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Analytical validation of Aspyre Clinical Test for Lung (Blood): A multiplexed PCR and pyrophosphorolysis-based assay for detecting actionable NSCLC variants in plasma cfDNA and cfRNA☆

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ABSTRACT

Background: Liquid biopsy is an important non-invasive method of sampling the molecular profile of tumors for patients to access personalized oncology therapeutics but can be challenging. NGS-based methods require high sample quality, high sequencing depth and associated cost, with complex workflows, while PCR assays are limited in variant coverage. Aspyre Clinical Test for Lung® (Blood) is a simplified genomic profiling assay for NSCLC that targets 114 variants in 11 genes (*ALK, BRAF, EGFR, ERBB2, KRAS, RET, ROS1, MET & NTRK1/2/3*) to robustly inform clinical management. The assay detects single nucleotide variants, insertions, deletions, gene fusions and exon skipping events from plasma-derived cfDNA and cfRNA simultaneously.

Method: Sensitivity, specificity, analytical accuracy and analytical precision at standard input levels (20 ng cfDNA and 42 ng cfRNA) were tested using a combination of contrived samples and extracts from clinical samples taken from both healthy volunteers and patients with NSCLC. The effects of potential interfering substances on assay performance were tested. Assay sensitivity and specificity were also assessed at lower sample input levels (5 ng cfDNA and 6 ng cfRNA).

Results: At standard input levels, median limits of detection were ≤0.25 % variant allele fraction for single nucleotide variants, ≤0.4 % variant allele fraction for insertions or deletions, ≤6 copies for gene fusions, and ≤100 copies *MET* exon 14 skipping events. The specificity from variant-free samples was 100 %. Tests of analytical accuracy yielded 100 % NPA and 94 % PPA between Aspyre Clinical Test for Lung (Blood) and either results from orthogonal NGS testing or expected outcomes of contrived samples. Results were 100 % replicable across multiple operators, reagent lots, days and equipment. At low input levels, median limits of detection were ≤0.8 % for single nucleotide variants and insertions/deletions, 6 copies for gene fusions and 100 copies for *MET* exon 14 skipping, with a false-positive rate of 0 %.

Conclusions: We present validation studies of Aspyre Clinical Test for Lung (Blood) using contrived and clinical samples. The technology is simple and fast, yet highly sensitive, specific, robust and reproducible with a turn-around time of two days. Aspyre Clinical Test for Lung (Blood) facilitates access to cost-effective, rapid, actionable molecular profiling of plasma for patients with NSCLC.

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1. Introduction

While overall mortality from cancer in the US has declined in recent years, disparities in incidence and treatment have grown [1]. Lung cancer incidence in women under 65 outstripped that in men in 2021, and lung cancer still has one of the least favorable prognoses with a higher mortality rate than any other cancer overall [1]. Inequalities in exposure to risk factors such as air pollution and poverty lead to disparities in occurrence and treatment outcomes between different ethnic and socioeconomic groups. While there is now a range of effective and well-tolerated targeted therapeutics, access is limited to patients who can achieve the requirements of an adequate and timely biopsy that has sufficient material at high enough quality for testing to achieve a definitive and prompt diagnosis prior to starting any therapy. Around 70 % of patients' tumors harbor potentially actionable alterations [2] yet only around 35 % of patients receive appropriate care options, leading to suboptimal outcomes [3]. There remains an urgent need for genomic profiling technologies and testing options that can significantly close clinical practice gaps such as those arising from high sample quality and quantity requirements, extended testing turnaround times, and test robustness.

Liquid biopsies are tests for mutational signatures of cancer performed on nucleic acids sampled from body fluids, most commonly blood. This nucleic acid may arise from several sources including circulating tumor cells, exosomes, microRNAs or cell-free nucleic acid. Blood liquid biopsies have gained traction in recent years as a method of sampling the variant profile of patients' tumors without being limited by sampling only a restricted portion of the tumor, which may harbor considerable spatial heterogeneity. Additionally, non-invasive testing methods are needed for patients who are too frail for tissue biopsies, or whose tumors are difficult to access. Due to the reduced proportion of tumor-derived material in liquid biopsy compared to a direct tumor tissue biopsy, liquid biopsies require tests of considerable sensitivity, with variant allele fractions (VAF) of 0.5 % or lower for variant profiling [4].

