# Analytical verification methods for the Oncomine Lung cfDNA Assay using the Ion S5 XL System

## Key highlights

- Investigate tumor heterogeneity and reoccurrence from a 10 mL liquid biopsy sample with accurate and repeatable results
- Obtain reliable results across multiple samples with the Ion Torrent™ Oncomine™ cfDNA Assays and the Ion S5™ platform
- Detect variants at 0.1% allele frequency using the Oncomine cfDNA Assays with consistency and reproducibility

## Introduction

The study of genetic information from cell-free DNA (cfDNA) may impact future oncology diagnosis and treatment. By enabling a noninvasive and cost-effective alternative to traditional biopsy samples, liquid biopsy may redefine the future of cancer care.

The Oncomine Lung cfDNA Assay helps detect somatic variants at low frequency (e.g., 0.1%) in cfDNA from plasma using Ion Torrent™ next-generation sequencing (NGS) systems. Here, assay performance is summarized with analytical verification methods that may provide useful information for researchers who plan to analyze liquid biopsy samples in their labs. The assay performance is demonstrated with multiple sample types across a broad input range, high assay reliability (reproducibility and repeatability), accuracy (sensitivity and specificity), and a simple workflow.

## Materials and methods

Multiple sample types were amplified using the Oncomine Lung cfDNA Assay to generate libraries for targeted sequencing. These multi-biomarker assays are optimized to help detect 169 hotspots in 11 key genes (Table 1).

**Table 1. Oncomine Lung cfDNA Assay targets.**

| Assay | Gene count | Gene names | Amplicons | Hotspots |
|---|---|---|---|---|
| Lung | 11 | *ALK*, *BRAF*, *EGFR*, *ERBB2*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, *PIK3CA*, *ROS1*, *TP53* | 35 | 169 |

The process of library generation followed standard protocols as outlined in the user guide to produce barcoded libraries. Eight libraries were multiplexed for templating on the Ion Chef™ System and subsequently sequenced on the Ion S5™ XL System using the Ion 530™ Chip Kit.

The cfDNA was extracted using the Applied Biosystems™ MagMAX™ Cell-Free DNA Isolation Kit from either banked late-stage non-small cell lung cancer (NSCLC) research samples or purchased whole blood from healthy donors. For matched solid-tumor DNA studies, DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissues using the Applied Biosystems™ RecoverAll™ Multi-Sample RNA/DNA Isolation Kit.

To evaluate sensitivity and specificity, two reference DNA mixes were generated from Thermo Scientific™ AcroMetrix™ Oncology Hotspot Control material, which contains 40 engineered variants covered by the Oncomine Lung cfDNA Assay, in a genomic DNA (gDNA) background (GM24385 from Coriell Institute for Medical Research). Both the AcroMetrix Oncology Hotspot Control material and GM24385 gDNA were fragmented by sonication to mimic the size of cfDNA.

Mixtures were prepared with the 40 variants present at either 0.1% or 0.5% allele frequencies. Variant frequencies from both control mixtures were verified by digital PCR (dPCR).

Data generated using the commercially available Multiplex I cfDNA Reference Standard Set (Horizon Discovery)—a widely available control material—help establish expectations for performance of the Oncomine Lung cfDNA Assay. The reference standard set contains mixes including eight engineered variants covered by the targeted library, present at 5%, 1%, 0.1%, or 0% frequencies in a known background.

# Results

## Sample type capacity

We demonstrated that libraries could be successfully generated from 0.25–50 ng of cfDNA, 5–20 ng of FFPE DNA, and 20–50 ng or 6,000 copies of control material [1] as summarized in Table 2. Table 3 shows typical library yield generated from 1–20 ng of cfDNA isolated from plasma in a healthy donor.

Two cfDNA samples, labeled S1 and S2, were isolated from banked cancer research plasma and further titrated as shown in Table 4. The higher-frequency (9.4%) epidermal growth factor receptor (*EGFR*) variant deletion in S1 was detected with a wide range of cfDNA input, down to 0.25 ng. The lower-frequency (0.2%) *KRAS* variant in S2 was successfully detected using 2–20 ng of cfDNA input. It is noteworthy that in S2, the high-frequency allele of *MET* in the same plasma can be detected consistently, suggesting the presence of a germline mutation in this research sample.

**Table 2. Sample types and input ranges.**

| | Sample type | | Control sample | | |
|---|---|---|---|---|---|
| | cfDNA | FFPE | (0–5.0%) | 0.1% mix* | 0.5% mix* |
| Input range | 0.25–50 ng | 5–20 ng | 20–50 ng | 6,000 copies | 6,000 copies |

* Mixture of AcroMetrix Oncology Hotspot Control and GM24385 DNA.

