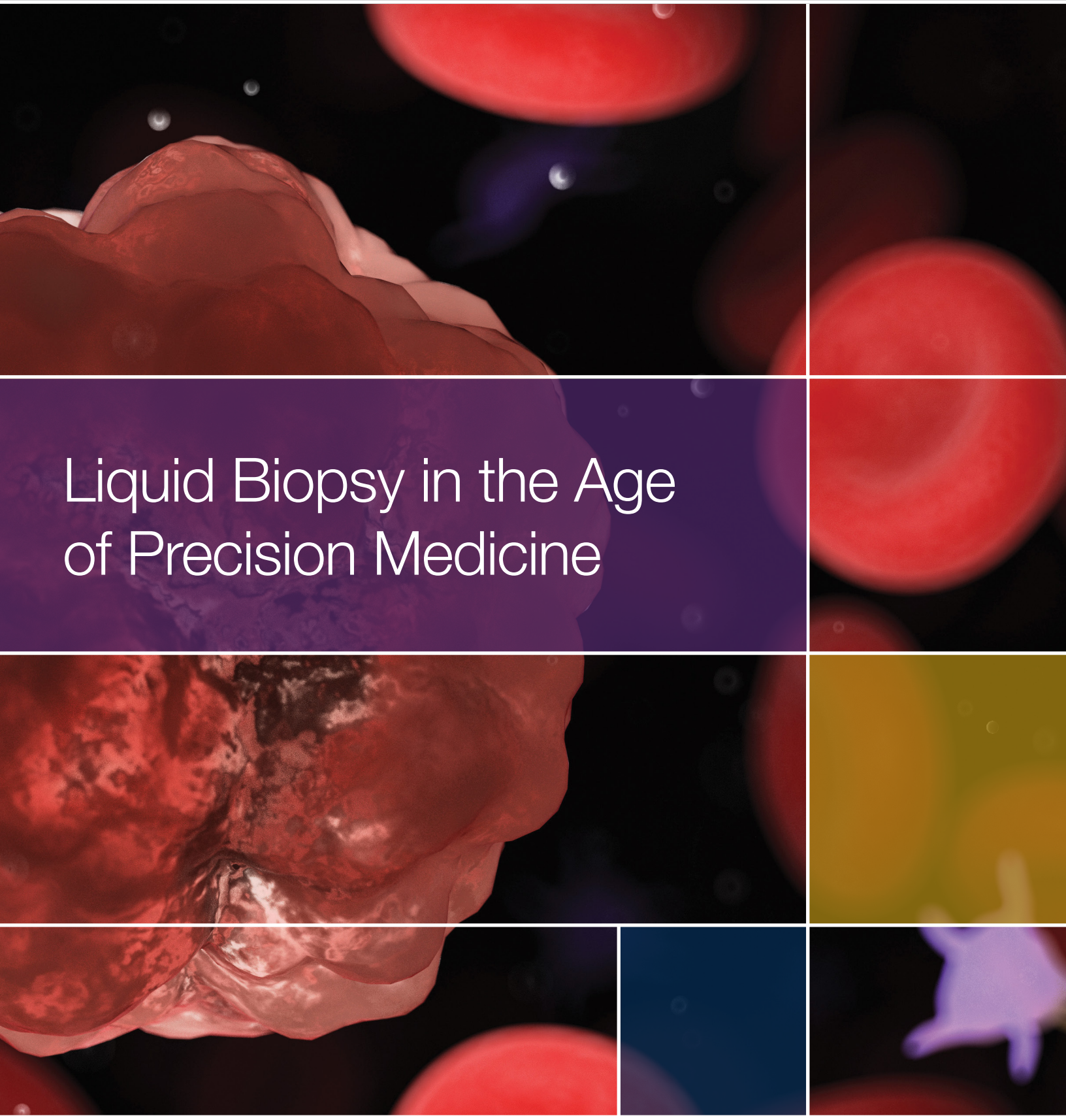


Liquid Biopsy in the Age of Precision Medicine

PRECISION for medicine®

Coinciding with the emergence of approved precision medicine modalities, interest in liquid biopsy has increased dramatically over the past decade. The techniques for isolating circulating tumor cells (CTCs) were first described in 1960. Since then, technology has progressed such that it is now possible to analyze the genetic material of CTCs at the single-cell level to study spatial and temporal dynamics in circulation.[1] Liquid biopsy has also expanded to include a variety of matrices beyond CTCs.

