liquid-matrix contact, this enabled efficient flow-through volume reduction. Although DNA loss was slightly higher for real bottom-phase samples than for spiked water, the purification step remains essential for removing excess salts and residual proteins that can interfere with downstream analyses. DNA retention in the matrix likely arises from entrapment within interstitial spaces by capillary forces [188], weak electrostatic interactions [244], or, in the case of plasma extracts, associations with salt and proteins.

When purification plates previously exposed to high-speed centrifugation were reused, DNA retention increased substantially. This suggests that mechanical stress during conditioning damages purification matrix particles, altering pore structure and binding capacity [264, 265]. While a second elution can recover most of the retained DNA, it also risks co-eluting residual contaminants, making the use of fresh plates preferable. In contrast, reducing the input-to-matrix ratio improved concentration performance without compromising recovery or purity, thereby providing a simple means to increase the final DNA concentration factor. Further tuning of this ratio could enhance performance, though too little matrix risks contaminant breakthrough, while excess matrix increases DNA retention. Overall, these insights demonstrate that physical handling parameters, rather than matrix chemistry alone, can substantially influence the final yield and concentration in the purification step.

The analytical performance of the ATPS extracts confirmed their suitability for amplification-based downstream analysis. qPCR spike-in experiments inspired by Devonshire et al. [15] showed no evidence of inhibition [266], and measured concentrations closely matched expected values, indicating efficient removal of potentially interfering plasma and phase-forming components. ALU-qPCR analysis of unspiked plasma further demonstrated that ATPS extracts contained predominantly short DNA fragments (<247 bp) [253], whereas silica-based extraction also retained longer fragments.

The resulting low DNA integrity index aligns with the characteristic size distribution of ctDNA in plasma [206], and electropherograms confirmed the efficient exclusion of longer fragments from the bottom phase. This selective recovery reduces gDNA contamination while efficiently retaining cfDNA [11]. The preferential recovery of shorter fragments suggests that the ATPS extraction system introduces a degree of size selectivity. Such enrichment may be advantageous for mutation-based liquid biopsy assays, as tumor-derived DNA fragments are typically shorter than background cfDNA [267–269]. Similar size-selective approaches have been reported to increase the relative abundance of tumor-derived variants [136, 238]. For applications requiring fully unbiased genome-wide fragment representation, the potential influence of extraction-related size selection should be evaluated in future studies.

To assess the compatibility of ATPS-derived extracts with sequencing workflows, proof-of-concept NGS experiments were performed in a clinical molecular pathology laboratory with experience in liquid biopsy diagnostics for solid tumors. Library preparation was consistently successful for all ATPS extracts, indicating the absence of inhibitory residues and sufficient cfDNA quality. Sequencing quality metrics were within expected ranges, enabling reliable variant calling at adequate depth. According to the sequencing laboratory, the library preparation and sequencing quality metrics obtained from ATPS-extracted samples were comparable to those typically observed for routine plasma samples processed in their laboratory using the Maxwell®RSC Rapid ccfDNA Kit (Promega), which had previously been internally benchmarked against a QIAamp-based extraction workflow (personal communication). Together, these results demonstrate that cfDNA purified using the developed ATPS-based workflow is compatible with downstream NGS analysis. However, this study does not include a direct, comprehensive comparison with established cfDNA extraction methods, and future studies should demonstrate that variant detection and library complexity are comparable.

Similar to the microfluidic PIBEX approach developed by Lee et al. [188], the presented workflow addresses several practical limitations associated with conventional silica-based extraction methods, such as the manual QIAamp Circulating Nucleic Acid Kit, including the risk of cross-contamination during multi-step handling, limited input volumes, and relatively high consumable costs [13]. Based on comparative analyses reported in the literature, the recovery efficiency achieved with the ATPS-based method falls within the range reported for commonly used magnetic bead-based systems, such as the Maxwell RSC ccfDNA Plasma Kit (Promega, 60%) and the MagMAX Cell-Free DNA Isolation Kit (Applied Biosystems, 50%) [14, 254]. Although further benchmarking against established workflows will be necessary, the simplicity of the phase-partitioning approach, its minimal handling requirements, and potential scalability suggest that the developed ATPS workflow represents a promising alternative to conventional adsorption-based cfDNA extraction strategies.

A limitation of the present study is that most experiments were performed using cfDNA reference material spiked into healthy plasma rather than patient-derived clinical samples. While this model system enabled controlled investigation of DNA partitioning behavior and extraction performance, clinical plasma samples may present additional complexities, including variable levels of genomic DNA contamination, nucleoprotein complexes, and heterogeneous cfDNA fragment length distributions [95, 270]. Consequently, future validation using patient-derived plasma samples will be necessary to fully assess the robustness and clinical applicability of the ATPS-based extraction workflow.

5.5 Conclusions

In conclusion, this study demonstrates the feasibility of an ATPS-based workflow for the extraction and purification of cfDNA-like fragments from plasma under mild, aqueous conditions. The process operates without chemical or enzymatic lysis, enables efficient protein removal, and yields high DNA purity compatible with PCR and NGS analyses, all within a short processing time using minimal equipment. This makes it suitable for routine and potentially automated use. Although a trade-off between recovery and concentration remains, and validation with patient-derived plasma is still required, the presented method represents a considerable step toward more accessible cfDNA extraction. Building on prior work, this study addresses earlier limitations in concentration efficiency and lysis, establishing a proof of concept for an alternative ATPS-based extraction workflow for amplification-based liquid biopsy applications.

Acknowledgments

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Supplementary data

Appendix **A5** contains the Supporting Figures associated with this chapter.

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Conclusion and outlook

This thesis aimed to investigate aqueous two-phase systems (ATPS) as an alternative platform for extracting short circulating cell-free DNA (cfDNA)-like fragments from plasma and to translate fundamental insights into a practical, analytically compatible workflow for liquid biopsy applications. To achieve this, a systematic approach was followed, progressing from controlled model systems to application in a complex biological matrix and ultimately to the development of an integrated extraction process. In doing so, this work addresses key challenges associated with cfDNA isolation, including low analyte concentrations, abundant plasma proteins, and the need for scalable, simplified sample preparation strategies.

The first study (Chapter 3) focused on establishing a fundamental understanding of short DNA fragment partitioning in polymer–salt ATPS. A multitude of system parameters, including polyethylene glycol (PEG) molecular weight, tie-line length, phase volume ratio, and the addition of neutral salt, were investigated, revealing consistent trends in DNA distribution. In particular, PEG molecular weight emerged as a key determinant of partitioning behavior, with lighter PEG chains favoring top-phase distribution while heavier PEG led to DNA enrichment in the bottom phase. Furthermore, adding neutral salts such as NaCl to systems containing PEG smaller than 600 g/mol enabled selective DNA transfer from the top phase to the protein-depleted bottom phase. These findings not only confirmed that short DNA fragments behave similarly to plasmid DNA in PEG/phosphate systems but also provided a basis for the rational selection of systems for further separation experiments. Because the experiments were conducted in well-defined model systems or simplified matrices, it remained unclear how well the observed partitioning behavior would translate to ATPS containing human plasma, where additional interactions with proteins and other biomolecules are expected. In particular, DNA–protein interactions and changes in phase behavior could alter partitioning and reduce selectivity. Addressing this uncertainty was therefore essential to assess the practical applicability of ATPS for cfDNA extraction and directly motivated the second study.