We recently reported the validation of Aspyre Clinical Test for Lung (Tissue) at the Biofidelity CAP/CLIA laboratory in Morrisville, North Carolina [5]. Aspyre Clinical Test for Lung is designed to interrogate 114 somatic variants including 77 single nucleotide variants (SNV) and insertions or deletions (indel) which are detected through DNA, and 36 gene fusions and *MET* exon 14 skipping variant which are detected through RNA [5]. All of these targets were chosen because they are clinically actionable or inform on prognosis [6], and are from 11 genes (*ALK*, *BRAF*, *EGFR*, *ERBB2*, *KRAS*, *MET*, *NTRK1/2/3*, *RET*, *ROS1*) recommended for first-line testing [6]. The test has a turnaround time of two days from sample receipt to result reporting. In this work, we report on the validation of Aspyre Clinical Test for Lung (Blood) (hereafter Aspyre Lung Blood) which performs genomic profiling on the same 114 variants and has an identical post-extraction laboratory workflow for samples of cell-free DNA (cfDNA) and cell-free RNA (cfRNA) derived from blood plasma, enabling tissue and blood samples to be processed on the same run (Fig. 1). The sensitivity, specificity, accuracy, precision and susceptibility to interfering substances of Aspyre Lung Blood were evaluated and are reported herein.

2. Results

2.1. The Aspyre Lung Blood assay analyzes cfDNA and cfRNA derived from blood

Aspyre is a unique technology based on pyrophosphorolysis (driving the DNA polymerase in reverse), which can proceed on perfectly complementary DNA duplexes, but is almost entirely blocked by mismatches. Using this exquisitely specific reaction, the Aspyre technology can reach single molecule sensitivity to detect target sequences, even in the presence of large amounts of background DNA [7]. Aspyre Lung is a panel of 114 biomarkers across 11 genes that covers all NCCN guideline-recommended genomic biomarkers for first-line targeted therapy of NSCLC, with a turnaround time of two days [5]. The assay analyzes DNA to detect single nucleotide variations (SNV), and insertions and deletions (indel), and RNA to detect gene fusions and *MET* exon 14 skipping. Standard input levels for Aspyre Lung Blood are 20 ng

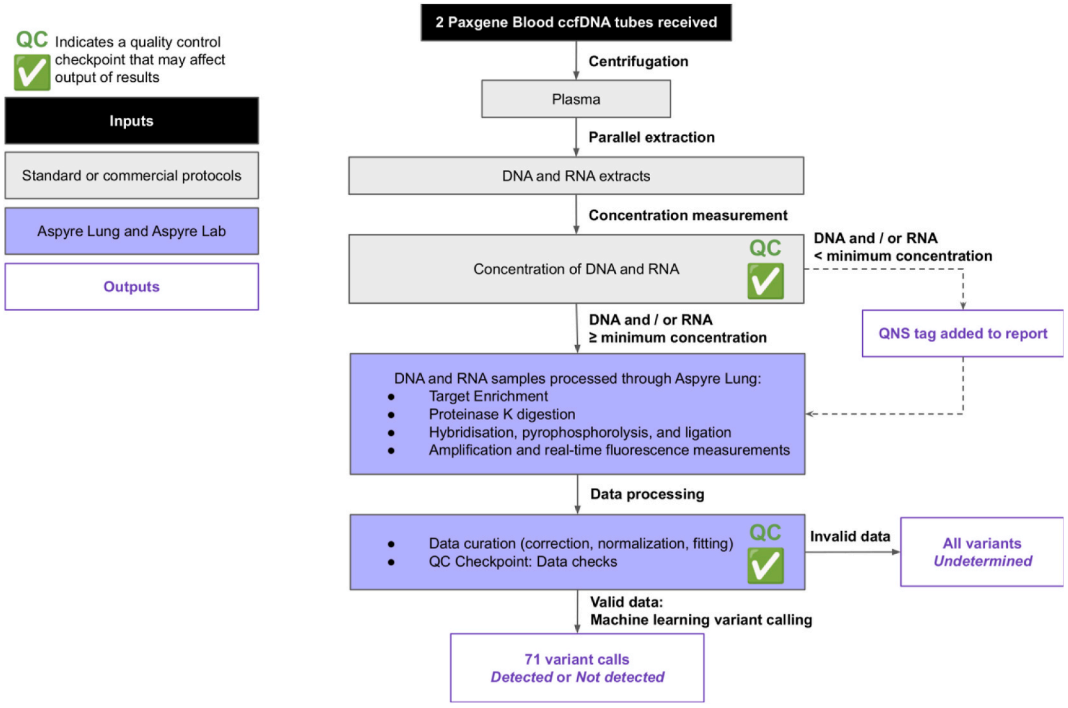

Fig. 1. Workflow of Aspyre Lung Blood. Shown are the assay inputs and outputs, sample processing, data processing and quality control stages. After the extraction of cfDNA and cfRNA there is no difference in handling of the nucleic acids. QC: quality control.

cfDNA and 42 ng matched cfRNA; low input levels are 5 ng cfDNA and 6 ng cfRNA. The procedure for both cfDNA and cfRNA is parallelized in a single assay workflow, and no equipment is required beyond a standard thermocycler and a fluorescent real-time thermocycler. Analysis occurs via a cloud-based pipeline to call presence or absence of variants in the panel, with no bioinformatics interpretation required.

