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ASPYRE-Lung: Validation of a Simple, Fast, Robust and Novel Method for Multi-Variant Genomic Analysis of Actionable NSCLC Variants in FFPE Tissue

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ASPYRE

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ABSTRACT

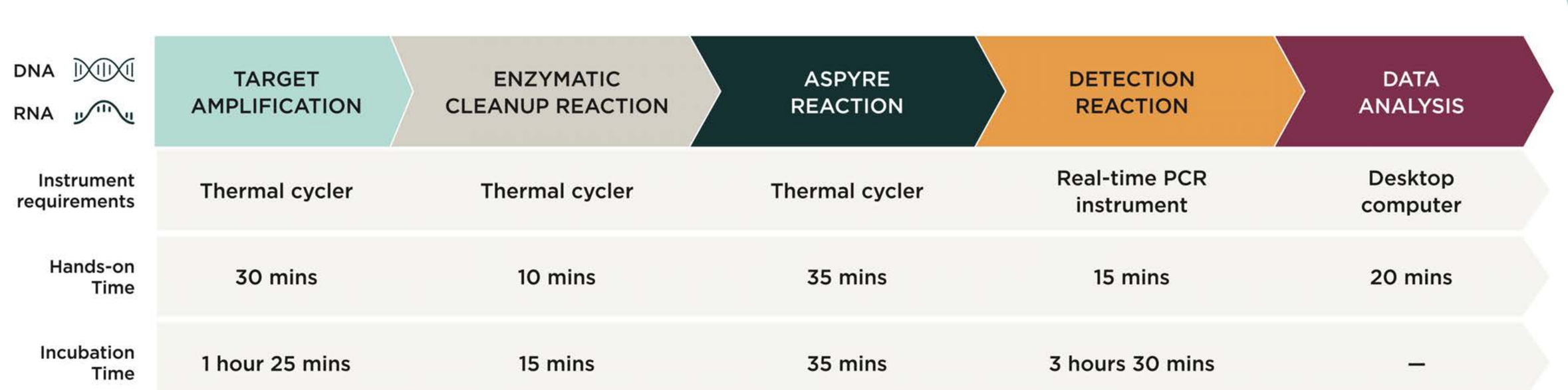
Introduction: Patients can only access the full potential of targeted drugs if they have biomarker testing. Current methods such as NGS are costly and challenging to interpret, while PCR assays are limited in the number of variants they can cover. We developed ASPYRE® (Allele-Specific PYrophosphorolysis REaction) technology to address the urgent need for rapid, accessible and affordable diagnostics informing actionable genomic target variants of a given cancer. The targeted ASPYRE-Lung® panel for NSCLC covers 114 variants in 11 genes (*ALK, BRAF, EGFR, ERBB2, KRAS, RET, ROS1, MET & NTRK1/2/3*) to robustly inform clinical management, based on practice guidelines. The assay detects single nucleotide variants, insertions, deletions, and gene fusions from tissue-derived DNA and RNA simultaneously.

Methods: We tested the limit of detection, specificity, analytical accuracy and analytical precision of ASPYRE-Lung 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 in FFPE lung tissue samples was determined to be ≤ 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. The analytical accuracy test yielded no discordant variants between ASPYRE-Lung and orthogonal testing (targeted capture NGS) or contrived samples, and results were replicable across operators, reagent lots, runs, and real-time PCR instruments with a high degree of precision. The technology is simple and fast, with a turnaround time of 2 days from sample receipt to result, with analysis via a cloud-based analysis algorithm and no further interpretation or bioinformatic analysis required.

Conclusion: ASPYRE-Lung has the potential to be transformative in providing rapid, actionable molecular profiling of tissue for patients with NSCLC.

ASPYRE-Lung ASSAY WORKFLOW



The steps of the ASPYRE-Lung assay workflow after nucleic acid extraction. The TAT from sample receipt to results out is completed at the Biofidelity Genomics Laboratory within 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 blank 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/RNATM FFPE miniprep kit (Zymo Research). Concentrations were determined by Qubit.

ASPYRE-Lung 20 ng DNA and 6 ng RNA were analyzed at the Biofidelity Genomics Laboratory, a CLIA site, using standardized protocols.