A growing body of research demonstrates the broad utility of liquid biopsy for a range of applications, from elucidating and confirming mechanism of action to stratifying patients and monitoring response to treatment. The 2020 approvals of 2 companion diagnostics that combine liquid biopsy and next-generation sequencing (NGS) ushered in a new era of mutation testing and biomarker profiling that targets multiple genes to guide treatment decision-making. With this paradigm shift, the myriad clinical applications of liquid biopsy are coming into focus.

In this white paper, we explore the opportunities and challenges associated with liquid biopsy. We examine the role of cell-free DNA (cfDNA) in genomic profiling and take a deep dive into CTC-based liquid biopsy and how it can be leveraged in all phases of drug development to support informed decision-making.

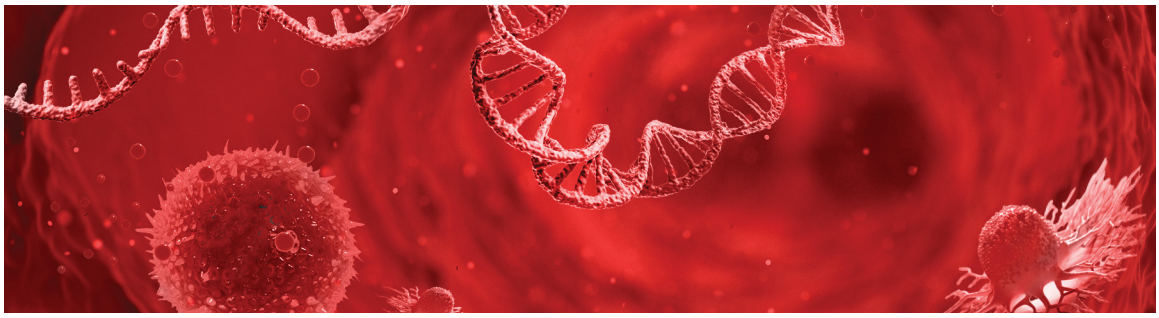
Background on Liquid Biopsy

The term “liquid biopsy” was originally reserved for the measurement of tumor cells or nucleic acids circulating in the blood. However, in recent years, the term has grown in popularity and broadened to include the measurement of a variety of biomarkers in bodily fluids such as urine, saliva, and cerebrospinal fluid (CSF). With recent advances in technology, liquid biopsies may be used to examine a spectrum of matrices, from CTCs and circulating tumor DNA (ctDNA) to soluble proteins, cfDNA, cell-free RNA (cfRNA), and exosomes.

CTCs are cancer cells that have migrated into the bloodstream. Interest in CTCs stems from increasing evidence that cancer cells undergo dynamic molecular changes in response to systemic therapy. Consequently, these cells hold promise as functional biomarkers of the metastatic cascade, for both academic research and clinical applications. As a surrogate for tumor tissue, CTCs can be used to evaluate protein expression, pharmacodynamic markers, mechanistic markers, and drivers of tumor progression. CTCs can also be used to detect biomarkers for patient stratification. For example, one study found that prostate cancer CTCs can demonstrate a high degree of phenotypic heterogeneity and that this heterogeneity was linked to decreased overall survival in metastatic castration-resistant prostate cancer (mCRPC).[2]

Research shows that ctDNA appears to be an accurate representation of the tumor itself. In a study comparing targeted sequencing of ctDNA and matched metastatic tissues in patients with mCRPC, gene alterations identified from ctDNA were highly concordant with tissue sequencing.[3] Further, in some cases, ctDNA sequencing revealed clinically relevant alterations that were not identified in the tumor biopsy.