2.2. The median limit of detection of Aspyre Lung Blood is 0.3 % VAF for SNV and indels, 6 copies for fusions, and 100 copies for MET exon 14 skipping

The LoD (limit of detection) was tested using reference samples of representative variants from each class of mutation to find the level at which a 95 % hit rate was achieved (LoD95) in a two-stage process. Each selected variant was first tested five times at three different VAFs (variants detected from DNA) or copy numbers (variants detected from RNA) in the estimation stage. Second, the lowest level with ≥95 % hit rate from the estimation stage was used for confirmation of the LoD95 by testing 20 replicates using a different reagent lot. During the estimation phase with standard input levels, all samples yielded 100 % positive results at the lowest levels tested, except for *EGFR* p. T790M, which had 100 % hit rate at the second lowest level tested (0.4 %, Supplementary Table 1). After the estimation phase, 6/7 DNA variants and 7/7 RNA variants were confirmed at the estimated levels with ≥17/20 replicates positive (as per CLSI EP17-A2 specifications with 20 replicates, upper one-sided 95 % confidence limit of 93.8 % [8]) (Table 1, Supplementary Fig. 1). The exception was *EGFR* p. L858R which did not achieve confirmation at 0.2 % VAF from the estimation stage but was confirmed at 0.3 % with 17/20 positive calls. Results were also aggregated by class and confirmed in every case. Therefore, the median LoD95 for Aspyre Lung Blood is ≤0.25 % VAF for SNV, ≤0.4 % VAF for indels (≤0.3 % across all cfDNA variants), ≤6 copies gene fusions (detected from cfRNA), and ≤100 copies *MET* exon 14 skipping events.

2.3. Aspyre Lung Blood is highly specific

Blood samples from 31 healthy volunteers with no history of cancer were evaluated by Aspyre Lung Blood in duplicate using two different reagent lots (Table 2). Results were used to calculate the false positive rate per sample and per variant. There were no positive calls for any sample at any of the 6840 variants analyzed by Aspyre Lung Blood (Table 2); therefore, the false positive rate per sample was 0 % (0–6 % Clopper-Pearson 95%CI). Expanding the test number to all reportable variants, the Clopper-Pearson 95 %CI drops to 0–0.09 %.

In addition, false-positive rates were calculated separately from all samples with known negative variants across all experiments performed as part of the assay validation at standard input levels (i.e. LoB plus contrived samples in sensitivity, precision, accuracy and interfering substances control reactions, Table 3). From these samples, a single false-positive *RET* fusion call was made from one of the interfering substance control replicates. It is possible that this was the result of cross contamination as a separate contrived sample containing a sample positive for a *RET* fusion was on the same run, nonetheless, it is included in these calculations.

2.4. Aspyre Lung Blood has high analytical accuracy

Analytical accuracy was assessed by testing seven DNA and seven RNA contrived control specimens at twice the LoD95 covering all variant classes, three clinically relevant double-variant contrived DNA samples, and cfDNA and cfRNA samples from patients previously

Table 2
Variants tested per sample during the LoB assessment. Independent replicate testing of 60 plasma samples from 31 donors were tested for all variants in the Aspyre Lung Blood assay. The 114 variants covered by the panel condense to 71 reportable variants; for example, *ERBB2* c.2313_2324dup and *ERBB2* c.2314_2325dup are both reported as *ERBB2* exon 20 p.Y772_A775dup, and all gene fusions that involve *NTRK1*, *NTRK2* or *NTRK3* are reported under "*NTRK1/2/3* fusion".

Category	Number (n)			
	Tested per Aspyre assay	Total Tested	Positive	FP rate (95 % CI)
Samples	1	60	0	0 (0–6)
Total nucleotide variants tested	114	6840	0	0 (0–0.05)
Reportable variants	71	4260	0	0 (0–0.09)
SNVs	26	1560	0	0 (0–0.2)
Indels + complex substitutions	31 + 20	3060	0	0 (0–0.1)
Fusions	36	2160	0	0 (0–0.2)
Exon skipping	1	60	0	0 (0–6)

Table 1
LoD95 confirmation data. Twenty replicate samples for each variant tested at levels determined by results from the initial LoD estimation. A minimum of 17/20 positive results were required for confirmation of LoD95 of each variant at the level tested. Confirmed median LoD95 for SNV was ≤0.25 % VAF, for indel was ≤0.4 % VAF, 6 copies for fusions, and 100 copies for *MET* exon 14 skipping. NA, not applicable.

