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Performance Evaluation of the Aspyre Clinical Test for Lung (Tissue) at Low Nucleic Acid Input for Simple, Fast and Robust Testing for Actionable NSCLC Variants in FFPE Tissue

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Aspyre® Lung

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ABSTRACT

Introduction: Patients can only access the full potential of targeted therapy if they complete biomarker testing. Current methods such as NGS are costly and difficult to interpret with long turnaround times, while PCR assays are limited in the number of variants they can cover. The Aspyre® (Allele-Specific PYrophosphorolysis REaction) technology has been developed to address the urgent need for rapid, accessible and affordable diagnostics informing actionable genomic target variants of a given cancer. The targeted Aspyre Clinical Test for Lung® panel for NSCLC covers 114 variants in 11 genes to inform clinical management based on practice guidelines. The assay detects single nucleotide variants, insertions, deletions, gene fusions and *MET* exon 14 skipping from tissue-derived DNA and RNA simultaneously.

The recommended input into Aspyre Lung for tissue is 20 ng of DNA, and 6 ng of RNA, respectively. However, FFPE tissue samples from fine needle aspirates, core needle biopsies or cytological specimens often yield low quantities of degraded nucleic acids of insufficiently high quantity to achieve even these modest levels. Being able to test for a large panel of variants using Aspyre Lung with lower inputs of nucleic acid allows for comprehensive biomarker profiling without exhausting tissue. It reduces the risk for the need for repeat biopsies and allows for optimal integration with additional clinical workflows.

Methods: To evaluate assay performance at low input we tested the limit of detection, specificity, analytical accuracy and analytical precision of Aspyre Lung at 5 ng of DNA and 1.5 ng of RNA respectively, using FFPE lung tissue samples from patients with NSCLC, variant-negative FFPE tissue, and FFPE-based contrived samples with controllable variant allele fractions.

Results: The sensitivity (LoD95) of Aspyre Lung with low input tissue samples was confirmed ≤ 3% variant allele fraction for single nucleotide variants and insertions or deletions, ≤ 100 copies for fusions, and ≤ 200 copies for *MET* exon 14 skipping. The specificity was 100% with no false positive results. Results were replicable across operators, reagent lots, runs, and real-time PCR instruments with a high degree of precision. Tests of assay accuracy against an orthogonal testing method using 29 NSCLC patient samples yielded 100% positive percent agreement and 100% negative percent agreement. These performance metrics are identical to those obtained for the initial analytical validation of Aspyre Lung at 20 ng DNA, and 6 ng RNA[1].

Conclusion: Aspyre Lung retains full performance at low nucleic acid input and can be used effectively and safely with 5 ng of DNA and 1.5 ng of RNA.

Aspyre Lung WORKFLOW

	Target Amplification	Enzymatic Cleanup Reaction	Aspyre Reaction	Detection Reaction	Data Analysis
DNA RNA					
Instrument Requirements	Thermal cycler	Thermal cycler	Thermal cycler	Real-time PCR instrument	Desktop computer
Hands-on Time	30 mins	10 mins	35 mins	15 min	20 mins
Incubation Time	1 hour 25 mins	15 mins	35 mins	3 hours 30 mins	-

The steps of the Aspyre Lung assay workflow after nucleic acid extraction. Typical TAT from sample receipt to final results is 2 days.

STUDY MATERIALS & METHODS

Contrived reference samples Variant-specific DNA (SNVs, indels) and RNA (gene fusions, *MET* exon 14 skipping) oligonucleotides were manufactured by commercial suppliers, quantified by dPCR, and spiked into background DNA or RNA extracted from variant-free FFPE tissue samples.

Clinical samples FFPE patient tissue blocks were obtained from commercial biobanks.

Ethical approval Institutional Review Board (IRB) or equivalent was obtained for sample use in diagnostics development by biobanks through collection sites. All data were de-identified so no patients could be identified by study personnel outside of the clinical trial site including the biobanks and the study authors.