In addition to ctDNA, circulating cfDNA encompasses all the different types of DNA in the bloodstream at any given time. While the total amount of cfDNA in the plasma and serum of patients with cancer varies among individuals, patients with cancer tend to have higher average levels of cfDNA than healthy people. Numerous studies have demonstrated high concordance between the mutations found in cfDNA and those found in solid biopsy samples.[5,6] Moreover, levels of plasma cfDNA may increase as tumors progress, and there is evidence the cfDNA acts as a signaling molecule that induces metastasis.[4] cfDNA has been extensively studied across many cancer types with promising results as a tool for detecting cancer, monitoring tumor mutations, determining treatment eligibility, and monitoring therapeutic response.[7]



Numerous studies have demonstrated high concordance between the mutations found in cfDNA and those found in solid biopsy samples.[5,6]

To date, most of the approved liquid biopsy clinical diagnostics use ctDNA or cfDNA. The Cobas Epidermal Growth Factor Receptor (EGFR) Mutation Test is an FDA-approved, PCR-based ctDNA assay used to help guide EGFR tyrosine kinase inhibitor therapy in non-small cell lung cancer (NSCLC). Notably, due to the possibility of false negative results, patients with a negative Cobas test must still undergo tissue biopsy. This example highlights the current state of liquid biopsy—these assays are often a complement to, not a replacement for, tissue biopsy.

In 2020, the FDA approved two cfDNA-based companion diagnostics that combine liquid biopsy with next-generation sequencing. The first, the Guardant360 CDx assay, detects mutations in 55 tumor genes and is used to identify specific EGFR mutations in patients with NSCLC to determine eligibility for treatment with osimertinib.[8] It also provides information on mutations in other genes that may influence treatment planning. The second, the FoundationOne Liquid CDx test, was initially approved as a companion diagnostic for drugs used to treat NSCLC with EGFR mutations and advanced metastatic prostate cancer with breast cancer gene 1 (BRCA1) and BRCA2 mutations. The FDA later approved the test for identifying[9]:

- BRCA1 and BRCA2 mutations in patients with ovarian cancer for treatment with rucaparib
- Anaplastic lymphoma kinase (ALK) rearrangements in patients with NSCLC for treatment with alectinib
- Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutations in patients with breast cancer for treatment with alpelisib
- BRCA1, BRCA2, and A-T mutated (ATM) mutations in patients with mCRPC for treatment with olaparib

Potential Advantages of Liquid Biopsy

When sampling of a tumor is difficult or not feasible, liquid biopsy offers an alternative. Given the scarcity of cfDNA or CTCs in a patient sample, collecting clinically meaningful information can be challenging. However, with successful enrichment and isolation, liquid biopsies offer several potential advantages:

Ease of collection

Low risk of side effects

Ability to perform serial testing across multiple timepoints, which has the potential to be used for real-time monitoring of therapy response

Opportunity to assess the whole-body burden of disease and response to therapy

Preservation of cellular contents allows for gene expression profiling at the single cell level and other downstream analyses

Further, in studies where tissue can be obtained for evaluation at a single point in time, the opportunity for repeat tumor sampling is unlikely and liquid biopsy would be a minimally invasive option.

Moreover, tumors may harbor segregated clones, which may be missed by biopsy but may be better represented by the capture of CTCs.

The ease of collection and ability to perform serial testing make liquid biopsy for CTCs an attractive option for use in clinical trials, either as a supplement or an alternative to tumor biopsy.

Utilizing cfDNA for Genomic Profiling

The optimal method for profiling cfDNA depends on the target mutation. If the mutation is known, digital droplet PCR (ddPCR) may be the most appropriate technology, as a customized assay can be created, enabling sensitive and specific detection of low-frequency mutations. If mutations are unknown, NGS is preferred for its massively parallel sequencing capability and its compatibility with low-quantity input DNA.

There are a number of commercially available assays, including the Oncomine Precision Assay and the TruSight Oncology 500 (TSO500) assay, which analyze variants across known cancer-related genes.

- The Oncomine Precision Assay analyzes 78 variants, including 45 mutations, 14 copy number variants (CNVs), and 18 fusion variants, across 50 key genes. This assay can be used for cfDNA and cfRNA and is run on the Ion Torrent™ Genexus™ System, which integrates and automates nucleic acid extraction and purification, library preparation, sequencing, and analysis reporting.
- The TSO500 includes pan-cancer biomarker content that supports identification of all relevant DNA and RNA variants implicated in a variety of solid tumor types on an Illumina platform. The assay also measures microsatellite instability (MSI) and tumor mutational burden (TMB) and is available in a high throughput version.