Variant type	Gene	Exon	Protein variant	COSMIC ID	Confirmation positive hit rate		
					Level	Positive calls (n = 20)	Aggregated positive/total tests*
DNA					**% VAF**		
SNV	*KRAS*	2	G12C	COSM516	0.2	20	77/80
	EGFR	21	L858R	COSM6224	0.3	17	
	EGFR	20	T790M	COSM6240	0.4	20	
	BRAF	15	V600E	COSM476	0.2	20	
Deletion	*EGFR*	19	E746_A750del	COSM6223	0.2	20	60/60
Insertion	*ERBB2*	20	Y772_A775dup	COSM20959	0.8	20	
	EGFR	20	A767_V769dup	COSM12376	0.4	20	
RNA Fusions					**Copy number**		
Fusion	*EML4-ALK*	E13_A20	NA	COSF408	6	20	119/120
	KIF5B-RET	K15_R12		COSF1232	18	20	
	CD74-ROS1	C6_R34		COSF1200	6	20	
	TPM3-NTRK1	T8_N10		COSF1329	6	20	
	QKI-NTRK2	Q6_N16		COSF1446	6	19	
	ETV6-NTRK3	E5_N15		COSF571	6	20	
RNA Exon Skipping					**Copy number**		
Exon skipping	*MET*	14	L982_D1028del	COSM29312	100	17	17/20

Table 3
False positive rate of Aspyre Lung Blood. Analysis was conducted over all samples with known-negative variants used across all experiments performed using standard input levels during analytical validation.

Level	Type	FP (n)	Total (n)	FP rate % (CI95)
Sample	DNA	0	384	0 (0–0.96)
	RNA	1	383	0.26 (0.01–1.45)
	Paired DNA/RNA	1	383.5	0.26 (0.01–1.44)
Variant	DNA	0	25,041	0 (0–0.01)
	RNA	1	1616	0.06 (0–0.44)
	Paired DNA/RNA	1	26,657	0.004 (0–0.02)

diagnosed with NSCLC. Inclusion criteria for the patient samples were sufficient material to perform Aspyre Lung Blood at standard input levels, orthogonal genomic test results, and VAFs at levels above the LoD95 of Aspyre Lung Blood. 32 cfDNA and cfRNA derived from 44 different patients were run (20 paired samples, 12 cfDNA without matched cfRNA, and 12 cfRNA without matched cfDNA). Contrived samples were tested at twice the LoD95 (Supplementary Table 2), and results of the clinical samples were compared to those expected from the orthogonal assay (Fig. 2 and Supplementary Table 3).

All contrived samples matched expected results, including four SNVs, three indels, six gene fusions, *MET* exon 14 skipping, and the three double variant samples (Table 4). There were ten patient-derived cfDNA samples with positive variant calls from orthogonal testing methods, and all yielded matched calls from Aspyre Lung Blood. There was a single patient sample that was positive by the orthogonal testing method for a *MET* exon 14 skipping mutation in the cfDNA; matched cfDNA and cfRNA from this patient were tested but the cfRNA did not yield a positive result by Aspyre Lung Blood. Unfortunately, there were no patients with cfRNA samples available from this prospective collection that were positive for gene fusions therefore this section of the accuracy assessment is entirely based on contrived samples.

2.5. Aspyre Lung Blood has high analytical precision

The analytical precision of Aspyre Lung Blood was measured using a combination of healthy volunteer and contrived samples. Ideally, three positive clinical samples from patients with NSCLC would be tested three times each, but blood draws from these patients generally do not yield sufficient material to make this possible, hence contrived samples were used. Runs were performed using two operators, two reagent lots, and two real-time PCR instruments. Reproducibility (inter-run precision) was assessed by comparing results from the four separate runs, and repeatability (intra-run precision) assessed by comparing results from three technical replicates within each run. All results were identical and matched the expected (Supplementary Table 4) yielding per-sample and per-variant PPA and NPA of 100 % (Table 5).

Table 4
Summary of analytical accuracy of Aspyre Lung Blood assessed using contrived and clinical samples. The PPA and NPA obtained across samples and variants for both clinical and contrived sample types and the associated 95 % confidence intervals are shown. PPA - positive percent agreement, NPA - negative percent agreement, NA - not applicable.

		Actual % (CI95)			
Level	Metric	Paired DNA & RNA	DNA only	RNA only	DNA Double Mutant
Sample	n	39	39	39	3
	PPA	94 (73–99.9)	100 (80–100)	88 (47–99.7)	100 (29–100)
	NPA	100 (91–100)	100 (91–100)	100 (91–100)	NA
Variant	n	1521	2574	195	198
	PPA	96 (80–99.9)	100 (80–100)	88 (47–99.7)	100 (54–100)
	NPA	100 (99.9–100)	100 (99.9–100)	100 (98–100)	100 (98–100)

2.6. Aspyre Lung Blood is not affected by common interfering substances present in nucleic acid extracts

Extraction procedures are generally optimized to remove substances

Table 5
Summary of analytical precision (repeatability and reproducibility) data. Shown are the positive and negative percent agreement values between runs of Aspyre Lung Blood, demonstrating 100 % reproducibility (inter-run precision) and repeatability (intra-run precision), with 95 % confidence intervals. Samples were assayed over four independent runs across four days by two operators using two real-time PCR instruments and two reagent lots. An expanded dataset from which this table is derived is shown in Supplementary Table 4.