**Table 3. Library yield generated with a serial dilution of cfDNA input from plasma collected from a healthy donor.** Blood was collected in EDTA tubes and plasma isolated with a double-spin protocol.

| cfDNA input (ng) | Library (pM) |
|---|---|
| 20 | 413.1 |
| 20 | 400.1 |
| 5 | 193.9 |
| 5 | 197.6 |
| 2 | 57.3 |
| 2 | 57.6 |
| 1 | 66.6 |
| 1 | 66.0 |

**Table 4. Detected variants from 0.25–20 ng cfDNA isolated from banked cancer research plasma.**

| | | cfDNA input (ng) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Variant | 20 | 10 | 5 | 2 | 1 | 0.5 | 0.25 |
| S1 | *EGFR* p.L747_E749delLRE | 9.4% | Not tested* | Not tested* | 10.4% | 14.7% | 16.5% | 7.9% |
| S2 | *KRAS* p.G12D | 0.2% | 0.3% | 0.1% | 0.2% | Not detected | | |
| | *MET* p.T1010I | 47.4% | 49.8% | 45.5% | 47.6% | 51.2% | | |

*These input amounts were not tested in this experiment to determine limits of detection.

## Assay reliability

To verify assay reliability—defined here as reproducibility and repeatability of assay results—samples were run at two independent sites, on three independent Ion S5 XL Systems for sequencing, and three independent Ion Chef Systems for templating.

To evaluate reproducibility, four users independently ran the same experiment with the same set of samples. Each individual sequenced eight Oncomine Lung cfDNA libraries using the recommended 6,000 copies of control material with allele frequency titrated to 0.5% or 0.1% in background GM24385 gDNA (labeled 0.5% mix and 0.1% mix, respectively) as input. Four replicate libraries were generated from each control mixture, and a total of eight libraries were multiplexed for templating and sequencing. Observed sensitivity and specificity are listed in Table 5, demonstrating similar results across sites, instruments, and users.

**Table 5. Assay reproducibility across multiple sites, instruments, and users.**

| Site | Individual | Sample | Sensitivity (%) | | | Specificity (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Individual user | User average (n = 4) | All users average (n = 16) | Individual user | User average (n = 4) | All users average (n = 16) |
| Site A | User 1 | 0.5% mix | 100 | 100 | 100 | 98 | 98.5 | 98 |
| | | | 100 | | | 99 | | |
| | | | 100 | | | 97 | | |
| | | | 100 | | | 100 | | |
| Site B | User 2 | | 100 | 100 | | 98 | 97.5 | |
| | | | 100 | | | 97 | | |
| | | | 100 | | | 98 | | |
| | | | 100 | | | 97 | | |
| | User 3 | | 100 | 100 | | 99 | 98.5 | |
| | | | 100 | | | 98 | | |
| | | | 100 | | | 99 | | |
| | | | 100 | | | 98 | | |
| | User 4 | | 100 | 100 | | 99 | 98.8 | |
| | | | 100 | | | 100 | | |
| | | | 100 | | | 99 | | |
| | | | 100 | | | 97 | | |
| Site A | User 1 | 0.1% mix | 95 | 90.3 | 90 | 99 | 98.8 | 98 |
| | | | 85 | | | 98 | | |
| | | | 88 | | | 99 | | |
| | | | 93 | | | 99 | | |
| Site B | User 2 | | 90 | 92.0 | | 99 | 98.8 | |
| | | | 88 | | | 99 | | |
| | | | 95 | | | 99 | | |
| | | | 95 | | | 98 | | |
| | User 3 | | 93 | 93.3 | | 99 | 98.5 | |
| | | | 90 | | | 97 | | |
| | | | 95 | | | 99 | | |
| | | | 95 | | | 99 | | |
| | User 4 | | 85 | 85.3 | | 99 | 98.3 | |
| | | | 98 | | | 98 | | |
| | | | 88 | | | 99 | | |
| | | | 70 | | | 97 | | |

Repeatability experiments were performed by subjecting a single multiplexed library to templating and sequencing on three consecutive days with the same user and instruments. The multiplexed library contained four replicate libraries generated from 6,000 copies of the 0.5% control mix, and four replicate libraries generated from 6,000 copies of the 0.1% control mix. We observed high repeatability using the Ion Chef System for templating and the Ion S5 XL System for sequencing of Oncomine Lung cfDNA libraries (Table 6).