Of note, NGS platforms vary, and it is important to determine which platform is most appropriate based on the sample type, the sensitivity of the system for specific mutations, and the amount of DNA required. Profiling information generated by cfDNA analysis can be combined with other data to find correlations and uncover new targets or biomarkers.

Oncomine Precision Assay

DNA HOTSPOTS			CNV	INTER-GENETIC FUSIONS		INTRA-GENETIC FUSIONS
AKT1	ESR1	MAP2K-	ALK	ALK	NTRK2	ALK
AKT2	FGFR1	MET	AR	BRAF	NTRK3	AR
AKT3	FGFR2	MTOR	CD274	ESR1	NUTM1	CD274
ALI<	FGFR3	NRAS	CDKN2A	FGFR1	RET	CDKN2A
AR	FGFR4	NTRK1	EGFR	FGFR2	ROS1	EGFR
ARAF	FLT3	NTRK2	ERBB2	FGFR3	RSP02	ERBB2
BRAF	GNA11	NTRK3	ERBB3	MET	RSP03	ERBB3
COK4	GNAQ	PDGFRA	FGFR1	NRG1		FGFR1
CDKN2A	GNAS	PIK3CA	FGFR2	NTRK1		FGFR2
CHEK2	HRAS	PTEN	FGFR3			FGFR3
CTNNB1	IDH1	RAF1	KRAS			KRAS
EGFR	IDH2	RET	MET			MET
ERBB2	KIT	ROS1	PIK3CA			PIK3CA
ERBB	KRAS	SMO	PTEN			PTEN
ERBB4	MAP2K1	TP53				

Challenges of CTC Analysis

CTCs circulate at an extremely low frequency of approximately 1 in 1 billion cells. Due to the extremely low concentration of CTCs in the bloodstream, a key challenge in CTC analysis is detection and characterization of these rare cells. Achieving high yield and high purity also remains an obstacle.

Approaches to CTC Isolation

A multitude of strategies have been developed for CTC isolation, and these techniques vary in their sensitivity and specificity.[10] Broadly, there are three major approaches to CTC isolation[10]:

- Positive Selection
- Negative Selection
- Selection-Free

Magnetic bead separation is a common method for CTC isolation. This method, which uses antibody-labeled ferroparticles to capture cells in a magnetic field, can be used for either positive or negative selection. CellSearch is the most widely used magnetic bead-based selection assay. This assay positively selects for putative CTCs based on expression of epithelial cell surface antigen EpCAM and then enumerates these cells based on positivity for cytokeratins and lack of CD45, a WBC marker.[11]

Microfluidic devices have also been used for positive selection of CTCs based on surface markers. In general, these devices consist of antibody-coated microposts that capture CTCs as blood flows through the device. One benefit of this approach is that many different CTC-specific antigens can be used simultaneously for cell capture.[10]

POSITIVE SELECTION	NEGATIVE SELECTION	SELECTION-FREE
Refers to enrichment methods that select for cells with CTC-like attributes—either physical properties or cell surface markers—that are not exhibited by other blood cell components. Physical properties that have been used for CTC isolation include size, deformability, density, and surface charge.	Involves depletion methods that select for and eliminate components that have white blood cell (WBC)-like properties.	Refers to methods that do not rely on positive or negative selection. Given the heterogeneity of CTC subpopulations, these marker-independent isolation techniques are crucial.

The Need for Label-free Techniques

Many CTC-enrichment approaches rely on the capture of epithelial cells using epithelial lineage markers such as EpCAM or N-cadherin, an anti-mesenchymal antibody.[10] For certain cancers—such as breast, colon, and prostate—there is abundant data linking the presence of circulating epithelial cells to more aggressive disease.[12] CTCs may exhibit downregulated expression of epithelial cell surface markers during epithelial-to-mesenchymal transition, however, highlighting a potential weakness of epithelial cell capture-based methodologies. Moreover, EpCAM is non-specifically expressed on normal epithelial cells and M2 polarized macrophages.[10]