Level	Metric	Actual % (CI95, Clopper-Pearson)
Sample	PPA	100 (90–100)
	NPA	100 (94–100)
Variant	PPA	100 (95–100)
	NPA	100 (99.91–100)

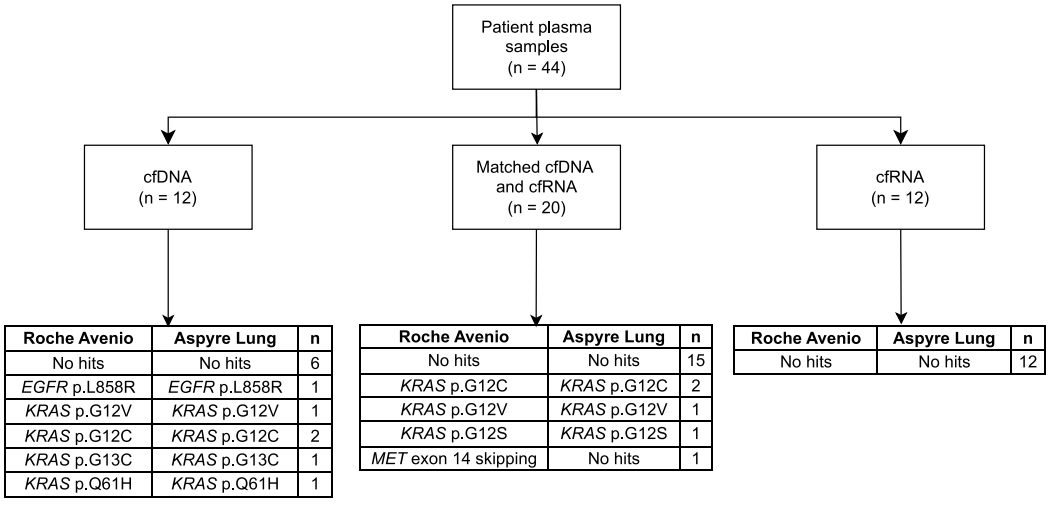

Fig. 2. Clinical samples used in the analytical accuracy validation. Shown are the results from the comparator assay (Roche Avenio ctDNA targeted kit V2) and Aspyre Lung Blood.

that might interfere with downstream procedures, however, the potential for carryover remains, particularly for aqueous soluble analytes. To test for the effects of this, contrived samples were assayed in five replicates after being spiked with two blood-derived substances previously shown to inhibit PCR-based reactions: hemoglobin or immunoglobulin G (Table 6). Water was used as a control. The levels of 'contaminants' used were based on literature values and are expected to be far in excess of actual levels achieved under standard conditions [9–11].

2.7. Aspyre Lung Blood can be run at low sample input levels (5 ng cfDNA and 6 ng cfRNA)

The circulating tumor fraction of blood varies widely between samples and is dependent on both the individual patient and their clinical characteristics [15]. To assess the sensitivity and specificity of Aspyre Lung Blood at low input levels, the LoD95 was estimated for 5 ng cfDNA (Supplementary Table 5) and confirmed for 5 ng cfDNA and 6 ng cfRNA (Supplementary Table 6). A comparison of the sensitivities of the assay at standard and low input sample levels is shown in Table 7. For targets detected from cfDNA, the confirmed LoD95 is generally higher than that for standard input as stochastic sampling of 5 ng cfDNA is less likely to contain as many amplifiable molecules of the target as 20 ng. As the absolute number of target copies in the cfRNA samples remains constant, the sensitivity (reported in absolute copies) is not affected by reduced input levels. Overall, the confirmed median LoD95 for SNV & indel was ≤0.8 % VAF, for gene fusions was ≤6 copies, and *MET* exon 14 skipping variant ≤100 copies.

The specificity at low input levels was also assessed from 35 healthy donor samples (Supplementary Table 7) and was also calculated from all samples with known negative variants across the LoD portion of the low input study (Table 8). There were no false-positive results seen in any variant-free sample at low input levels, supporting the use of down to 5 ng cfDNA and 6 ng cfRNA sample when standard input levels are unavailable.

3. Discussion

Patients who are newly diagnosed with NSCLC require rapid genomic profiling to assess eligibility for targeted therapy, which is both more cost-effective and has improved outcomes compared to non-targeted chemotherapy [12,13]. These studies demonstrate the sensitivity, specificity, accuracy and precision of Aspyre Lung Blood, a simplified genomic profiling assay performed at Biofidelity's CAP/CLIA laboratory in North Carolina, with a two-day turnaround time (96.2 % of blood samples reported out in under two days from sample receipt). The assay has parallel workflows for DNA and RNA and yields information on 114 somatic mutations across 11 genes, all of which have implications for choice of therapy or prognosis.

