# See.d®

# Standardizing the Pre-Analytical Workflow for Integrated Liquid Biopsy

## Executive Summary

Liquid biopsy is rapidly redefining oncology by enabling minimally invasive access to tumor-derived biomarkers, including circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs). These analytes provide complementary molecular and cellular insights into tumor evolution, therapeutic response, and minimal residual disease. However, despite strong clinical momentum, widespread implementation remains limited by a fundamental barrier: variability in pre-analytical sample handling.

Differences in blood processing timing, plasma separation methods, and manual cellular workflows introduce significant pre-analytical variability that can adversely affect assay sensitivity, reproducibility, and overall data quality. These challenges are particularly pronounced in the analysis of ctDNA and CTCs, as nucleic acid degradation, sample degradation and cellular loss, may compromise downstream analytical performance.

To address these limitations, Tethis developed See.d an automated pre-analytical system designed to standardize whole blood processing at the point of collection. The system enables simultaneous generation of high-quality plasma and preserved nucleated cell preparations on proprietary SmartBioSurface® (SBS) slides, supporting integrated molecular and cellular profiling from a single sample.

## Standardizing the Liquid Biopsy Workflow

Blood is a complex biological matrix in which clinically relevant tumor-derived signals coexist with abundant normal cellular components. While ctDNA circulates as fragmented DNA released through apoptosis, necrosis, and other cellular processes, CTCs represent intact malignant cells shed into the bloodstream and implicated in metastatic dissemination.

Although both analytes are now well established in translational oncology, the successful implementation of ctDNA- and CTCs-based assays is strongly dependent on the quality and consistency of pre-analytical processing. Conventional workflows rely on manual handling steps and centralized laboratory processing, often resulting in transport-related delays that can compromise both plasma integrity and cellular preservation. These limitations introduce variability that is difficult to control across clinical sites and studies.

See.d was developed to directly address these gaps by shifting pre-analytical processing closer to the point of blood collection and replacing manual workflows with a fully automated system that ensures standardized sample preparation regardless of operator or site.

## Automated Dual-Output Processing from a Single Blood Sample

See.d processes fresh whole blood collected in standard K2EDTA tubes within a defined pre-analytical window of six hours from collection. Through an integrated automated workflow, the system performs plasma separation, red blood cell lysis, and recovery of the nucleated cell fraction, which is subsequently deposited onto SBS slides and fixed for downstream analyses.

From a single 8 mL blood sample, See.d generates a dual output consisting of up to ~2.5 mL of plasma suitable for cell-free nucleic acid analysis and ten SBS slides, each designed to contain approximately 2.5 million nucleated cells (Fig. 1). This dual-compartment output enables parallel analysis of plasma-derived and cellular biomarkers within a unified and standardized workflow.

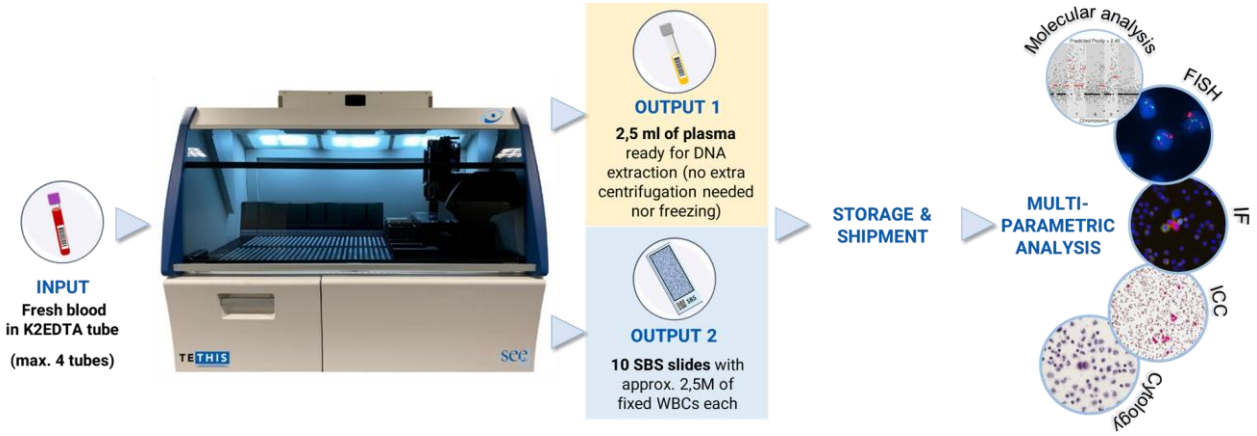


*Figure 1. Standardized pre-analytical workflow with See.d.*

A key advantage of the system for cytological and rare cell analyses is the preservation of the entire nucleated cell population. By avoiding enrichment or depletion steps based on physical or molecular characteristics, the workflow minimizes selection bias and maintains access to heterogeneous cellular populations present in blood. This enables comprehensive evaluation of rare cell types, CTCs, CTC clusters, and immune cell populations, while preserving their morphological and molecular features for downstream analysis.