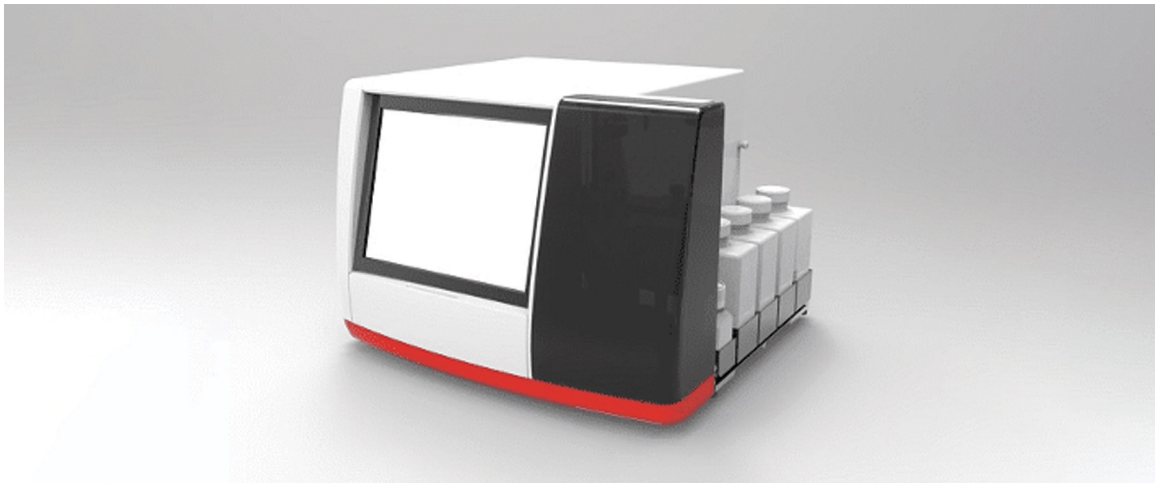
To detect a wider variety of CTCs more sensitively and specifically, other approaches are needed, eg, selection-free, morphology-driven methodologies. These greater, more agnostic techniques include flow cytometry, high-throughput microscopy, and reverse transcription polymerase chain reaction (RT-PCR) or digital droplet PCR (ddPCR). A key advantage of these approaches is that, since there is no selection step, there is no loss of CTCs. Yet, each of these methods may also be associated with pitfalls. For instance, flow cytometry requires cells to be constituted in a single cell suspension, so any relevant biological information associated with CTC clusters is lost.[10]

After CTCs are isolated, they may be further characterized using *in vitro* or *in vivo* functional assays that provide additional insight into their behavior. For example, the Metastasis-Initiating-Cells (MIC) assay tests CTCs for their ability to invade a cell adhesion matrix. The EPISPOT assay detects protein secreted by CTCs. CTCs can also be injected into xenotransplantation models to study the development of metastases.[10]

Selecting Biological Markers for CTC Detection

To date, there is no standard or consensus on the “right” or “best” marker for detecting a CTC. Ideally, the CTC marker would be expressed on every CTC, but not on any other cells in the blood sample, and it would be expressed throughout the progression of the disease. The most commonly used markers are epithelial lineage markers for positive selection, nuclear markers for negative selection of red blood cells and platelets, counterstain markers for negative selection, and disease-specific markers. To be effective, disease-specific markers should be expressed in much higher levels in cancer cells than in normal cells. However, it has been shown that de-differentiation and subsequent loss of tumor-specific markers may occur in aggressive cancers that have CTCs.[13]

ApoStream CTC Platform



The ApoStream Instrument

ApoStream is Precision for Medicine's proprietary CTC platform, co-developed by the National Cancer Institute. Using a dielectrophoresis-based, antibody-independent separation approach, ApoStream isolates and enriches CTCs, facilitating any type of downstream analysis. The technology exploits multiple differences between target cells and blood, and can also be used to isolate other rare cell types such as stem cells, progenitor and differentiated immune cells, including CAR-T cells and other difficult-to-identify immune cell populations.

With ApoStream, enriched CTCs remain intact and can be integrated with any downstream assay—multiplex immunofluorescence (mIF), next generation sequencing (NGS), fluorescence *in situ* hybridization (FISH) or ISH, *in vitro* assays, and even animal models (see Figure 1).