**Table 6. Assay repeatability across multiple sites, instruments, and users.**

| | | Sensitivity (%) | | | Specificity (%) | | |
|---|---|---|---|---|---|---|---|
| Sample | Repeat | Single point | Run average (n = 4) | All runs average (n = 12) | Single point | Run average (n = 4) | All runs average (n = 12) |
| 0.5% mix | Run 1 | 100 | 100 | 100 | 99.2 | 99.6 | 99.6 |
| | | 100 | | | 100 | | |
| | | 100 | | | 99.2 | | |
| | | 100 | | | 100 | | |
| | Run 2 | 100 | 100 | | 100 | 99.4 | |
| | | 100 | | | 99.2 | | |
| | | 100 | | | 99.2 | | |
| | | 100 | | | 99.2 | | |
| | Run 3 | 100 | 100 | | 100 | 99.6 | |
| | | 100 | | | 100 | | |
| | | 100 | | | 99.2 | | |
| | | 100 | | | 99.2 | | |
| 0.1% mix | Run 1 | 97.5 | 97.5 | 98.3 | 100 | 99.8 | 99.8 |
| | | 97.5 | | | 100 | | |
| | | 97.5 | | | 99.2 | | |
| | | 97.5 | | | 100 | | |
| | Run 2 | 100 | 99.4 | | 99.2 | 99.8 | |
| | | 100 | | | 100 | | |
| | | 97.5 | | | 100 | | |
| | | 100 | | | 100 | | |
| | Run 3 | 100 | 98.1 | | 100 | 99.8 | |
| | | 95 | | | 99.2 | | |
| | | 97.5 | | | 100 | | |
| | | 100 | | | 100 | | |

## Assay sensitivity and specificity

To evaluate assay sensitivity and specificity, two users conducted experiments at a single site. We utilized the fragmented AcroMetrix Oncology Hotspot Control material described above in the design of the repeatability experiment. The 0.5% and 0.1% control mixes were verified by dPCR for allele frequencies. We also used the control mix verification to measure the actual number of DNA copies/µL of material.

We observed high average sensitivity and specificity in this assay, with 99.5% average specificity across all experiments (Table 7).

Using control data as shown in Table 7, a mean sensitivity of 92.2%, specificity of 99.7%, and PPV of 99% were reported for variant detection at 0.1% frequency. We also report a mean sensitivity of 100%, specificity of 99.6%, and PPV of 98.8% for variant detection at 0.5% frequency. The data here are based on the expected 40 true positives and expected 129 true negatives from our control experiments, considering the total number of 169 hotspots targeted by the Oncomine Lung cfDNA Assay. We used the following equations to calculate these metrics:

Sensitivity = TP/(TP + FN)
Specificity = TN/(TN + FP)
PPV = TP/(TP + FP)

TP: true positive, TN: true negative, FP: false positive, FN: false negative

**Table 7. Assay sensitivity and specificity with the AcroMetrix Oncology Hotspot Control.**

| Sample | Sensitivity (%) | Average sensitivity | Specificity (%) | Average specificity | PPV (%)* | Average PPV* |
|---|---|---|---|---|---|---|
| 0.5% mix | 100 | 100 | 99.2 | 99.6 | 97.6 | 98.8 |
| | 100 | | 100 | | 100 | |
| | 100 | | 99.2 | | 97.6 | |
| | 100 | | 100 | | 100 | |
| 0.1% mix | 97.5 | 92.2 | 99.2 | 99.7 | 97.6 | 99 |
| | 90 | | 100 | | 100 | |
| | 97.5 | | 100 | | 100 | |
| | 90 | | 100 | | 100 | |
| | 85 | | 99.2 | | 97.1 | |
| | 100 | | 100 | | 100 | |
| | 100 | | 99.2 | | 97.6 | |
| | 77.5 | | 100 | | 100 | |

* PPV: positive predictive value

## Confirmation with cfDNA reference standards

Commercial cfDNA reference standards contain well-calibrated, low allele frequency variants at eight hotspot locations in key genes targeted by the Oncomine Lung cfDNA Assay (Table 8). The cfDNA reference standards include six single-nucleotide variants (SNVs) from five genes, one insertion, and one deletion.

## Assay accuracy (cross-verification)

Consistency of allele frequency at ~0.1% was detected in all three Oncomine cfDNA Assays, and were confirmed using orthogonal dPCR (Table 9). Using the cfDNA reference standards, we evaluated assay accuracy across three disease-specific Oncomine cfDNA Assays—targeting lung, breast, and colon cancer samples, respectively. Results were obtained using NGS on the Ion S5 XL System, and dPCR on the Applied Biosystems™ QuantStudio™ 3D Digital PCR System.