Nucleic acid extraction Nucleic acid from 12 µM curls was extracted using the Quick-DNA/RNA™ FFPE miniprep kit (Zymo Research). Concentrations were determined by Qubit.

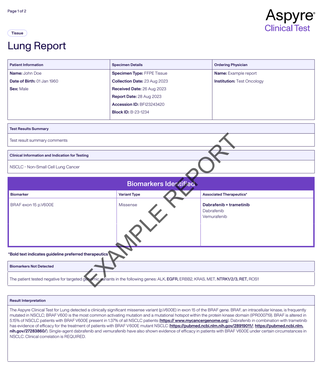
Aspyre Lung 5 ng DNA and 1.5 ng RNA were analyzed at the CAP CLIA laboratory of Biofidelity Inc., using standardized protocols.

Data analysis Data from real-time PCR instruments were downloaded and analyzed using custom cloud-based Aspyre Lab software. All variant calling was blinded to results from available orthogonal analyses.

INTRODUCTION TO Aspyre

Comprehensive genomic testing of *EGFR*, *BRAF*, *ALK*, *RET*, *ROS1*, *ERBB2*, *KRAS*, *NTRK1, NTRK2, NTRK3* and *MET* is indicated in patients with NSCLC. The detection of abnormalities in these genes informs the use of targeted therapeutic agents. We have previously described development and analytical validation of a novel method, Aspyre, for rapid and low-cost detection of single nucleotide variants, insertions, deletions and complex events from DNA and fusions and exon skipping from RNA[1]. Here, we describe analytical validation of this assay at low nucleic acid input, including testing of sensitivity (LoD95), specificity, analytical accuracy and analytical precision.

Genes and variants covered by Aspyre Lung tissue. A total of 114 genomic variants are assessed and aggregated as actionable variant calls where appropriate for treatment guidance e.g. *NTRK*-fusion positive call is aggregated from multiple potential fusion pairs.


Aspyre Clinical Test

Lung Report

Patient Information | Specimen Details | Ordering Physician

Test Results Summary

Clinical Information and Indication for Testing

NSCLC - Non-Small Cell Lung Cancer

Biomarkers Identified

Biomarker	Variant Type	Associated Therapeutics*
BRAF exon 15 p.V600E	Missense	Dabrafenib + trametinib; Dabrafenib; Vemurafenib

*Bold text indicates guideline-preferred therapeutic(s).

Biomarkers Not Detected

The patient tested negative for targeted biomarkers in the following genes: ALK, EGFR, ERBB2, KRAS, MET, NTRK1/2/3, RET, ROS1

Result Interpretation

The Aspyre Clinical Test for Lung detected a clinically significant missense variant (p.V600E) in exon 15 of the BRAF gene. BRAF, an intracellular kinase, is frequently mutated in NSCLC. BRAF V600 is the most common activating mutation and a mutational hotspot within the protein kinase domain (PROSITE). BRAF is altered in ~1-3% of all NSCLC patients (https://www.mycancergenome.org). Dabrafenib in combination with trametinib has evidence of efficacy for the treatment of patients with BRAF V600E mutant NSCLC (https://pubmed.ncbi.nlm.nih.gov/28990857/; https://pubmed.ncbi.nlm.nih.gov/27266560/). Single-agent dabrafenib and vemurafenib have also shown evidence of efficacy in patients with BRAF V600 under certain circumstances in NSCLC. Clinical correlation is RECURRED.

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Aspyre Clinical Test

Lung Report

Patient Information – BP2324G430

Biomarkers Evaluated

Methods and Limitations

Disclaimer

Lab Director Signature

Laboratory Director

Methods and Limitations

1. Silina A.L. et al. 2021. Single-copy detection of somatic variants from solid and liquid biopsy. Sci Rep. 10(1):6068.
2. Gray E.R. et al. 2022. Ultra-sensitive molecular detection of gene fusions from RNA using Aspyre. BMC Med Genomics. 15(1):215.