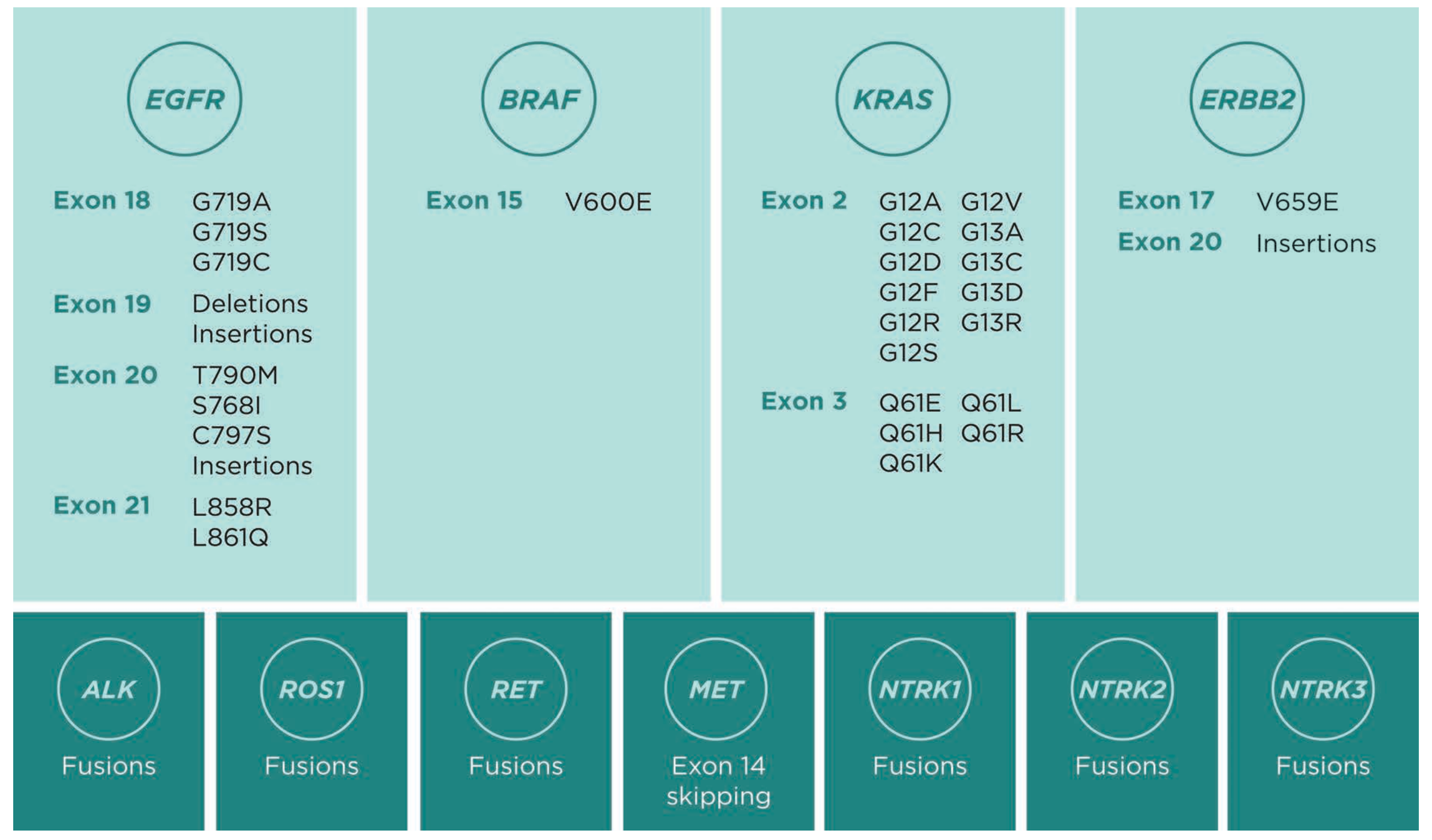
Orthogonal testing DNA was sequenced through targeted capture and sequencing (Roche Avenio Targeted Assay, Illumina NextSeq 500).

Interfering substances Low variant-containing contrived samples were spiked with molecular biology-grade ethanol or guanidinium thiocyanate at concentrations mimicking potential carryover during the extraction process, before testing with ASPYRE-Lung.