Accordingly, the second study (Chapter 4) investigated DNA partitioning and recovery in plasma-containing PEG/phosphate ATPS. The most notable difference from model systems was the formation of a protein-rich interphase, which partially affected phase separation and DNA distribution, especially in systems that required the addition of a neutral salt to recover DNA in the bottom phase. Nevertheless, the expected trends were confirmed in selected plasma-containing PEG 1000 systems, enabling high DNA recovery in the salt-rich bottom phase while significantly reducing protein content. This partitioning behavior proved reproducible across different types of blood collection tubes and samples from individual donors. Beyond confirming the applicability of ATPS to DNA isolation from plasma, this study also addressed practical considerations for further process development. Process efficiency was increased by using solid PEG and salts rather than pre-dissolved solutions for phase formation, enabling higher plasma input without additional dilution. The scalability of this approach was validated by processing 4 mL of plasma at once, which corresponds to the volume of plasma from a single blood draw. Furthermore, a method to purify the salt-rich DNA extracts was necessary to make them suitable for downstream analysis. To this end, a reverse elution step was introduced, allowing DNA to be recovered and slightly concentrated in the flow-through while retaining salts and removing residual protein. These findings established the feasibility of ATPS-based DNA capture from plasma while revealing remaining challenges, including the potential need for a lysis step to improve protein removal and the insufficient final DNA concentration in the extracts, as sequencing-based downstream analyses place high demands on input material. Addressing these issues formed the basis for the subsequent study.

The third study (Chapter 5) therefore focused on integrating individual process steps into a coherent, optimized ATPS-based extraction workflow and on evaluating its analytical performance. First, the need for a dedicated lysis step before or after ATPS formation was critically assessed. The results showed that protein removal was unaffected by lysis. They also suggested that the high salt concentrations in the bottom phase may promote the dissociation of DNA–protein complexes, enabling efficient DNA recovery without additional lysing reagents. These findings support workflow simplification by omitting time-consuming enzymatic or chemical treatments. In addition, strategies to increase DNA concentration while maintaining recovery were explored. Serially adding fresh PEG and plasma to the salt-rich bottom phase from a previous ATPS increased concentration but also caused cumulative losses. This trade-off between recovery and concentration is a key consideration for future process optimization, particularly for low-abundance analytes such as cfDNA. Furthermore, operational parameters, including mixing conditions before phase separation, centrifugation time, and system handling, were investigated to shorten the process while maintaining robustness. These optimization experiments revealed that process performance is strongly influenced not only by system composition but also by practical execution. In particular, complete dissolution of the components and thorough mixing before phase separation were found to be crucial for consistent DNA recovery. Importantly, the developed workflow was evaluated for compatibility with downstream analytical methods. The extracted DNA showed no evidence of PCR inhibition, confirming its suitability for amplification-based analyses. Additionally, the extracts exhibited a fragment-size distribution characteristic of cfDNA, and tests with a spiked DNA ladder revealed a selective reduction in high-molecular-weight DNA. This size selectivity may be advantageous for liquid biopsy applications, as diagnostically relevant fragments are enriched while background interference is reduced. Although the overall recovery of sequencing-ready DNA was slightly lower than that achieved with a conventional adsorption-based extraction kit, the ATPS-based process demonstrated clear advantages in processing time, minimal equipment requirements, and operational simplicity. The successful generation of sequencing libraries and the detection of target mutations in

a synthetic cfDNA reference set using next-generation sequencing further confirmed the feasibility of the developed workflow.

Taken together, the results of this thesis demonstrate that ATPS are a viable and versatile platform for extracting cfDNA-like fragments from plasma. Moreover, this work provides a comprehensive framework for understanding and using liquid–liquid extraction systems for cfDNA isolation by linking fundamental partitioning studies to application-oriented process development. The findings show that selective capture of short DNA fragments, substantial protein removal, and compatibility with downstream molecular analyses can be achieved through a simplified, scalable workflow. At the same time, several aspects remain to be addressed to fully realize this method's potential. While the studies presented demonstrate feasibility using model DNA fragments and reference cfDNA material, validation with clinical samples is required to assess performance under realistic conditions, including varying DNA concentrations and fragment lengths, and to detect tumor-derived DNA. Given the simplicity of phase separation and the use of mild conditions, which offer clear advantages for scalable implementation, integrating aqueous two-phase extraction into automated, high-throughput platforms is a promising avenue for future development to broaden the range of applications.

In conclusion, this thesis establishes ATPS as a promising alternative to conventional solid-phase extraction methods for cfDNA-like fragments. Although further optimization and validation are needed, the work demonstrates that liquid–liquid extraction strategies can simplify sample preparation workflows without compromising analytical compatibility. By addressing both fundamental and applied aspects, this research lays a foundation for future advances that improve the accessibility, scalability, and efficiency of liquid biopsy technologies.

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Abbreviations

Acronym	Meaning
ATPS	aqueous two-phase systems
bp	base pairs
BP	bottom phase
cfDNA	circulating cell-free DNA
ctDNA	circulating tumor DNA
Ct	cycle threshold
Da	Dalton
ddPCR	droplet digital polymerase chain reaction
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraacetic acid
EO-PO	ethylene oxide-propylene oxide
gDNA	genomic DNA
HSA	human serum albumin
IgG	immunoglobulin G
IP	interphase
kbp	kilo base pairs
LOD	limit of detection
MW	molecular weight
NGS	next-generation sequencing
PAA	polyacrylate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pDNA	plasmid DNA
RFU	relative fluorescence unit
qPCR	quantitative polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Continued on next page

Acronym	Meaning
ssDNA	single-stranded DNA
TAE	tris-acetate-EDTA
TLL	tie-line length
TP	top phase
UV	ultraviolet
UV/Vis	ultraviolet/visible
VAF	variant allele frequency
V_R	volume ratio

A3

Appendix Chapter 3

Partitioning behavior of short DNA fragments in polymer/salt aqueous two-phase systems