An industry leader in the use of mIF on CTCs, Precision for Medicine has developed a proven workflow for CTC phenotyping and biomarker profiling (see Figure 2).

Figure 1. **CTC isolation and enrichment enables multiple assays from a single tube**

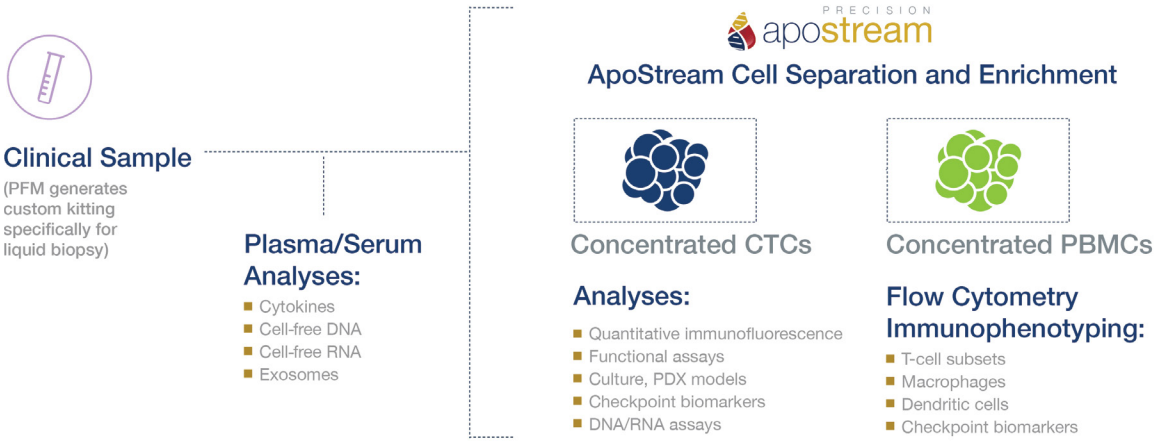
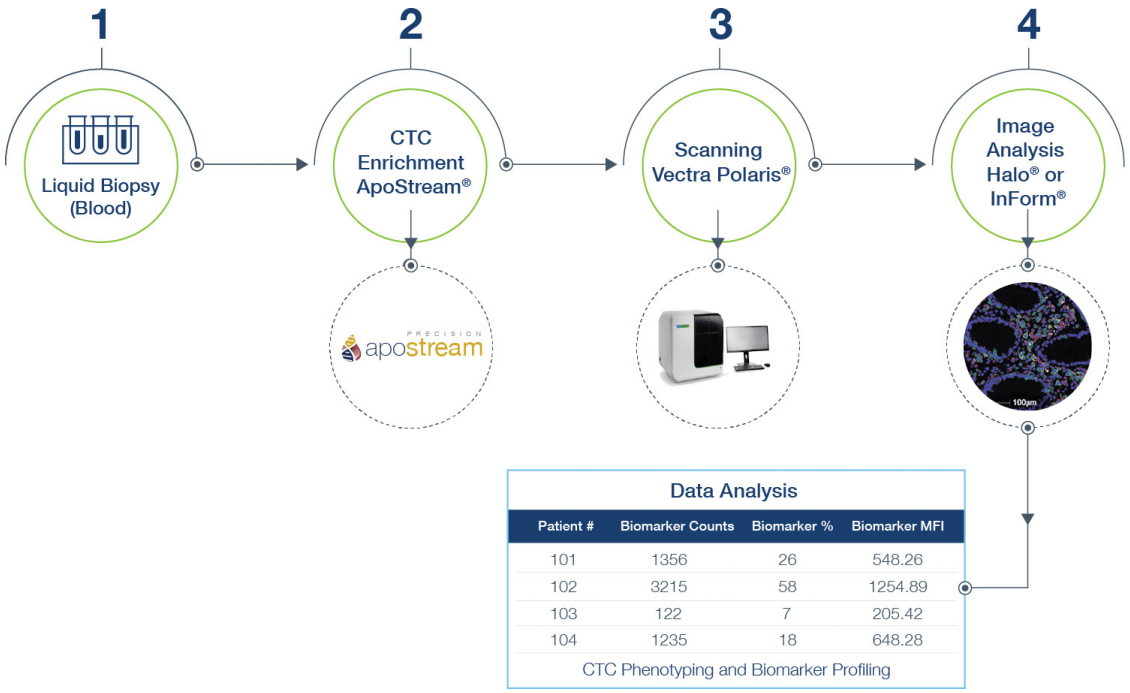