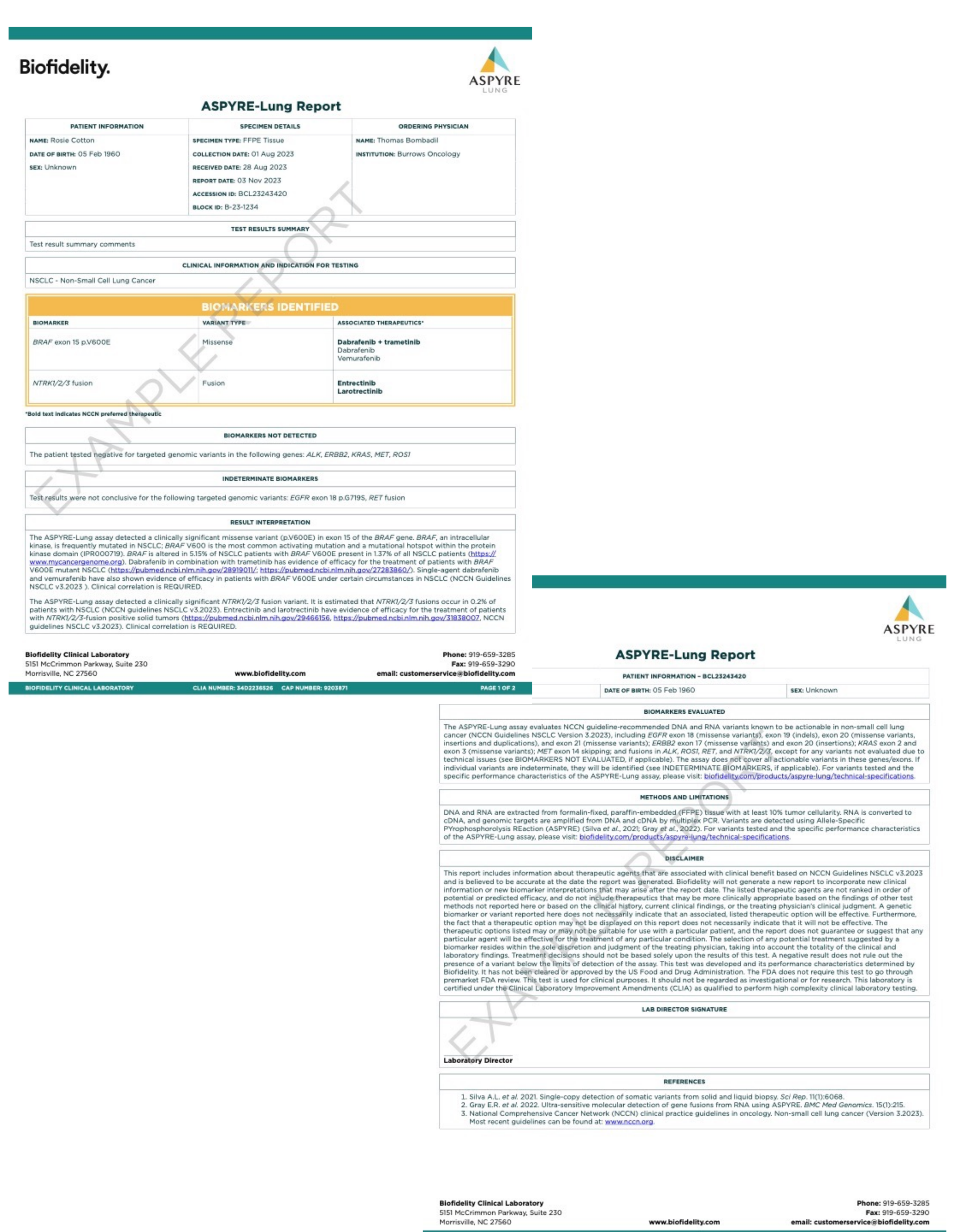
Data analysis Data from real-time PCR instruments were downloaded and analyzed using custom cloud-based ASPYRELab v1.0.0 software. All variant calling was blinded to results from orthogonal analyses.

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 of a novel method, ASPYRE, for rapid and low-cost detection of single nucleotide variants, insertions, deletions and complex events from DNA[1] and fusions and exon skipping from RNA[2]. Here, we describe analytical validation of this assay including testing of sensitivity (LoD95), specificity, analytical accuracy, analytical precision and resiliency to potential interfering substances.



Genes and variants covered by the ASPYRE NSCLC tissue panel. 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.



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, and an interpretation of results.

RESULTS

Variant type	Gene	Total positive/ total tests*
DNA		
SNV	*KRAS* exon 2 G12C COSM526	80/80
	EGFR exon 21 L858R COSM6224	
	EGFR exon 20 T790M COSM6240	
	BRAF exon 15 V600E COSM476	
Deletion	*EGFR* exon 19 E746-A750del COSM6223	60/60
Insertion	*ERBB2* exon 20 Y772-A775dup COSM20959	
	EGFR exon 20 A767-V769dup COSM12376	
RNA Fusions		
Fusion	*EML4-ALK* E13_A20 COSM408	118/120
	KIF5B-RET K15_R12 COSF1232	
	CD74-ROS1 C6_R36 COSF1200	
	TMP3-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. Results of testing 20 replicate contrived samples at levels pre-determined by an LoD estimation (range 3-10% VAF for SNV/InDel, 100-300 copies for gene fusions and 200-800 copies for *MET* exon 14 skipping). Confirmed LoD95 by mutation class (SNV, indel, fusion and *MET* exon 14 skipping) 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. NA, not applicable.