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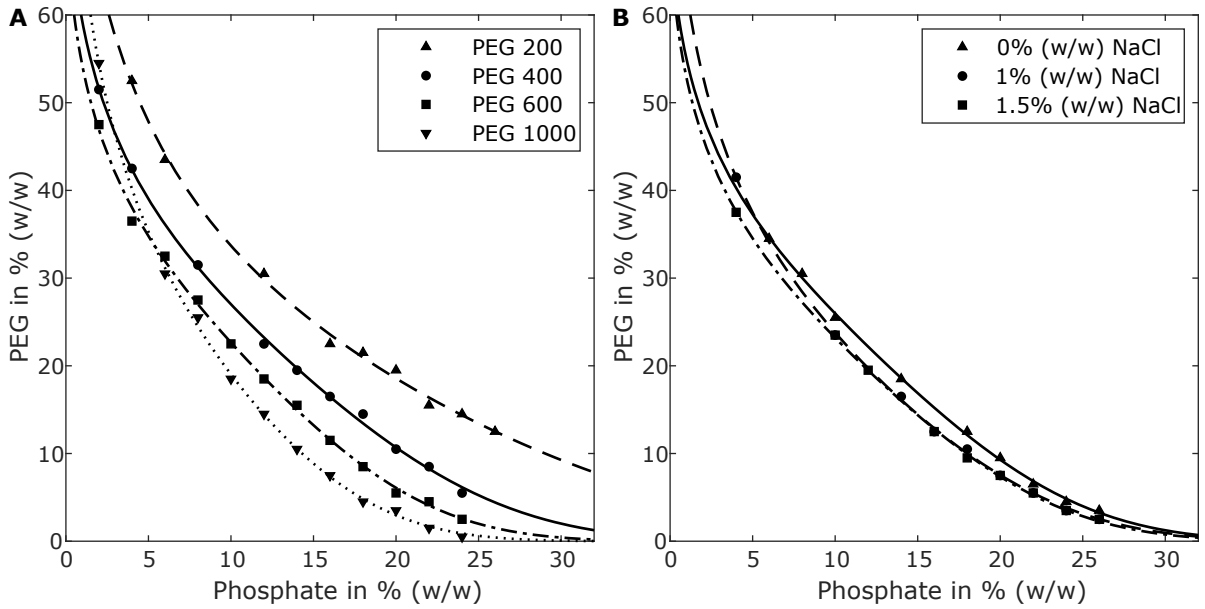


Figure A3.1 Binodal curves for (A) PEG/phosphate ATPS at pH 7.4 with various PEG molecular weights and (B) PEG 400/phosphate ATPS with NaCl addition. Binodal data was measured on the liquid handling station and fitted using Equation 3.1.

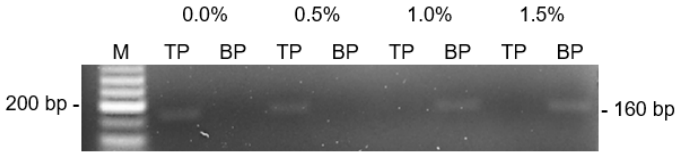


Figure A3.2 Agarose gel electrophoresis of top phase (TP) and bottom phase (BP) samples from a 17.7% (w/w) PEG 400/17.3% (w/w) phosphate ATPS with increasing NaCl concentrations (0.0%, 0.5%, 1.0%, and 1.5% (w/w)). Lane M is a DNA bp marker. Systems were spiked with 600 ng/mL DNA and samples were desalted before electrophoresis.

Table A3.1 Data values of 160 bp DNA fragment recoveries shown in Figure 3.1 and total recoveries (mean ± SD). Composition, PEG molecular weight (MW), and tie-line length (TLL) are indicated for each ATPS.

PEG MW	**ATPS comp.** in % (w/w)		**TLL**	**DNA recovery** in %		
	PEG	**Phosphate**	in % (w/w)	**Top phase**	**Bottom phase**	**Total**
200	16.9	23.6	27.9	91.4 ± 8.3	0.1 ± 1.0	91.5 ± 9.3
	17.8	23.7	33.7	101.6 ± 3.4	0	101.6 ± 3.4
	19.1	23.8	39.7	94.3 ± 5.0	0	94.3 ± 5.0
	20.2	24.7	46.9	99.6 ± 3.1	0.2 ± 1.8	99.8 ± 4.9
	21.1	24.9	50.3	90.0 ± 2.7	0.3 ± 1.2	90.2 ± 3.9
	21.8	25.5	54.1	91.1 ± 5.0	0.5 ± 0.7	91.6 ± 5.7
400	17.7	17.3	33.5	97.8 ± 2.4	0	97.8 ± 2.4
	19.7	18.4	45.0	72.9 ± 2.8	0	72.9 ± 2.8
	20.0	19.6	48.5	76.6 ± 2.0	0.1 ± 0.7	76.7 ± 2.7
	20.8	20.8	52.6	78.2 ± 4.8	0	78.2 ± 4.8
	22.4	22.5	58.8	55.9 ± 3.5	1.2 ± 1.0	57.0 ± 4.5
	24.9	24.3	66.4	36.4 ± 3.8	0	36.4 ± 3.8
600	16.2	15.1	30.5	51.5 ± 1.3	47.3 ± 1.5	98.8 ± 2.9
	18.5	16.1	42.0	1.5 ± 0.6	129.8 ± 4.0	131.2 ± 4.6
	19.3	16.5	44.9	0.2 ± 0.7	121.5 ± 2.9	121.7 ± 3.6
	19.9	17.9	48.9	0.9 ± 0.7	106.7 ± 4.3	107.5 ± 5.0
	21.7	18.6	53.7	0.3 ± 0.5	31.1 ± 4.0	31.4 ± 4.5
	24.2	20.6	61.4	17.7 ± 1.0	0.3 ± 0.5	18.0 ± 1.5
1000	17.1	12.9	31.3	0.2 ± 1.9	82.6 ± 2.3	82.8 ±4.2
	19.5	13.8	41.1	1.2 ± 1.4	89.4 ± 2.8	90.6 ± 4.3
	22.1	14.4	48.6	0	57.3 ± 9.6	57.3 ± 9.6
	23.5	14.8	52.1	0	72.1 ± 3.1	72.1 ± 3.1
	24.2	15.4	54.3	1.2 ± 1.5	26.5 ± 2.3	27.7 ± 3.7
	26.4	16.3	59.8	0	14.2 ± 1.1	14.2 ± 1.1

A4

Appendix Chapter 4

Extraction and purification of short DNA fragments from plasma using aqueous two-phase systems for liquid biopsy