The SBS slides consist of nanostructured $TiO_2$ films characterized by nanoscale topographical features and porosity that mimic those of typical extracellular matrix structures. These properties support the adhesion and retention of non-adherent cells while preserving cellular morphology and compatibility with a broad range of downstream analytical techniques, including immunofluorescence (IF), immunocytochemistry (ICC), cytology, fluorescence in situ hybridization (FISH), and laser capture microdissection. Together, these capabilities support not only the detection of rare cells but also their multimodal characterization at the single-cell level.

## Preservation of Plasma Integrity and cfDNA Quality

The performance of See.d in plasma processing was evaluated across multiple healthy donor (HD) samples, demonstrating highly consistent plasma recovery from whole blood with a mean yield of 2.2 ± 0.1 mL (Fig. 2a).

Importantly, cfDNA fragment size distribution profiles obtained from See.d-processed plasma were comparable to those obtained using standard clinical laboratory procedures, indicating that automated processing does not compromise nucleic acid integrity (Fig. 2b). Notably, these profiles were preserved following extended storage at 4 °C and simulated transport at room temperature, demonstrating that the processed plasma remains suitable for downstream cfDNA extraction under flexible handling and logistical conditions. The stability of the processed plasma further supports the applicability of See.d in decentralized and resource-limited settings.

Quantitative analysis of synthetic DNA recovery further confirmed the robustness of the workflow. Digital PCR (dPCR) measurements showed recovery rates equivalent to those achieved with standard manual processing methods (Fig. 2c), even after storage of the automatically collected plasma at 4 °C and simulated transport at room temperature.

Collectively, these findings demonstrate that automated plasma preparation using See.d preserves both the structural integrity and analytical utility of cfDNA.