**Table 8. Low-frequency variants in five genes, at eight hotspot locations in cfDNA reference standards (Horizon Discovery).**

| Reference standard | | *EGFR* | | | | *KRAS* | *NRAS* | *NRAS* | *PIK3CA* |
|---|---|---|---|---|---|---|---|---|---|
| **Frequency** | **Input** | p.E746_A750delELREA | p.L858R | p.T790M | p.V769_D770insASV | p.G12D | p.A59T | p.Q61K | p.E545K |
| **0.1%** | 30 ng | 0.23% | 0.00% | 0.00% | 0.16% | 0.18% | 0.00% | 0.17% | 0.19% |
| | | 0.21% | 0.00% | 0.05% | 0.14% | 0.11% | 0.00% | 0.08% | 0.10% |
| **0.1%** | 50 ng | 0.06% | 0.17% | 0.06% | 0.10% | 0.22% | 0.17% | 0.15% | 0.10% |
| | | 0.17% | 0.10% | 0.09% | 0.05% | 0.06% | 0.08% | 0.00% | 0.18% |
| **1%** | 10 ng | 0.72% | 1.07% | 0.75% | 0.74% | 1.14% | 1.15% | 1.15% | 2.29% |
| | | 0.99% | 1.03% | 0.37% | 1.03% | 0.63% | 1.95% | 1.03% | 1.26% |
| **5%** | 5 ng | 5.58% | 3.66% | 4.40% | 2.83% | 5.94% | 4.82% | 6.54% | 6.13% |
| | | 4.52% | 4.86% | 6.32% | 3.97% | 6.34% | 6.11% | 6.94% | 5.29% |

**Table 9. Verification of 0.1% detection sensitivity across Oncomine cfDNA Assays (lung, breast, and colon cancer samples).**

| Gene | Variant | Oncomine cfDNA Lung Assay | Oncomine cfDNA Breast Assay | Oncomine cfDNA Colon Assay | Digital PCR |
|---|---|---|---|---|---|
| ***KRAS*** | p.G12D | 0.20% | 0.20% | 0.15% | 0.27% |
| | | 0.17% | 0.14% | 0.14% | 0.24% |
| ***PIK3CA*** | p.E545K | 0.15% | 0.14% | 0.26% | 0.02% |
| | | 0.11% | 0.06% | 0.16% | 0.19% |
| ***EGFR*** | p.E746_A750delELREA | 0.12% | NA* | NA* | 0.10% |
| | | 0.11% | NA* | NA* | 0.12% |
| ***EGFR*** | p.L858R | 0.07% | 0.14% | NA* | 0.05% |
| | | 0.06% | 0.06% | NA* | 0. 28% |

* These hotspots are not included in the indicated Oncomine cfDNA Assay hotspot file.

## Assay workflow verification

We observed high concordance between variants detected in FFPE samples and cfDNA assays, from six independently matched late-stage NSCLC research samples (Table 10). As expected, higher variant frequencies were observed in the FFPE solid tumor samples, and significantly lower frequencies measured in the matched cfDNA samples. Sample sets included FFPE solid tumor sample and 2–4 mL plasma from blood collected in EDTA tubes.

**Table 10. Assay accuracy from matched FFPE and plasma samples.**

| Sample | Variant | FFPE | cfDNA |
|---|---|---|---|
| 1 | *EGFR*-L858R | **71.42%** | **2.62%** |
| 2 | *TP53*-R158L | **51.89%** | **4.32%** |
| 3 | *MET*-T1010I | 43.87% | 51.75% |
| | *KRAS*-G12C | **34.62%** | **0.28%** |
| 4 | NA | Not detected | Not detected |
| 5 | *EGFR*-L858R | **58.44%** | **7.28%** |
| | *MET*-T1010I | 41.93% | 48.72% |
| | *TP53*-Y220C | **35.54%** | **1.93%** |
| 6 | *TP53*-R158L | **10.19%** | **1.26%** |

**Bold numbers** indicate allele frequencies as determined using the Oncomine Lung cfDNA Assay for the indicated somatic variants. Non-bold numbers show frequencies of germline variants that were also detected in the targeted libraries.

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## Conclusions

From analytical verification of the Oncomine Lung cfDNA Assay, we observed accurate measurement of low-frequency variants in cfDNA isolated from healthy donor plasma and late-stage NSCLC research samples. The Oncomine Lung cfDNA Assay performs over a wide range of input amounts. High sensitivity and specificity for detecting variants at levels of 0.1% frequency in cfDNA standard materials was observed with consistent reproducibility and repeatability. The low allele frequency of 0.1% was confirmed using NGS across three different Oncomine cfDNA Assays (lung, breast, and colon cancer samples), with orthogonal verification using dPCR.

Find out more at **thermofisher.com/cfdna-assays**

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