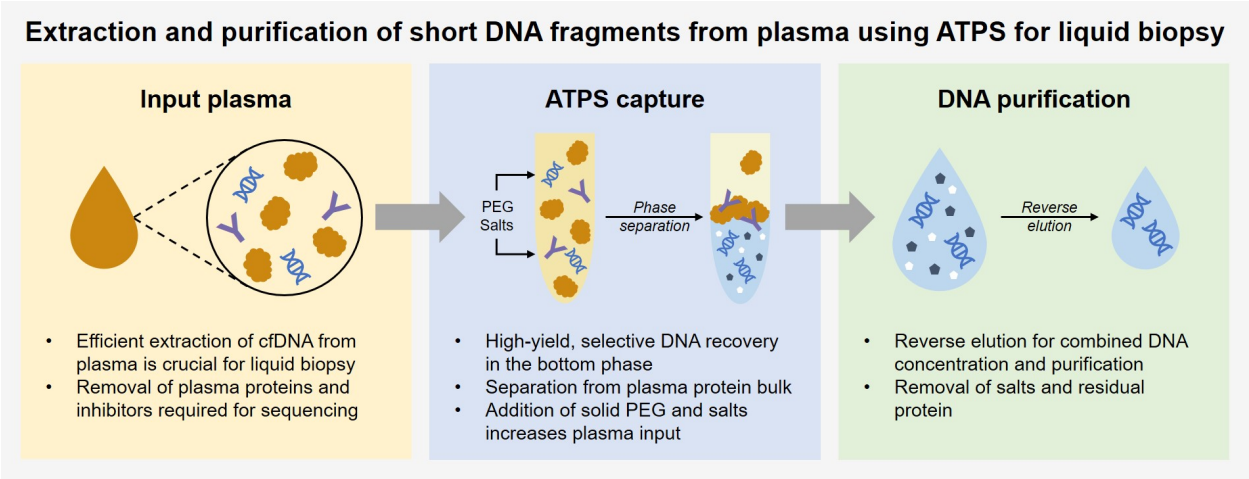
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Graphical abstract



Supplementary material

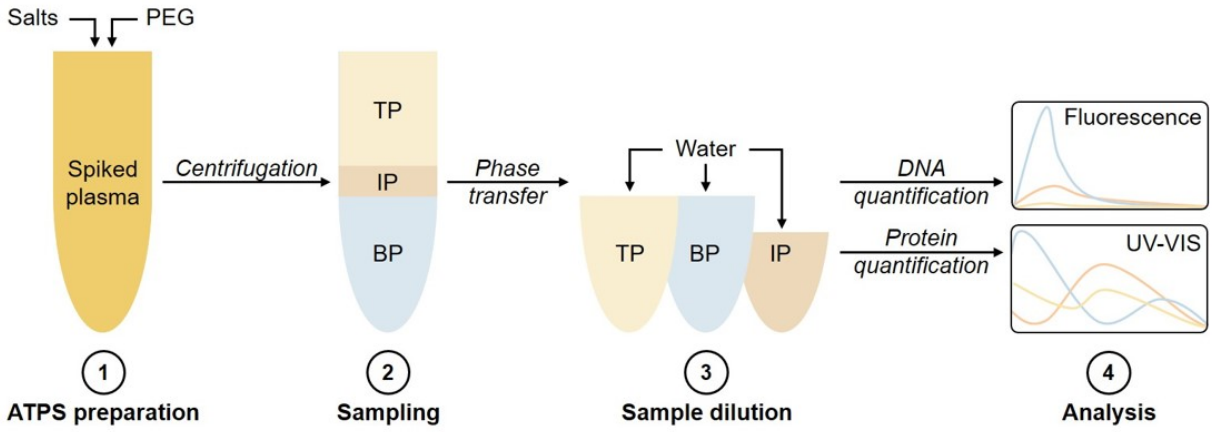


Figure A4.1 Schematic overview of the experimental workflow for partitioning experiments with plasma-containing ATPS. System preparation, phase separation, sampling of the top phase (TP), interphase (IP), and bottom phase (BP) and subsequent analysis are depicted.

Table A4.1 Binodal coefficients a, b, and c, and tie-line slope (TLS) of the investigated PEG/phosphate ATPS at pH 7.4. A detailed description of binodal and tie-line data acquisition is given in Meutelet et al. (2024).

ATPS	**a**	**b**	**c**	**TLS**
PEG 400/Phosphate	82.33	-0.330	-0.00007	-1.29
PEG 400/Phosphate/1.5% (w/w) NaCl	73.64	-0.333	-0.00010	n/a
PEG 1000/Phosphate	111.24	-0.504	-0.00017	-1.94

Table A4.2 System compositions in % (w/w) and corresponding stock solution volumes in µL to prepare ATPS with a theoretical total volume of 600 µL and pH 7.4 investigated in this study. *70% (w/w) PEG in water; **40% (w/w) phosphate salts in water, pH 7.4; ***25% (w/w) NaCl in water.

PEG MW	**PEG**		**Phosphate**		**NaCl**		**Water**		**Plasma**	
	%	µL*	%	µL**	%	µL***	%	µL	%	µL
400	17.7	161.8	17.3	222.7	0	0	41.4	35.6	24.0	179.9
400	17.7	161.8	17.3	222.7	1.5	35.6	39.9	0	24.0	179.9
1000	19.5	172.0	13.8	171.0	0	0	29.0	0	37.7	257.0

Table A4.3 Amount of solid phase-forming components in mg and volume of spiked plasma in µL used to prepare ATPS with a theoretical total volume of 600 µL and pH 7.4. The corresponding amounts of PEG 1000 and phosphate are specified in % (w/w). The expected phase volume ratio (V_R) is indicated for the five system points on the same tie-line.

PEG %	**Phosphate** %	**PEG 1000** mg	**NaH_2PO_4** mg	**K_2HPO_4** mg	**Plasma** µL	**Water** µL	V_R
19.5	13.8	133.0	21.31	72.45	454.7	0	n/a
19.0	12.5	127.6	19.1	65.2	412.4	47.3	1.0
22.0	11.0	146.7	16.7	57.0	412.4	34.0	1.4
25.0	9.5	165.6	14.3	48.9	412.4	21.2	2.1
27.0	8.5	178.0	12.8	43.5	412.4	12.6	3.1
30.0	7.0	196.5	10.5	35.6	412.4	0	5.3

Table A4.4 Manually determined phase volumes in µl of the top phase (TP), bottom phase (BP), and interphase (IP) of the investigated PEG/phosphate ATPS with a theoretical total volume of 600 µL. The corresponding amounts of PEG and phosphate are specified in % (w/w). *IP volume after redissolution with 100 µL water.