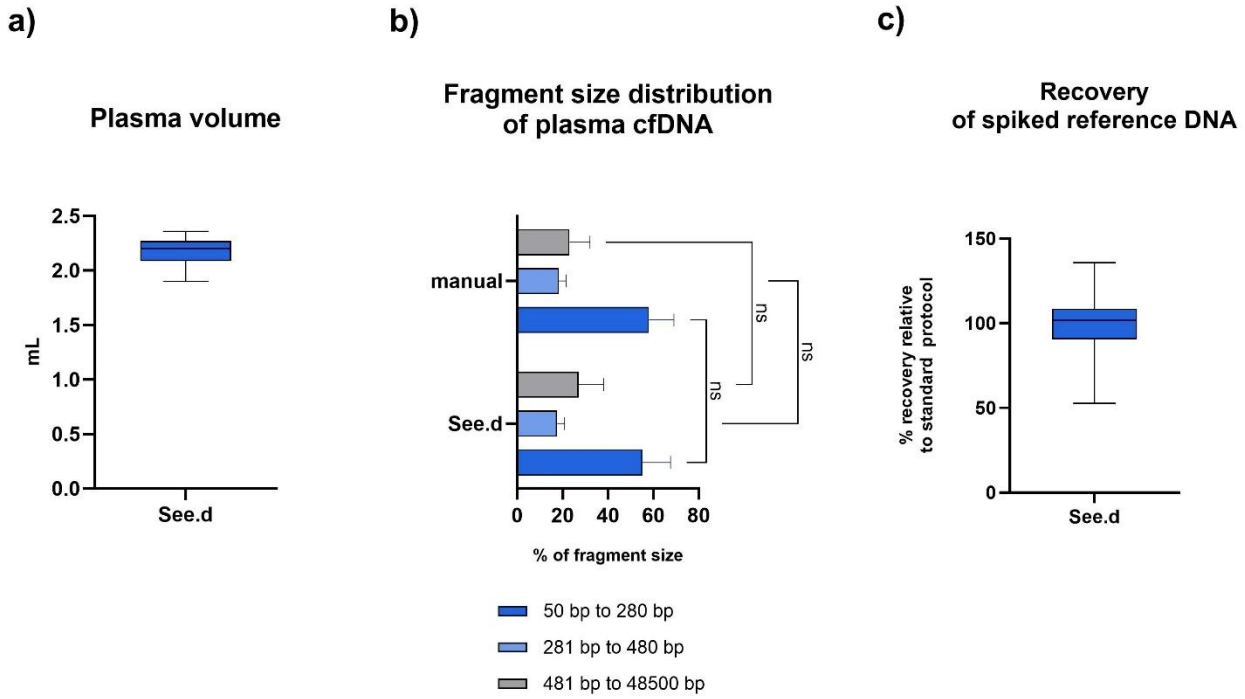


***Figure 2. Analysis of See.d collected plasma:*** *a) Box plots of plasma volumes collected by See.d from 30 HD whole blood samples (8 mL each). b) Column plots showing mean ± SD of cfDNA across three fragment size ranges extracted from plasma obtained from all 30 HD samples, processed either manually according to clinical SOPs or using automated See.d processing. Automatedly collected plasma samples were stored at 4 °C and room temperature prior to cfDNA extraction and quantification. No statistically significant difference was observed (two-way ANOVA with Šídák's multiple comparisons test, $p > 0.05$). c) Box plots showing percentage recovery of spiked reference DNA in See.d-processed plasma relative to SOP-processed plasma, determined by dPCR. Plasma samples collected using the automated workflow were stored at 4 °C and room temperature prior to reference DNA quantification.*

## Reproducible Recovery of Nucleated Cells on SBS Slides

In parallel with plasma processing, See.d enables standardized deposition of nucleated cells onto SBS slides. Across multiple donor samples, the system consistently generated SBS slides with a mean nucleated cell count of approximately 2.4 ± 0.19 million cells per slide (Fig. 3a). Quantitative image analysis confirmed the expected edge effects related to fluid dynamics during deposition, whereas the central regions of the slides exhibited highly uniform cellular density (Fig. 3b).

This uniformity is a critical prerequisite for downstream single-cell imaging and characterization, as it minimizes sampling bias, ensures consistent analytical conditions across regions of interest, and maximizes the number of cells that can be reliably analyzed.

Cellular morphology was preserved under different storage conditions, including both frozen and simulated transport environments, indicating robustness of the fixation and stabilization process. Following full IF staining, quantitative image-based metrics further confirmed preservation of nuclear integrity across conditions (Fig. 3c).