Liquid biopsies have emerged recently as a potential source of information about the tumor that is sampling site agnostic and therefore less affected by tumor heterogeneity [14]; however, highly sensitive assays are required to detect variants in cfNA [4]. The median LoD95 of Aspyre Lung Blood run at standard input levels for detection of SNV and indels is ≤0.3 % VAF; for gene fusions ≤6 copies, and for *MET* exon 14 skipping events, ≤100 copies. Levels of cfNAs vary in patients depending on several different factors [15], but the lack of a capture step in the Aspyre workflow is an advantage as the PCR amplification proceeds directly from extracted nucleic acid with no loss of material due to capture efficiencies that are less than 100 % [16,17]. At low input levels,

Table 7
Comparison of confirmed LoD95 of Aspyre Lung Blood run using standard input levels (20 ng cfDNA and 42 ng cfRNA) or low input levels (5 ng cfDNA and 6 ng cfRNA). LoD95 was estimated and confirmed using the same process described for standard input levels and is shown as VAF (%) or copy number (c) for cfDNA and cfRNA respectively. Estimation and confirmation run results are shown in Supplementary Tables 5 and 6 respectively. Std – standard. *< 85 % hit rate, but as a class the RNA fusions still passed the confirmation threshold with 116/120 positive calls.

Contrived Variant	Expected call	Std input LoD95	Low input LoD95
DNA		**20 ng**	**5 ng**
COSM516	*KRAS* exon 2 p.G12C	0.2 %	0.4 %
COSM6224	*EGFR* exon 21 p.L858R	0.3 %	0.8 %
COSM6240	*EGFR* exon 20 p.T790M	0.4 %	1.6 %
COSM476	*BRAF* exon 15 p.V600E	0.2 %	0.4 %
COSM6223	*EGFR* exon 19 p.E746_A750del	0.2 %	0.4 %
COSM20959	*ERBB2* exon 20 p.Y772_A775dup	0.8 %	1.2 %
COSM12376	*EGFR* exon 20 p.A767_V769dup	0.4 %	0.8 %
RNA Fusions		**42 ng**	**6 ng**
COSF408 (*EML4-ALK* E13-A20)	*ALK* Fusion	6c	6c
COSF1200 (*CD74-ROS1* C6-R34)	*ROS1* Fusion	6c	6c
COSF1329 (*TPM3-NTRK1* T8-N10)	*NTRK* Fusion	6c	6c
COSF1446 (*QKI-NTRK2* Q6-N16)	*NTRK* Fusion	6c	6c*
COSF571 (*ETV6-NTRK3* E5-N15)	*NTRK* Fusion	6c	6c
COSF1232 (*KIF5B-RET* K15-R12)	*RET* Fusion	18c	18c
RNA Exon Skipping		**42 ng**	**6 ng**
COSM29312 (p.L982_D1028del)	*MET* exon 14 skipping	100c	100c

Table 8
False positive rate of Aspyre Lung Blood performed at low input levels. Analysis was conducted over all variant-free samples used at low-input levels performed during LoD or LoB assessments of the analytical validation.

Level	Type	FP (n)	Total (n)	FP rate % (CI95)
Sample	DNA	0	404	0 (0–0.91)
	RNA	0	265	0 (0–1.4)
	Paired DNA/RNA	0	334.5	0 (0–1.1)
Variant	DNA	0	26,319	0 (0–0.014)
	RNA	0	1119	0 (0–0.33)
	Paired DNA/RNA	0	27,438	0 (0.013)

Table 6
Contaminants carried over from blood plasma extraction do not interfere with the Aspyre Lung Blood assay. Two DNA and two RNA contrived samples at twice the LoD95 were spiked with hemoglobin or immunoglobulin G to mimic carryover from blood-derived substances that are not removed during sample extraction. Shown are the positive calls made compared to total runs. NA, not applicable. *One control replicate yielded a false-positive for a *RET* fusion combined with a false negative for *ROS1* fusion and was repeated on suspicion of contamination.

						Total positive/total replicates		
Analyte	Gene	Exon	Protein Variant	COSMIC ID	VAF/Copies	control	1 μg/μL hemoglobin	150 pg/μL IgG
DNA	*KRAS*	2	G12C	COSM516	0.4 %	5/5	5/5	5/5
DNA	*EGFR*	19	E746_A750del	COSM6223	0.4 %	5/5	5/5	5/5
RNA	*EML4-ALK*	E13_A20	NA	COSF408	12 copies	5/5	5/5	5/5
RNA	*CD74-ROS1*	C6_R34	NA	COSF1200	12 copies	5/6*	4/5	5/5

confirmed sensitivities for each variant were around two-fold times those at standard input levels despite the level of target cfDNA decreasing by four-fold, with an assay-wide SNV/indel at 0.8 % VAF, and detection of variants from cfRNA remaining unchanged. This reflects the stochastic probability of sampling target molecules present in 20 ng cfDNA compared to 5 ng.