ATPS	**PEG** %	**Phosphate** %	**TP** µL	**BP** µL	**IP*** µL
PEG 400/Phosphate	17.7	17.3	325	265	102
PEG 400/Phosphate/NaCl	17.7	17.3	340	255	100
PEG 1000/Phosphate	19.5	13.8	290	240	130
PEG 1000/Phosphate	19.0	12.5	315	230	125
PEG 1000/Phosphate	22.0	11.0	345	190	125
PEG 1000/Phosphate	25.0	9.5	395	145	130
PEG 1000/Phosphate	27.0	8.5	420	115	135
PEG 1000/Phosphate	30.0	7.0	445	80	140

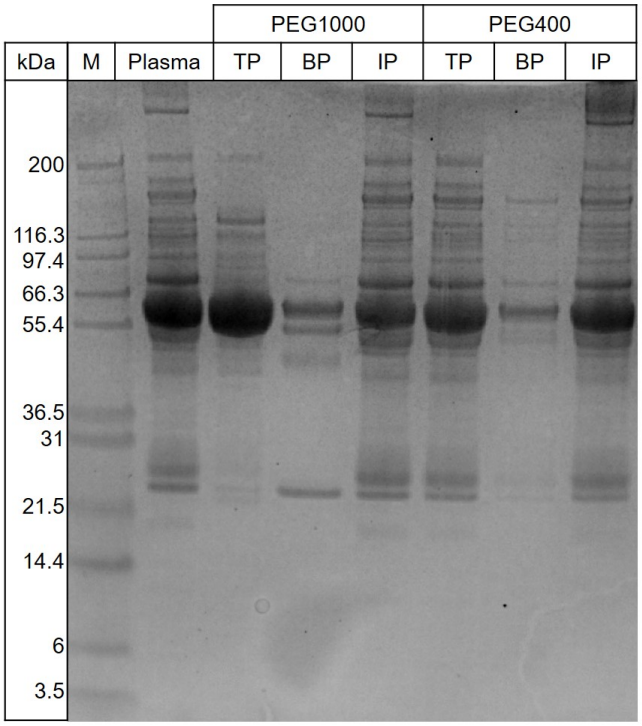


Figure A4.2 SDS-PAGE gel scan of PEG 1000 and PEG 400/phosphate/plasma ATPS top phase (TP), bottom phase (BP), and interphase (IP) samples. Pooled plasma from Streck tubes was included as control. Lane M is a molecular weight marker in kDa. Samples were diluted tenfold in water.

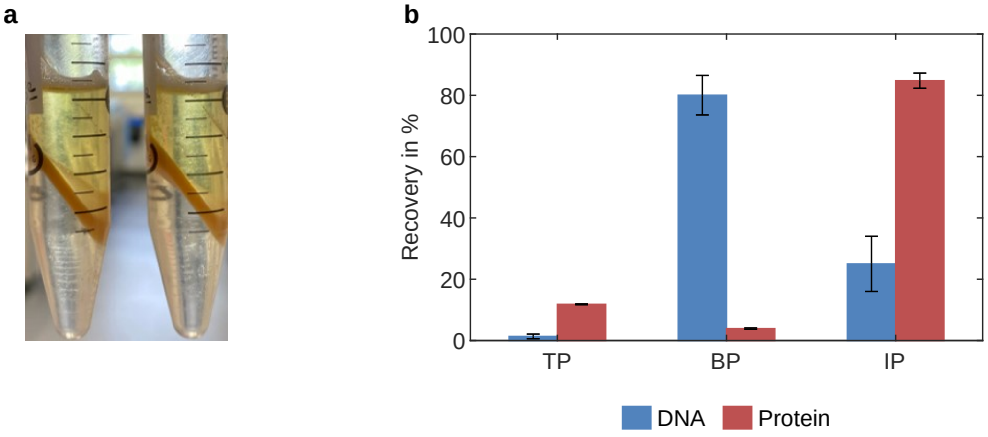


Figure A4.3 (a) Scaled-up ATPS with a plasma input volume of 4 mL; (b) 160 bp DNA and plasma protein recoveries in the top phase (TP), bottom phase (BP), and interphase (IP) of a scaled-up 19.5% (w/w) PEG 1000/13.8% (w/w) phosphate/66.7% (w/w) plasma ATPS made with solid components.

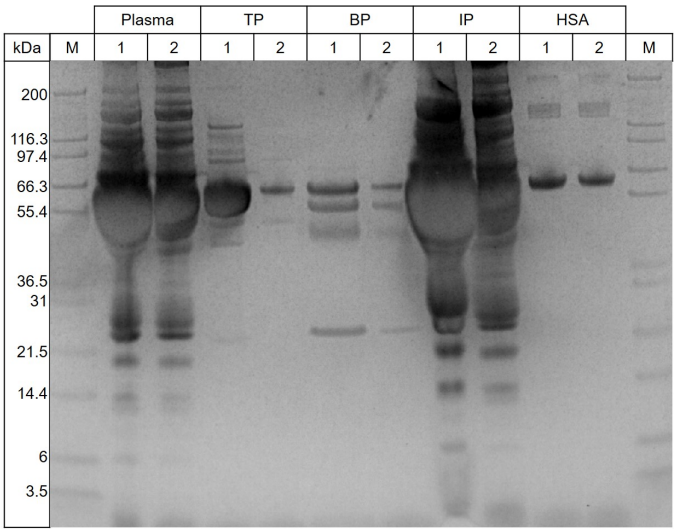


Figure A4.4 SDS-PAGE gel scan of PEG 1000/phosphate/plasma ATPS top phase (TP), bottom phase (BP), and interphase (IP) samples before (1) and after (2) reverse elution. Pooled plasma from Streck tubes was included as control. Lane M is a molecular weight marker in kDa.

A5

Appendix Chapter 5

Proof of concept for aqueous two-phase system-based extraction of cell-free DNA from plasma for liquid biopsy applications

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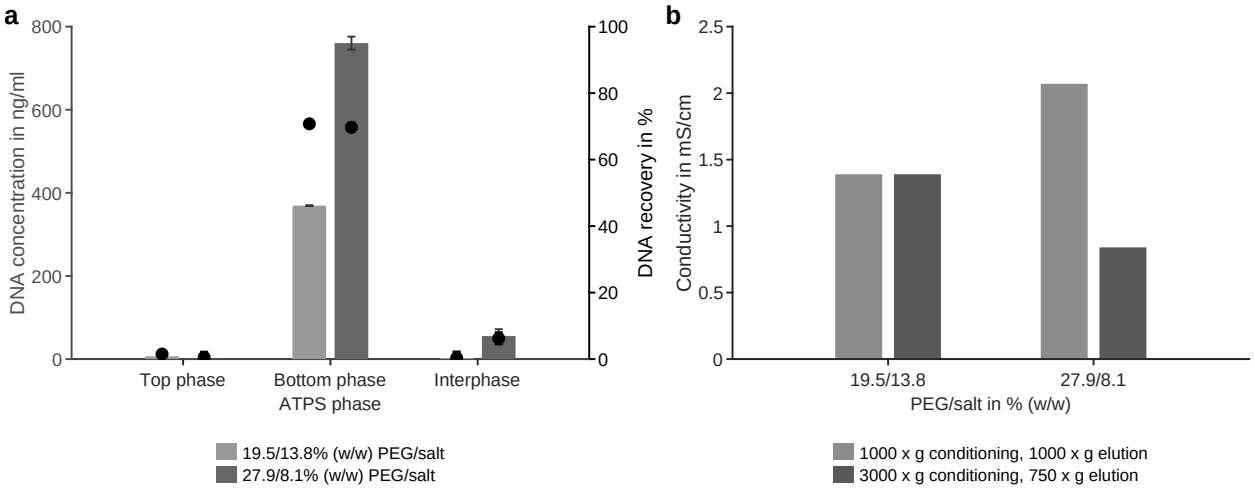


Figure A5.1 Effect of system composition and purification protocol on DNA partitioning and sample conductivity. (a) DNA concentration (left axis, bars) and recovery (right axis, dots) in the top phase, bottom phase, and interphase for two ATPS compositions with different PEG/salt ratios. Samples were purified using the standard reverse elution protocol (1000 × g for conditioning and elution). Data represent mean ± SD of technical triplicates. (b) Conductivity of bottom-phase samples from the two ATPS compositions after purification with either the standard or optimized reverse elution protocol (3000 × g conditioning, 750 × g elution). Measurements were performed once per sample.