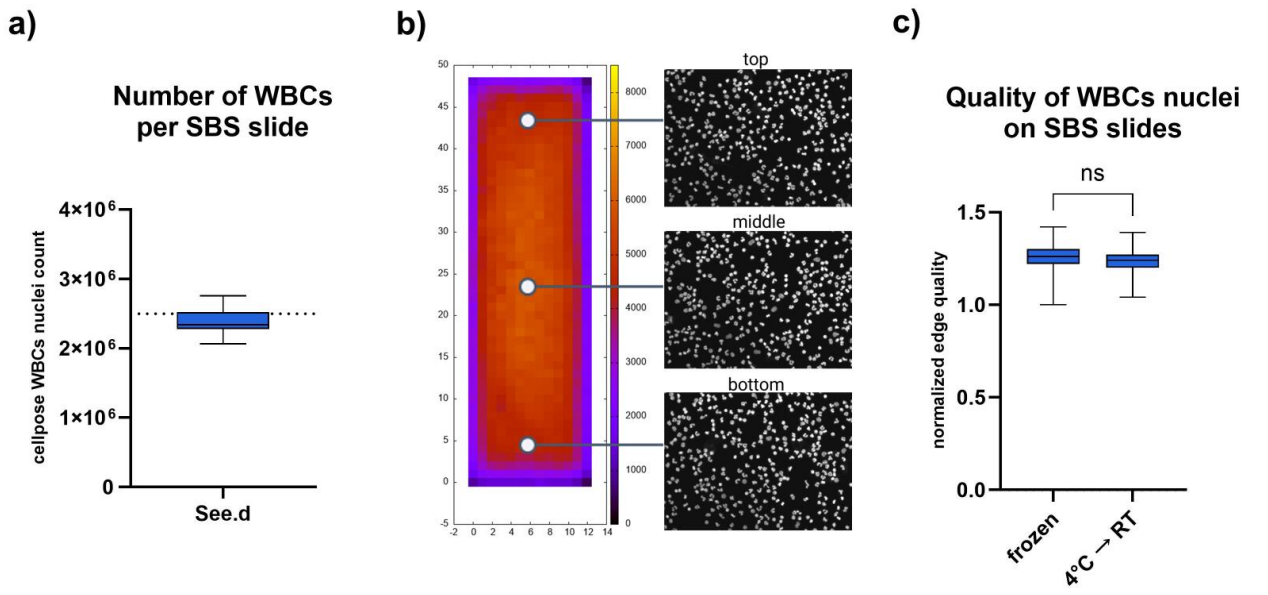


***Figure 3. Analysis of blood nucleated cells purified by See.d and seeded on SBS slides****: a) Box plot showing the mean WBCs count per SBS slide, calculated from 10 SBS slides per each of the 30 HD samples. b) Representative heat map of WBCs distribution across an SBS slide. Each field of view (FOV) is color-coded from dark purple to bright yellow, indicating increasing numbers of nuclei detected by automated imaging and cell-counting software; c) Box plots of the normalized edge quality parameter, quantifying the sharpness and continuity of WBCs nuclear boundaries as an indicator of preserved cellular morphology. This parameter was calculated using an automated image analysis algorithm applied to slides derived from 30 HD samples. No statistically significant differences were observed between slide storage conditions (unpaired t-test, p > 0.05). Abbreviations: WBCs, white blood cells.*

To evaluate performance in the context of rare cell detection, model tumor cells were spiked into whole blood prior to processing. Cells were pre-labelled with commercial fluorescent CFSE dye and subsequently identified by fluorescence microscopy using a validated image-based classification algorithm (Fig. 4).

See.d demonstrated high and reproducible recovery efficiency of spiked cells following automated processing, with an average recovery of 88 ± 15% across 30 independent spike-in experiments, supporting its suitability for rare event detection workflows such as CTC analysis.

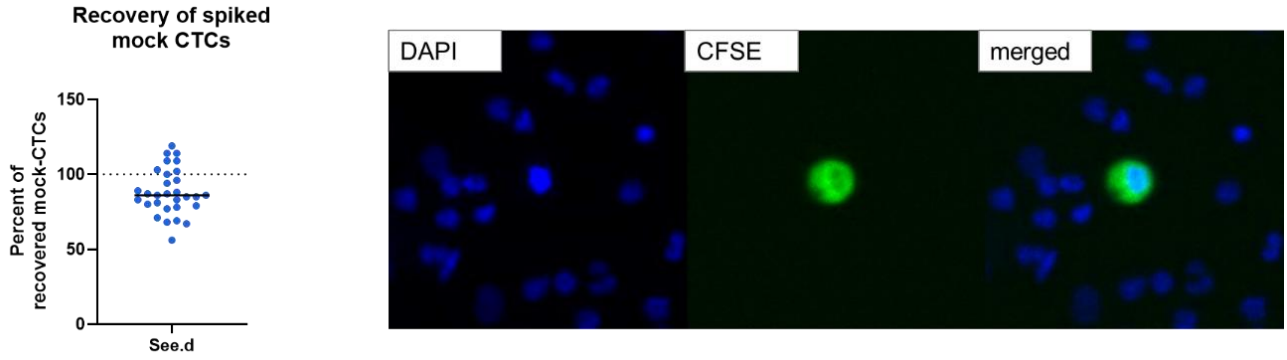


***Figure 4. Analysis of spiked model tumor cells in whole blood processed by See.d****. Left panel: percent recovery of spiked HeLa S3 cells into whole blood of 30 HD samples and processed in automation by See.d. Right panel: representative fluorescence microscopy image of a HeLa S3 cell pre-labelled with a commercial CFSE dye.*

## Multimodal Characterization of CTCs in Clinical Samples

The translational relevance of the system was further evaluated in a proof-of-concept study involving a patient with metastatic breast cancer. Ten SBS slides containing nucleated cells prepared by See.d from a blood sample were subjected to IF staining targeting epithelial and HER2 markers, together with hematopoietic exclusion markers.

Automated image analysis combined with expert review identified a population of 43 CTCs characterized by expression of epithelial markers or co-expression of epithelial and HER2 markers, in the absence of hematopoietic signature, consistent with a tumor-associated phenotype (see Table 1).

Following IF analysis, SBS slides were shown to be compatible with conventional H&E staining. Illustrative H&E images revealed preserved cellular morphology and features consistent with a malignant phenotype, including nuclear hyperchromasia and prominent nucleoli (see Fig. 5).

Subsequently, sequential FISH analysis targeting *ERBB2* revealed gene amplification in the majority of analyzed cells, confirming their malignant origin and demonstrating concordance between protein expression and genomic alteration in a subset of the circulating tumor population. Importantly, heterogeneity was also observed, with distinct subpopulations exhibiting discordant phenotypic and genotypic features, reflecting the biological complexity of CTCs.