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EXAMPLE REPORT

The Aspyre Lung patient report. The two-page report includes a summary of patient and sample details, a summary of test results including biomarkers identified, biomarkers not detected and indeterminate biomarkers, in addition to an interpretation of results.

RESULTS

Variant type	Gene, Exon, Protein Variant, COSMIC ID	Total positive/ total tests**
DNA		
SNV	*KRAS* exon 2 G12C COSM516	218/220
	EGFR exon 21 L858R COSM6224	
	EGFR exon 20 T790M COSM6240 *	
	BRAF exon 15 V600E COSM476	
	EGFR exon 18 G719A COSM6239 *	
	EGFR exon 18 G719S COSM6252 *	
	EGFR exon 20 C797S COSM6493937 *	
	EGFR exon 20 C797S COSM5945664 *	
Deletion	*EGFR* exon 19 E746_A750del COSM6223	80/80
Insertion	*ERBB2* exon 20 Y772_A775dup COSM20959	
	ERBB2 exon 20 H773dup COSM12377 *	
	EGFR exon 20 A767_V769dup COSM12376	
RNA Fusions		
Fusion	*EML4-ALK* E13_A20 COSF408	119/120
	KIF5B-RET K15_R12 COSF1232	
	CD74-ROS1 C6_R34 COSF1200	
	TPM3-NTRK1 T8_N10 COSF1329	
	QKI-NTRK2 Q6_N16 COSF1446	
	ETV6-NTRK3 E5_N15 COSF571	
RNA Exon Skipping		
Exon skipping	*MET*	20/20

LoD95 confirmation data. LoD95 at low input was estimated for 6 DNA variants (marked with *) at a range between range 3-10% VAF. Results were then confirmed by testing 20 replicates of freshly prepared contrived samples. Variants whose LoD95 was not estimated beforehand were run at 20 replicates at their respective LoD95 determined for the initial standard-input Aspyre Clinical Test for Lung FFPE validation (3% for indels, 100 copies for gene fusions and 200 copies for *MET* exon 14 skipping). Confirmed LoD95 by mutation class were ≤ 3% VAF for SNV and Indel, ≤ 100 copies for fusions, and ≤ 200 copies for *MET* exon 14 skipping.

** Results were aggregated across the given variant class.

Level	Metric	Actual % (CI95)
Sample	PPA	100 (86-100)
	NPA	100 (92-100)
Variant	PPA	100 (86-100)
	NPA	100 (99.89-100)

Summary of analytical precision (repeatability and reproducibility) data. Three contrived and three NSCLC patient samples were run in duplicate across four independent runs over four days by two operators using two real-time PCR instruments and two reagent lots. Shown are the positive and negative percent agreement values between runs of Aspyre Lung, aggregated over DNA and RNA on sample and variant level respectively, demonstrating 100% reproducibility (inter-run precision) and repeatability (intra-run precision).

		Number (n)		
Category	**Tested per Assay**	**Total Tested**	**Positive**	**FP rate**
Samples	1	60 DNA 59* RNA	0	0
Nucleotide Variants	114	6840	0	0
SNVs	26	1560	0	0
Indels + complex substitutions	31 + 20	3060	0	0
Fusions	36	2124	0	0
Exon Skipping	1	59	0	0
Reportable Variants	71	4260	0	0

Limit of Blank (LoB) data. DNA and RNA from 30 FFPE variant-free samples were tested twice using two reagent lots (one replicate per specimen per reagent lot). Data were analyzed by calculating the false positive (FP) rate per sample and per variant (FP rate = false positive calls/total tests). Numbers above show tests per assay, total number of tests, positive calls, and the FP rate for all samples and for each variant category. While 114 nucleotide variants are tested within Aspyre Lung, results are aggregated to 71 reportable variants as 1) several nucleotide variants produce the same reported protein alteration and 2) multiple fusion partners or breakpoints result in a single gene fusion call. There were no positive calls for any of the 30 samples tested, and there were no positive calls for any variant analysed by the test. Therefore, the FP rate was 0% (0-6% Clopper-Pearson 95%CI) and the LoB was zero. Considering number of opportunities tested per variant rather than number of samples, the 95%CI tightens to a reportable variant 95%CI of 0 - 0.09%.