Table A5.1 ATPS compositions used for DNA capture in the bottom phase (BP). Tie-line length (TLL), PEG 1000, salt, and plasma contents are given in % (w/w). For a plasma input of 1000 µl, the corresponding masses of PEG and phosphate salts (mg) and the resulting bottom phase volume are reported.

TLL	**PEG**	**Salt**	**Plasma**	**PEG 1000**	**NaH_2PO_4**	**K_2HPO_4**	**Plasma**	**BP volume**
% (w/w)	% (w/w)	% (w/w)	% (w/w)	mg	mg	mg	µl	µl
41	27.9	13.8	66.7	292.5	46.9	159.3	1000	500
34	19.5	8.1	64.0	448.7	29.5	100.0	1000	250
31	30.0	7.0	63.0	490.5	26.1	88.4	1000	185

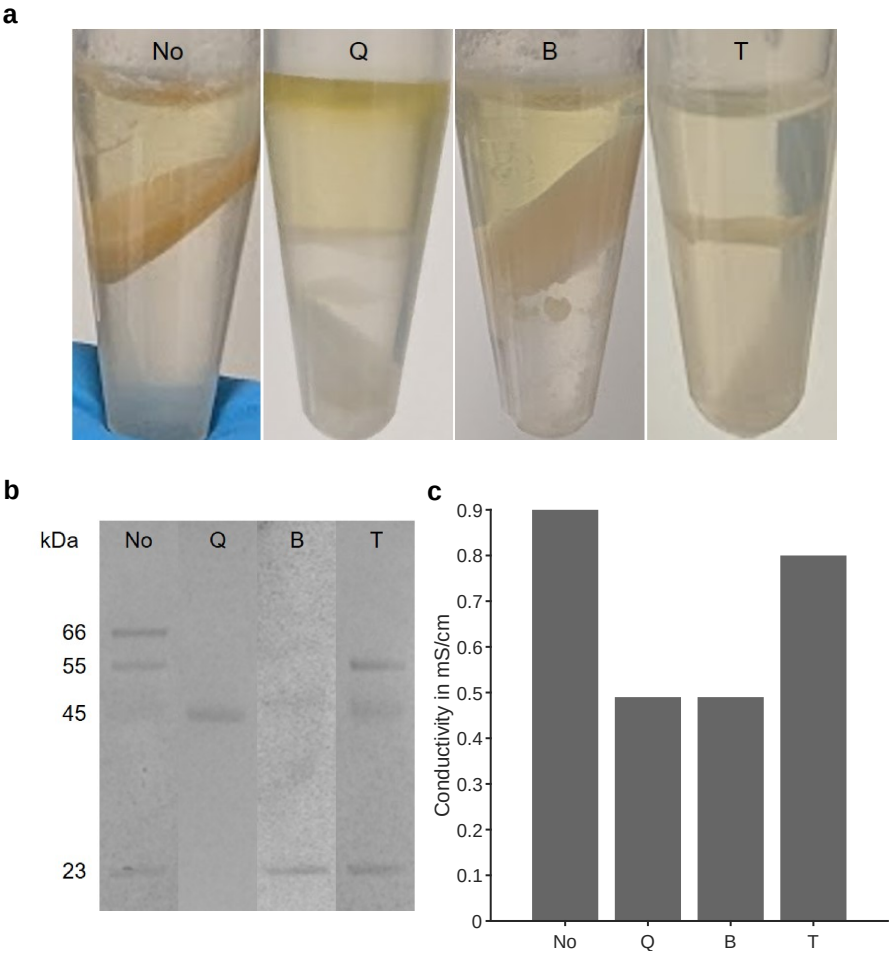


Figure A5.2 Impact of pre- and post-ATPS capture lysis on ATPS formation, protein partitioning, and sample conductivity. (a) ATPS formation after pre-capture lysis using enzymatic (Q), buffer-based (B), or reducing (T) methods, compared with a non-lysed reference (No). (b) SDS-PAGE analysis of protein present in the bottom phase after post-capture lysis. ATPS composition was 19.5/13.8% (w/w) PEG/salt. Samples were purified using the optimized reverse elution protocol. (c) Conductivity of bottom-phase samples after post-capture lysis under the four conditions indicated. Data is reported for a representative experiment.