Together, these results demonstrate the feasibility of sequential multimodal characterization of CTCs on SBS slides, integrating immunophenotypic, morphological, and genomic analyses within a single workflow.

| Phenotype | Total number of CTCs | FISH *ERBB2* amplified | FISH *ERBB2* wild type |
|---|---|---|---|
| Epithelial + | 25 | 20 | 5 |
| Epithelial/HER2 + | 18 | 17 | 1 |
| Epithelial + and Epithelial/HER2 + | **43** | **37** | **6** |

***Table 1. Detection of CTCs with epithelial and HER2 phenotypes, along with their ERBB2 gene status as assessed by FISH.***

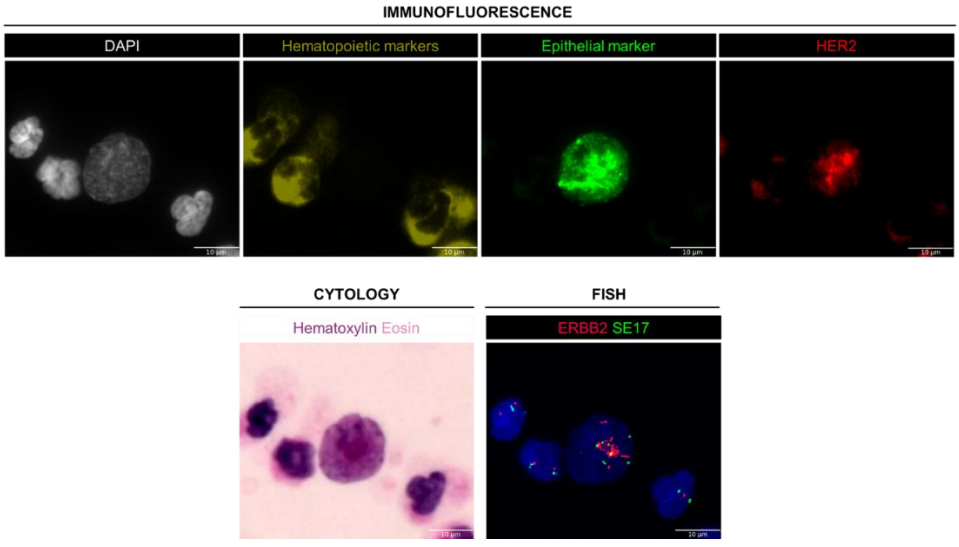


***Figure 5. Multimodal analysis in metastatic breast cancer**: IF with hematopoietic, epithelial, and HER2 markers was followed by H&E staining and FISH on the same slide. Note that for the FISH staining, multiple red signals indicate amplification of the ERBB2 gene, while green signals correspond to the probe hybridizing to the centromere of chromosome 17.*

## Towards a Standardized Foundation for Integrated Liquid Biopsy

The clinical adoption of liquid biopsy is driven by the need to generate reproducible, high-quality data across institutions and workflows. While considerable progress has been made in assay development, pre-analytical variability remains one of the most critical barriers to standardization.

By automating whole blood processing at the point of collection, See.d introduces a controlled and reproducible pre-analytical environment that preserves both plasma-derived and cellular analytes within a single integrated workflow. The ability to simultaneously generate cfDNA-compatible plasma and high-quality cellular preparations on SBS slides enables a unified analytical framework for multi-analyte liquid biopsy.

While the present study focuses on plasma generation for cfDNA analysis and nucleated cell recovery, the standardized plasma processing workflow may also support additional downstream biomarker analyses, including proteomic and metabolomic applications. Further studies will be required to characterize performance across these analyte classes.

The non-selection-based approach further ensures unbiased representation of circulating cellular populations, expanding the analytical landscape beyond conventional enrichment-based methodologies. In combination with compatibility for immunofluorescence, cytology, FISH, and AI-assisted image analysis, the platform supports a scalable foundation for multimodal precision oncology.

The ongoing feasibility study (NCT06097156) at the European Institute of Oncology (IEO, Milan) is evaluating the use of the See.d instrument and SBS slides for standardized preparation of plasma and cytological samples from whole blood. Through the analysis of samples from healthy volunteers and metastatic breast cancer patients, the study assesses workflow feasibility, instrument performance, and the preliminary analytical characterization of cfDNA and CTCs, providing an important foundation for future development of integrated liquid biopsy workflows.