* One RNA sample inadvertently processed at 15 ng (not 1.5 ng), impacting 1 replicate in these data, and 4 replicates from the precision study (see left). Replicates were removed from all pertinent analyses.

		Actual % (CI95)	
Level	**Metric**	**DNA & RNA single variant[1]**	**DNA Double variant[2]**
Sample	PPA	100 (91-100)	100 (40-100)
	NPA	100 (91-100)	100 (23-100)
Variant	PPA	100 (91-100)	100 (63-100)
	NPA	100 (98.6-100)	100 (98.60-100)

[1]n= 39 samples, 41 positives, [2]n= 3 samples, 6 positives

Summary of analytical accuracy of Aspyre Lung assessed using contrived and clinical samples. Analytical accuracy at low input was assessed using 12 DNA and 7 RNA single variant contrived samples covering all variant classes at 2x LoD, 3 DNA double-variant contrived samples, and 29 FFPE NSCLC patient specimens. Shown are PPA and NPA obtained per sample and per variant across samples for DNA & RNA combined, and associated 95%CI.

DISCUSSION

Around 55% of tissue samples fail to provide results from current Next Generation Sequencing (NGS)-based genomic testing to patients with NSCLC[2], leading to inadequate patient care. Our data from this validation demonstrates that Aspyre Lung is highly sensitive and specific with its exceptional performance characteristics maintained even at inputs as low as 5 ng DNA and 1.5 ng RNA. This is considerably below the nucleic acid input typically required from NGS-based assays and has thus the potential to address tissue-limited (QNS) specimens.

Patient access to critical biomarker testing remains poor (<50% of patients in the US[3]) with gaps due to the cost, complexity and long turnaround times associated with NGS-based testing. In contrast, the Aspyre Lung workflow is simple with only four reagent transfer steps after DNA/RNA extraction that require no more than standard lab equipment. Typical turnaround time from sample receipt to results is 2 days. The Aspyre report is simple to interpret and adapted for display in electronic medical records. Given the breadth of coverage and technical performance characteristics, Aspyre can address numerous gaps in current biomarker testing practice.

SUMMARY

In this study, we demonstrate that Aspyre Lung FFPE Tissue assay has **excellent analytical sensitivity at low nucleic acid input (5 ng DNA and 1.5 ng RNA)**, comparable to current NGS-based testing solutions with

- ≤ 3% VAF for SNV and indels from DNA
- ≤ 100 copies for gene fusions from RNA
- ≤ 200 copies *MET* exon 14 skipping from RNA.

The assay has **100% specificity** and is **highly reproducible and repeatable** across different operators, reagent lots, runs, days and qPCR instruments.

Aspyre ®
- combines the benefits of multi-gene testing with rapid turnaround time
- has simple bioinformatics, and supports easier clinical decision making (only actionable or prognostic markers are tested)
- Results are analyzed via a cloud-based algorithm and no further bioinformatic analysis or interpretation is required.

For further queries or discussion of this poster, please head to the CellCarta booth 3445

REFERENCES

1. Evans *et al.* 2024. ASPYRE-Lung: Validation of a simple, fast and novel method for multi-variant genomic analysis of actionable NSCLC variants in FFPE tissue. *Front. Oncol.* 14:1420162.

2. Hagemann et al. 2015. Clinical next-generation sequencing in patients with non-small cell lung cancer. Cancer. 121: 631-639.

3. Sadik *et al.* 2022. Impact of clinical practice gaps on the implementation of personalized medicine in advanced non-small-cell lung cancer. *JCO Precision Oncology* 6:e2200246

Data from this study are currently being prepared for publication.

*All authors are or were employees of Biofidelity Inc. or Biofidelity Ltd. and may have a financial interest including salary, equity, options, and intellectual property.