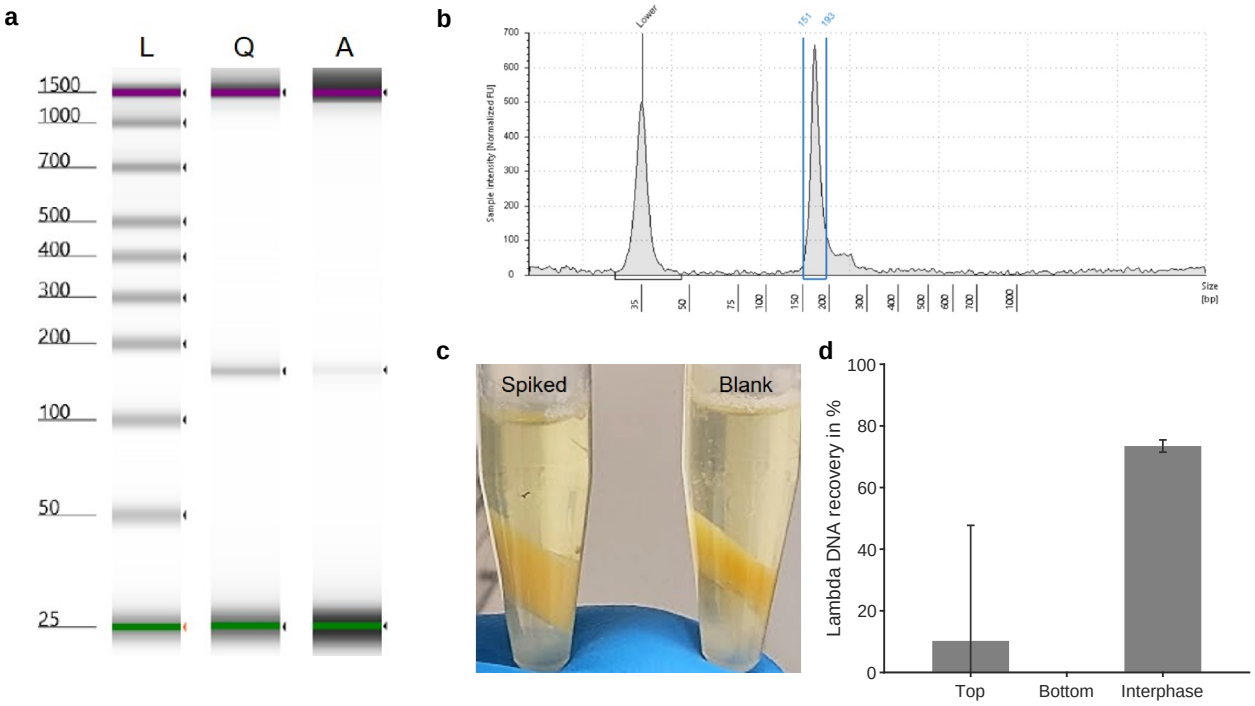


Figure A5.3 Effect of DNA fragment length on recovery and phase formation. (a) TapeStation analysis of 160 bp DNA extracted from human plasma using the ATPS-based process (A) and the manual silica-based QIAamp Circulating Nucleic Acid Kit (Q). Fragment-length analysis was performed on a representative sample with a D1000 ScreenTape; lane L is the internal ladder. For the ATPS workflow, DNA was recovered from the bottom phase. (b) Electropherogram of 160 bp DNA extracted from human plasma using the ATPS-based process. Analysis was performed on a representative sample with a cell-free DNA ScreenTape. (c) Phase formation of a 27.9/8.1% (w/w) PEG/salt ATPS with and without spiked lambda-DNA. (d) Recovery of lambda-DNA in the top phase, bottom phase, and interphase of the same ATPS. Data represent mean ± SD of technical triplicates.

Table A5.2 Quality metrics of NGS library preparation for ATPS-extracted cfDNA samples. Samples include DNA-free plasma spiked at 5% variant allele frequency (VAF) (spiked) and 0% VAF controls (blank). Reported library quality attributes include the number of unique fragments, the average number of unique start sites per GSP2, the on-target deduplication ratio, the median DNA fragment length, and the mean and median bases and targets coverage for extracts obtained using a single ATPS step (ATPS 1) or two consecutive ATPS steps (ATPS 2). Data is reported for a representative experiment.

Sample	Unique fragments	Unique start sites	On-target dedupl. ratio	Median fragment length	Mean base coverage	Median base coverage	Mean target coverage	Median target coverage
ATPS 1, spiked	2,783,484	261.81	3.05:1	103 bp	4414.78	3946.00	4027.88	3712.00
ATPS 1, blank	3,466,640	281.51	3.35:1	106 bp	5640.04	5076.00	5079.58	4687.00
ATPS 2, spiked	3,983,902	282.43	2.91:1	108 bp	6588.30	5895.00	5905.71	5524.33
ATPS 2, blank	3,057,152	265.57	2.91:1	104 bp	4945.40	4341.00	4464.17	4080.45

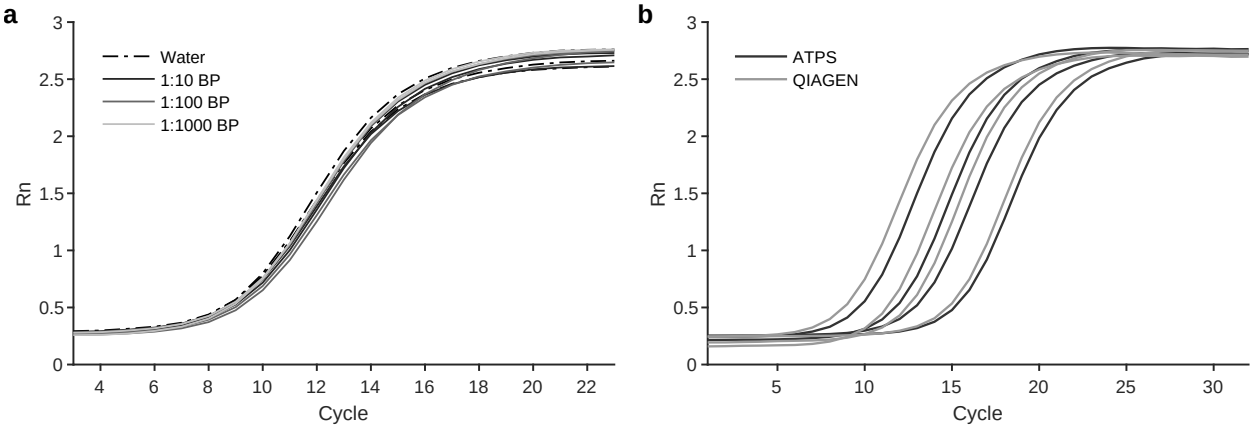


Figure A5.4 Assessment of qPCR amplification performance and potential matrix inhibition. (a) Amplification curves for 160 bp DNA spiked at 5 ng/mL into water and into a dilution series of the ATPS bottom phase extracts (BP) to evaluate potential matrix inhibition of PCR amplification. Fluorescence intensity is plotted against cycle number. Each sample was measured in technical triplicate, and all amplification curves are shown. (b) Amplification curves for 160 bp DNA spiked at different concentrations and extracted using the ATPS-based workflow or the silica-based QIAGEN kit. Due to differences in recovery efficiency between the extraction methods, DNA concentrations are not identical, and the plot is intended for qualitative comparison of amplification performance rather than direct quantitative comparison. Measurements were performed in technical triplicate, but only one representative curve per sample is shown for clarity.

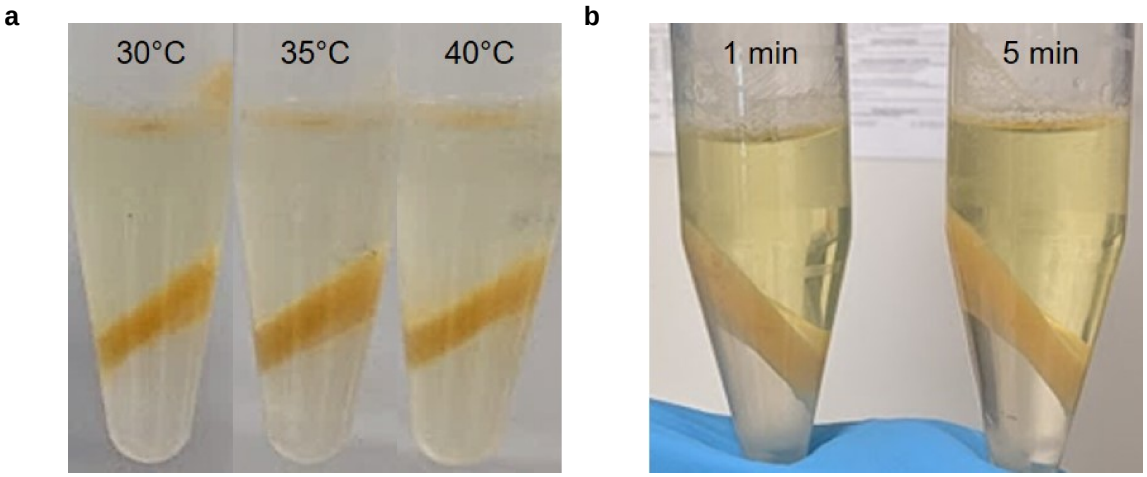


Figure A5.5 Effect of process parameters on ATPS formation. (a) Phase formation after incubation at different temperatures (30, 35, and 40 °C), followed by centrifugation at 8000 × g for 5 min. (b) Phase formation after centrifugation at 8000 × g for 1 or 5 min, following incubation at room temperature.