The sensitivity of Aspyre Lung Blood at standard input levels is on a par with established liquid biopsy assays such as the Guardant360 or FoundationOne Liquid, though these have slightly higher input levels of cfDNA required at ≥30 ng vs 20 ng for Aspyre Lung. At low input (5 ng cfDNA), however, Aspyre Lung Blood is more sensitive than the Guardant360 at 0.8 % VAF compared to 1.8 % for SNV, and 0.8 % VAF compared to 2.65 % for indel respectively [18]. FoundationOne Liquid CDx does not list outcomes for cfDNA input under 30 ng. All molecular tests have strengths and weaknesses, which allow different types and sizes of cancer care facilities to pick and choose those testing workflows that best align to patient care pathways. The rapid turnaround time and focus on recommended first-line actionable variants of Aspyre Lung position it well as a first-line diagnostic tool with the option for further extensive and comprehensive testing in the event of a negative result. Assays with longer turnaround times such as the Guardant360 and FoundationOne Liquid include variants with approved therapeutics that are for indications other than NSCLC (e.g. *FGFR*, *NRAS*) which could be prescribed off-label, and include assessment of copy number variation. However, both assays lack cfRNA components which can be critical for determination of whether gene fusions that are inferred from rearrangements in cfDNA are actually pathogenic or not [19].

The assessment of assay accuracy was divided into two parts, testing contrived samples and clinical NSCLC patient samples for SNVs from cfDNA. Contrived samples alone were used for detection of indels from cfDNA and gene fusions from cfRNA, as plasma samples positive for indels and gene fusions confirmed by orthogonal testing were unavailable from our prospective sample collection or from biobanks. This is a significant limitation of the current study. All results from contrived samples matched the expected results for all variant classes covered by Aspyre Lung Blood, and for samples containing two variants. Accuracy testing using samples from patients previously diagnosed with NSCLC yielded 100 % concordance for the ten SNV-positive samples, compared to orthogonal testing methods. A single patient sample that was positive for *MET* exon 14 skipping was available, however, this particular variant is detected by Aspyre Lung Blood through cfRNA and through the orthogonal test by inference of the effects of a mutation in cfDNA. This patient sample had an associated cfDNA VAF of 2.74 % (but no equivalent copy number for cfRNA); testing of the matched FFPE-derived RNA sample yielded a positive result by Aspyre Clinical Test for Lung (Tissue) in early 2023. Tissue and plasma samples were extracted in parallel experiments in December 2021 and the orthogonal test run in early 2022, therefore the negative result from the cfRNA could be due to degradation of extracted cfRNA material in the interim between the extraction (December 2021) and various assay runs performed for this study (August 2024). The negative result could also be due to low transcript expression from the variant copies of the *MET* genes, which would affect detection by Aspyre Lung Blood but not the orthogonal assay.

Five samples were used to test the precision of the assay; each of the three positive samples contained one variant detected from cfDNA, and one detected from cfRNA. All replicates within and across runs for each sample produced results that matched expected calls, with 100 % PPA and 100 % NPA. Similarly, spiking positive and negative samples with hemoglobin and immunoglobulin G which could potentially be carried over from blood through the nucleic acid extraction process did not show any results that would indicate interference with Aspyre technology. Our previous study looked at contamination from chemicals used in the actual extraction process that are found in many molecular biology kits (guanidine thiocyanate and ethanol) and did not find any negative impact [5], supporting the conclusion that the assay chemistry is robust. Future plans include completion of testing of more clinical samples including further clinical validation studies to pursue FDA approval of reagent kit products for Aspyre Lung Blood and Aspyre Lung Tissue.

4. Conclusions

Many testing options for somatic variants are available, yet over 60 % of patients with advanced NSCLC do not benefit from precision oncology [3]. The reasons for this are multifactorial but largely stem from the limitations of existing methodologies. Aspyre technology leverages the benefits of a simple, PCR-based workflow with the high accuracy borne of the unique usage of pyrophosphorolysis. Combining this with high-order multiplexing of real-time isothermal amplification allows for a test that covers all genes recommended for first-line testing [6] yet has no more complexity in the workflow than a qPCR-based assay, while also yielding the high sensitivity required for a blood-based precision oncology test. In this study, we demonstrated that Aspyre Lung Blood has high sensitivity at either standard or low input levels of sample and is highly specific. The assay is robust to different batches of reagents, different operators, and different real-time PCR instruments, without being affected by potential carry-over of endogenous blood-derived products or reagents used during extraction. While analysis of tumor tissue biopsies remains the gold standard in most care pathways, there are patients who are too frail, unwell, or from whom it is just not possible to obtain sufficient material for routine assays. For these patients, having access to a cost-effective and rapid screening assay that can analyze plasma before being considered for neoadjuvant immuno-chemotherapy enhances their options.