Figure 2. **Workflow for mIF on CTCs**



Leveraging CTC-Based Liquid Biopsy Across the Clinical Development Continuum: ApoStream Case Studies

ApoStream has been used for CTC isolation in a multitude of case studies, with objectives ranging from pharmacodynamic assessment and dosing strategy to characterization of heterogeneous CTC subpopulations and patient selection for clinical trial enrollment. Below are a few examples demonstrating the value of CTC-based liquid biopsy at different stages of clinical development.[10]

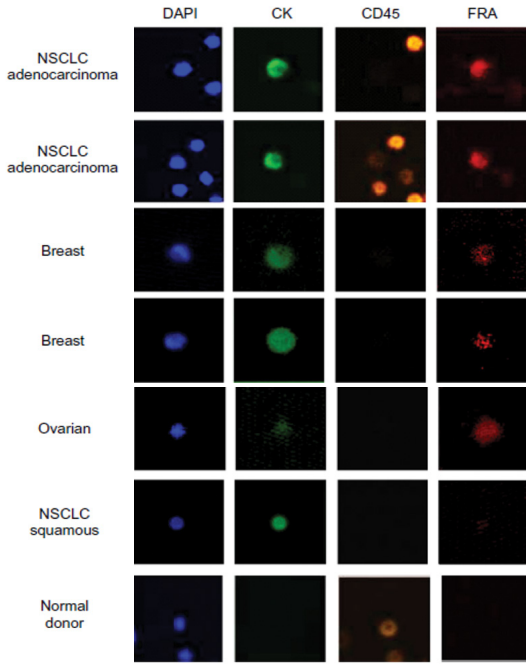
Phase 1

During early phase clinical trials, liquid biopsy may be used for examining mechanism of action or predicting response. In one study, ApoStream was used for isolating CTCs from 10 ml anticoagulated blood samples obtained from 5 patients with pancreatic cancer. The objective of this study was to assess whether CTC numbers, phenotypic features, and/or early apoptotic response to treatment with a putative apoptosis-inducing investigative drug could be used to predict clinical response. Qualitative immunofluorescence was performed on CTCs stained for drug-related markers and a panel of epithelial or mesenchymal markers, CD45, and 4′,6-diamidino-2-phenylindole (DAPI, a nuclear counterstain). Researchers found that CTCs could be obtained in sufficient numbers for multiparameter phenotyping to identify features that could potentially serve as selective, predictive, or prognostic biomarkers.[14]

Phase 2

In phase 2 studies, circulating tumor markers can be used for selecting patients, or evaluating pharmacodynamic markers or markers of resistance. In one study, laser scanning cytometry using highly selective antibodies was used to interrogate CTCs isolated with ApoStream to identify folate receptor alpha (FRα)-positive cells. FRα is highly expressed in several epithelial cancers, and its expression may be useful as a potential diagnostic and therapeutic target in certain solid tumors. This study demonstrated that FRα-positive CTCs could be isolated from patients with metastatic cancers, including non-small cell lung cancer, breast cancer, and ovarian cancer. The ability to detect FRα-positive cells may have clinical utility as a real-time liquid biopsy for detecting and monitoring FRα levels in cancer patients.[15]

Figure 3. **FRα-positive CTCs isolated using ApoStream®**



Phase 3

In phase 3 studies, validated liquid biopsies may play a role in predicting response to treatment. In the BEACON trial, preplanned exploratory analyses were conducted to identify CTC biomarkers that might predict response to etirinotecan pegol (EP), a pegylated topoisomerase 1 (Top1) inhibitor. ApoStream was used to isolate CTCs from the blood of 656 patients with metastatic breast cancer. Following enrichment, mIF was performed to measure expression of a variety of candidate response biomarkers. Researchers found that CTC Top1 expression after EP treatment might be useful for identifying those patients who were most likely to have an overall survival benefit.[16]

An End-to-End Solutions Provider

Precision for Medicine leverages the combined power of trials, labs, and data to drive development. By integrating clinical trial execution with deep scientific knowledge, specialty laboratory expertise, and advanced data sciences, Precision for Medicine maximizes insights into patient biology and accelerates the pace of discovery and approval. Over the course of more than two decades of running successful oncology, rare disease, and advanced therapy trials, Precision for Medicine has developed a unique blend of proprietary technologies, flexible processes, and creative problem-solving abilities that help advance the most challenging clinical development programs.