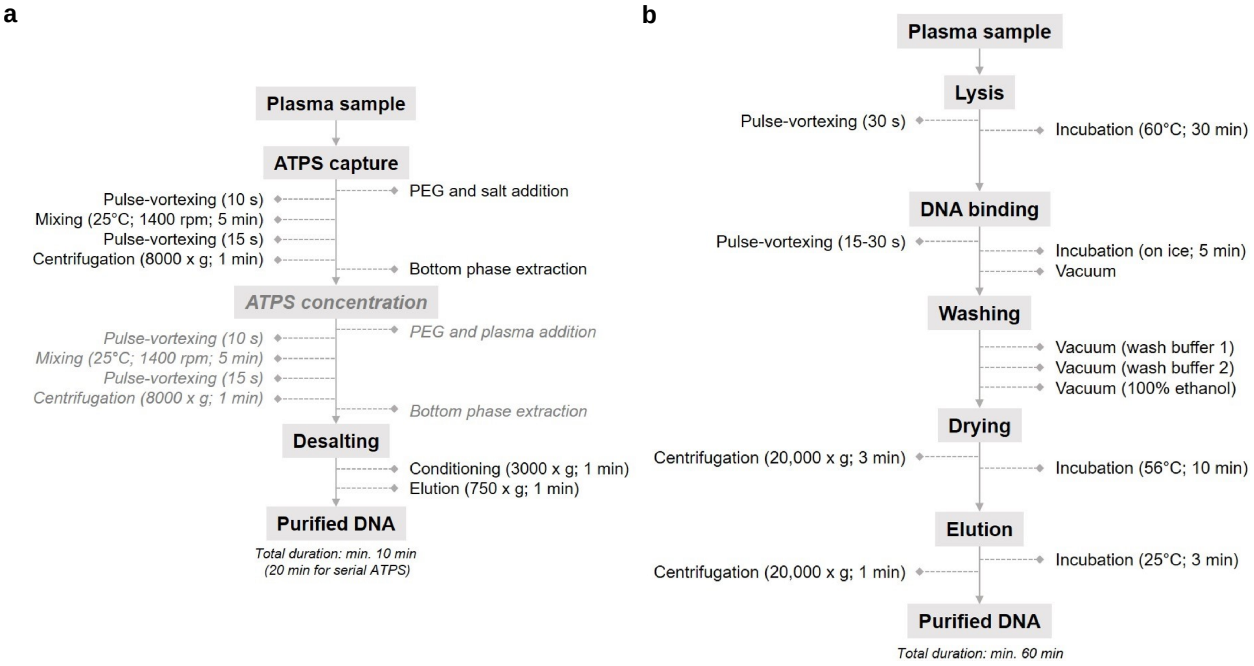


Figure A5.6 Schematic comparison of (a) the ATPS-based extraction workflow and (b) the QIAamp Circulating Nucleic Acid Kit protocol from plasma sample to purified, sequencing-ready DNA.

Title	Partitioning behavior of short DNA fragments in polymer/salt aqueous two-phase systems
Journal	Biotechnology Journal
Authors	Rafaela Meutelet, Lea J. Bisch, Benedikt C. Buerfent, Markus Müller, Jürgen Hubbuch[*] [*]corresponding author
Position	The content of this paper has been included in Chapter 3 of the dissertation.

Author	**Contribution**	
Meutelet, Rafaela	• conceptualization • investigation • project administration • writing – original draft	• formal analysis • methodology • data visualisation • writing – review & editing
Bisch, Lea J.	• formal analysis • methodology	• investigation • writing – review & editing
Buerfent, Benedikt C.	• project administration • supervision	• resources • writing – review & editing
Müller, Markus	• project administration • supervision	• resources • writing – review & editing
Hubbuch, Jürgen	• conceptualization • supervision	• project administration • writing – review & editing

Title	Extraction and purification of short DNA fragments from plasma using aqueous two-phase systems for liquid biopsy
Journal	Separation and Purification Technology
Authors	Rafaela Meutelet, Lea J. Bisch, Benedikt C. Buerfent, Johannes Oldenburg, Timo Hess, Heiko Rühl, Jürgen Hubbuch[*] [*]corresponding author
Position	The content of this paper has been included in Chapter 4 of the dissertation.

Author	**Contribution**	
Meutelet, Rafaela	• conceptualization • validation • investigation • writing – original draft • data visualisation	• methodology • formal analysis • data evaluation • writing – review & editing • project administration
Bisch, Lea J.	• validation • writing – review & editing	• investigation
Buerfent, Benedikt C.	• conceptualization • writing – review & editing	• resources • supervision
Oldenburg, Johannes	• resources • supervision	• writing – review & editing
Hess, Timo	• conceptualization • writing – review & editing	• resources
Rühl, Heiko	• resources	• writing – review & editing
Hubbuch, Jürgen	• conceptualization • supervision • funding acquisition	• writing – review & editing • project administration

Title	Proof of concept for aqueous two-phase system-based extraction of cell-free DNA from plasma for liquid biopsy applications
Journal	Scientific Reports
Authors	Rafaela Meutelet, Benedikt C. Buerfent, Timo Hess, Julia Teply-Szymanski, Paul Jank, Johannes Oldenburg, Heiko Rühl, Jürgen Hubbuch[*] [*]corresponding author
Position	The content of this paper has been included in Chapter 5 of the dissertation.

Author	**Contribution**	
Meutelet, Rafaela	• conceptualization • investigation • data evaluation • writing – original draft	• methodology • formal analysis • data visualisation • writing – review & editing
Buerfent, Benedikt C.	• conceptualization • supervision	• resources • writing – review & editing
Hess, Timo	• conceptualization • investigation • writing – review & editing	• resources • validation
Teply-Szymanski, Julia	• investigation • supervision	• validation • writing – review & editing
Jank, Paul	• conceptualization • writing – review & editing	• resources
Oldenburg, Johannes	• resources • writing – review & editing	• supervision
Rühl, Heiko	• resources	• writing – review & editing
Hubbuch, Jürgen	• conceptualization • supervision • funding acquisition	• resources • project administration • writing – review & editing