5. Methods

5.1. Contrived samples

DNA and RNA variants were selected to represent the most common variants across SNV and indels from the following exons: *BRAF* exon 15; *EGFR* exons 18, 19, 20, 21; *KRAS* exons 2, 3; and *ERBB2* exons 17, 20; and the most common *ALK, RET, ROS1, NTRK1/2/3* fusions alongside *MET* exon 14 skipping. Contrived control oligonucleotides made from DNA (SNV, indel) or RNA (fusions, *MET* exon 14 skipping) were manufactured (DNA from Eurofins, Wolverhampton, UK, RNA from IDT, Leuven, Belgium), and quantified by digital PCR (QIAcuity Digital PCR system, Qiagen) at Biofidelity Ltd (Cambridge, UK). To create the background of contrived samples, gDNA or RNA extracted from FFPE variant-free tonsil tissue was quantified by dPCR (DNA) and Qubit (RNA). To create variant-positive contrived samples, control oligonucleotides were spiked in and serially diluted to the appropriate concentrations, and immediately frozen at −20 °C (DNA) or −80 °C (RNA).

5.2. Clinical samples

Samples from volunteers without any known cancer diagnosis were procured from a commercial biobank (BioCollections, USA). Blood samples from patients with a confirmed NSCLC diagnosis were obtained from commercial biobanks (Geneticist, Tissue Solutions, Reprocell, BocaBio, Cureline, VitroVivo, iSpecimen). All blood samples were collected into Paxgene ccfDNA blood tubes (PreAnalytiX, Hombrechtikon, Switzerland).

5.3. Ethical approval

Institutional Review Board (IRB) or equivalent approval was obtained for the use of samples in diagnostics development by biobanks through participating collection sites. All patients provided written informed consent, and data were de-identified so no patients or volunteers could be identified by study personnel outside of the clinical trial

site including the biobanks and the study authors.

5.4. Nucleic acid extraction

Whole blood samples were collected in Paxgene Blood ccfDNA tubes and processed to plasma by double centrifugation within 3 days of collection according to the manufacturer's instructions. Plasma samples were stored at −80 °C. cfDNA and cfRNA were extracted using the cfDNA/cfRNA Serum and Plasma kit (Zymo Research, US), concentrations were quantified using a Qubit™ 1x dsDNA or RNA high sensitivity kit (ThermoFisher) and stored at −80 °C until further usage.

5.5. Aspyre reaction

The Aspyre reaction has been described for clinical samples [5] and was performed as carried out previously except with standard input levels of 20 ng cfDNA per PCR reaction and 42 ng cfRNA per RT-PCR reaction. Low input experiments were conducted using 5 ng cfDNA and 6 ng cfRNA per reaction.

5.6. Orthogonal testing of clinical samples

Extracted cfDNA from blood samples was analyzed using targeted enrichment (Roche Avenio ctDNA Targeted Kit V2) and sequencing (NextSeq 500 or NextSeq 2000, Illumina) by Glasgow Polyomics (University of Glasgow, UK), according to the manufacturer's guidelines. Analysis was performed by the Roche Sequencing Solutions team (Mannheim, Germany).

5.7. Interfering substances

Four contrived reference samples of *KRAS* p. G12C (COSM516) at 0.4 % VAF, *EGFR* exon 19 p. E746_A750del (COSM6223) at 0.4 % VAF, *EML4-ALK* (COSF408) and *CD74-ROS1* (COSF1200) at 12 copies prepared in cfDNA- or cfRNA-like background were spiked with hemoglobin (MilliporeSigma) or immunoglobulin G (MilliporeSigma) at concentrations mimicking potential carryover during the extraction process (hemoglobin at 1 μg/μL, immunoglobulin G at 150 pg/μL).

5.8. Data analysis

Data were downloaded from QuantStudio 5 RealTime PCR System (Thermofisher) instruments running Design and Analysis 2 software (v2.6.0). The Raw Data CSV produced by this software was analyzed using custom Aspyre Lab software v1.3.1 (standard input studies) or 1.4.0 (low input studies). This cloud-based web application takes the Raw Data CSV as input and performs corrections, normalizations, fitting, controls, checks, and provides variant calls as outputs. Variant calls are made using a support vector machine learning algorithm trained on several thousand samples, and take the form of detected or not detected, unless controls and checks fail in which case variants are undetermined. All variant calling was blinded to results from orthogonal analyses. The Aspyre Lab results were then further collated and analyzed using Google Sheets and custom Python analysis scripts.

Data availability

A dataset supporting the conclusions of this article is available in the NCBI SRA repository, https://www.ncbi.nlm.nih.gov/sra/PRJNA1235103.

Author contributions

Methodology: RTE, ASG, BWB, SB.

Conducted experiments: RTE, KEK, EGZ, JNB, CK, MBR, CK, ASW, KvB, ERG, JMM, RNP, CX, MSJ, SAb, JAS.

Generation of reagents: JMM, AC, SAn, IT, CHH, DN, JJ, PB, ASW, Formal analysis: RNP, AT, SAb, RTE, KP, BWB, SB.

Study Supervision: WJL, BWB, KP, SB.

Writing - Original Draft Preparation: ERG.

Writing - Review & Editing: RTE, ERG, JPG, WJL, BWB.

Ethical approval

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

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Declaration of competing interest

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Appendix A. Supplementary data

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