Level	Metric	Actual % (CI95)
Sample	PPA	100 (90-100)
	NPA	100 (91-100)
Variant	PPA	100 (92-100)
	NPA	100 (99.87-100)

Summary of analytical accuracy of ASPYRE-Lung assessed using contrived and clinical samples. Shown are the PPA and NPA obtained for each sample and for each variant across both clinical contrived sample types, for DNA & RNA combined and the associated 95% confidence intervals.

			TP / total Replicates			
			5 % Ethanol	Guanidine thiocyanate		
Analyte	Gene	VAF/ Copies		5 mM	10 mM	20 mM
DNA	*KRAS* exon 2 G12C COSM516	6%	5/5	5/5	5/5	5/5
DNA	EGFR exon 21 L858R COSM6224	6%	5/5	5/5	5/5	5/5
RNA	EML4- ALK E13_A20 COSF408	200 copies	5/5	5/5	5/5	5/5
RNA	KIF5B- RET K15_R12 COSM1232	200 copies	5/5	5/5	5/5	5/5

Contaminants carried over from FFPE sample extraction do not interfere with the ASPYRE-Lung assay. Two DNA and two RNA contrived samples at twice the LoD95 were spiked with ethanol or guanidinium thiocyanate to mimic carryover from substances used during sample extraction. Shown are the positive calls made compared to total runs from the subsequent ASPYRE-Lung assay runs. NA, not applicable.

		Number			
Category	Nucleic acid	Samples	Neg variant calls / sample	Positive	Total negative calls
LoD estimation	DNA	105	65	0	6825
	RNA	95	4	0	380
LoD confirmation	DNA	140	65	0	9100
	RNA	140	4	0	560
Analytical Precision	DNA	24	66	0	1584
	RNA	24	5	0	120
Analytical Accuracy (single mutant)	DNA	7	65	0	455
	RNA	7	4	0	28
Analytical Accuracy (double mutant)	DNA	3	64	0	192
LoB	DNA	60	66	0	3960
	RNA	60	5	0	300
Total					**23504**

Negative variant calls from samples tested during AV. A single 'Reportable Variant' may cover multiple nucleotide variants where the associated therapeutics are identical: ASPYRE-Lung tests for 114 variants, and outputs 71 potential calls. The negative calls from all experiments performed during assay validation were combined to estimate a **false positive rate of 0% (CI95 0-0.0157%)**

Sample-level metric	Actual % (CI95)
PPA	100 (86-100)
NPA	100 (93-100)

Summary of analytical precision (repeatability and reproducibility) data. Shown are the positive and negative percent agreement values between runs of ASPYRE-Lung, aggregated over DNA and RNA, demonstrating 100% reproducibility (inter-run precision) and repeatability (intra-run precision). Samples were assayed in four independent runs across four days by two operators using two real-time PCR instruments and two reagent lots.

DISCUSSION

Our data from this sample set demonstrates that ASPYRE-Lung is highly sensitive and specific with performance characteristics appropriate for use in a clinical laboratory. We believe that ASPYRE-based testing can provide more rapid and efficient genomic evaluation of individuals with NSCLC as the full ASPYRE-Lung assay (from sample receipt to reporting) is routinely completed in our CLIA laboratory within 2 days. This provides rapid genomic evaluation relative to current workflows.

Many NGS panel-based tests are available, yet patient access to critical biomarker testing remains poor (<50% of patients in the US[3]) and gaps remain due to the cost, complexity and long turnaround times associated with NGS. In contrast, ASPYRE-Lung testing processes are simple and fast with only four reagent transfer steps after DNA/RNA extraction that require no more than pipetting and standard lab equipment. ASPYRE results are also simpler to interpret and much more readily adapted for display in an electronic medical record in discrete data fields than a multi-page NGS report. Given its breadth of coverage and technical performance characteristics we believe that ASPYRE can address numerous gaps in current NSCLC biomarker testing practices.

SUMMARY

ASPYRE ®
- is a novel technology based on pyrophosphorolysis of oligonucleotide probes perfectly hybridized to sample-derived target DNA or cDNA (RNA) sequences.
- combines the benefits of multi-gene testing with rapid TAT
- has simple bioinformatics, and with easily interpretable clinical decision making (only actionable markers are tested)

In this study, we demonstrate that ASPYRE-Lung FFPE Tissue assay has **excellent analytical sensitivity**, comparable to current NGS-based testing solutions:
- ≤ 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** with no false positive results out of 23,504 negative calls made from all samples run.
The assay is also **highly reproducible** and repeatable across different operators, reagent lots, runs, days and qPCR instruments.
No interfering effects from carry over of extraction reagents were detected.

REFERENCES

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Data from this study have been prepared and submitted for publication.

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