Conclusion

Liquid biopsy offers an attractive alternative or adjunct to tissue biopsy. Until recently, the clinical applications of liquid biopsy were limited to assays targeting single genes. The approvals of two multi-gene liquid biopsy tests that use next-generation sequencing represent a great leap forward in precision medicine. Still, the approvals for both tests call for use in conjunction with tissue biopsy to rule out false negatives, unless biopsy is not feasible.

The full potential of liquid biopsy is yet to be realized. Researchers are actively investigating the use of liquid biopsy for detecting cancer, screening for disease recurrence, monitoring treatment, and assessing residual disease at the molecular level. As technologies continue to advance, the clinical applications of liquid biopsy will continue to expand across therapeutic areas, supporting the next revolutions in precision medicine and personalized healthcare.

Darren Davis, PhD

Senior Vice President

Visionary leader with more than 25 years of distinguished biotechnology and clinical translational research experience. Founded ApoCell in 2004 and later was instrumental in developing and commercializing the ApoStream® rare-cell liquid biopsy technology. Globally recognized cancer researcher and the author of more than 100 peer-reviewed publications. Dedicated and committed to improving the lives of patients with debilitating diseases.

Jesus Garcia, PhD

Scientific Liaison

Tissue and liquid biopsy expert with extensive experience in a wide range of histopathology assays and digital pathology solutions. Part of the implementation of new technologies at MD Anderson Cancer Center in collaboration with immuno-oncology leaders. Currently focused on partnering with biopharma to develop tissue and liquid biopsy biomarker strategies for clinical trials, and to implement digital pathology and AI in the drug development process.

Jie Yang, PhD

Scientific Liaison

Immunologist by training with extensive industry expertise in designing translational assays for biomarker-guided clinical trials. Conducted postdoctoral research on immuno-oncology at MD Anderson Cancer Center. Led biomarker assay development and collaborated on the implementation of new technologies for pre-clinical and clinical studies conducted by pharmaceutical and biotech companies for drug development.

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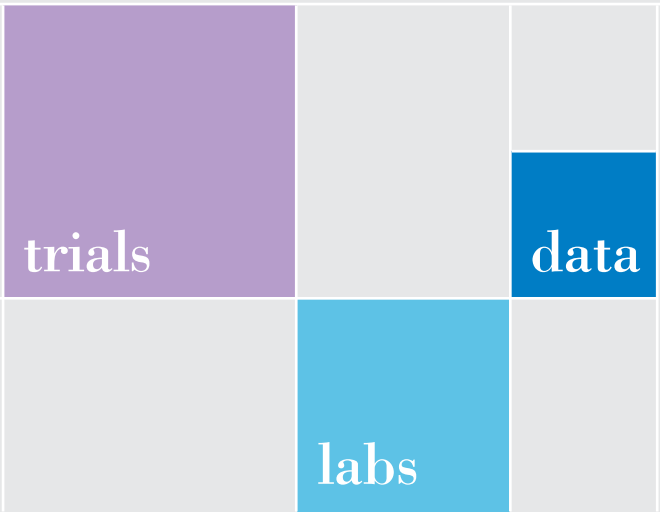

Precision for Medicine supports life sciences companies in the use of biomarkers essential to targeting patients more precisely and effectively. Precision applies novel biomarker approaches to clinical research that takes advantage of the latest advancements in science and technology, focusing predominantly on genomics, immune-response assays, global specimen logistics, biomarker analytics, companion diagnostics, and clinical trial execution. Precision for Medicine is part of Precision Medicine Group, with more than 1,450 employees in 25 locations in the US, Canada, and Europe.

To speak with a Precision scientist about the comprehensive suite of immune-monitoring solutions and which may be best for your study, send an email to: info@precisionformedicine.com.

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