Original Article

Evaluation of the ASPYRE-Lung targeted variant panel: a rapid, low-input solution for non-small cell lung cancer biomarker testing and experience from three independent sites

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Background: Many patients with non-small cell lung cancer (NSCLC) lack access to highly effective approved targeted therapeutics due to multiple gaps in biomarker testing. Challenges in comprehensive molecular testing include complexities associated with the need to assess the presence of multiple variants, costs of running multiple sequential assays per sample, high assay quality control (QC) failure rates, clinical need for rapid turn-around time (TAT) to initiate therapy, and insufficient tissue samples. The ASPYRE-Lung NSCLC assay addresses gaps in multiplexed testing by simultaneously analyzing DNA and RNA, detecting 114 actionable genomic variants across 11 genes, consistent with current NSCLC treatment guidelines. This study was to assess the ease of adoption and performance of ASPYRE-Lung in third-party laboratories, comparing concordance across sites and with orthogonal methods.

Methods: ASPYRE-Lung was established at two academic centers with multiple operators per site. Assay concordance was evaluated across three sites using 77 patient samples [61 derived from formalin-fixed paraffin-embedded (FFPE) tissue and 16 from cytology specimens].

Results: Reproducibility for all 77 samples yielded a positive percent agreement (PPA) of 100% and negative percent agreement (NPA) of 99.99%. Concordance with next-generation sequencing (NGS)-based methods across all three sites was high with PPA of 97.2% and NPA of 99.96%.

Conclusions: ASPYRE-Lung assay is a cost-effective, easy to adopt testing method requiring no specialized expertise or complicated bioinformatics, with the potential to inform genomic data on small tissue samples, thus enabling all patients with NSCLC to undergo biomarker testing in a timely manner and

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benefit from appropriate targeted therapies.

Keywords: Non-small cell lung cancer (NSCLC); targeted therapy; biomarker; molecular

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Introduction

Lung cancer has a poor prognosis when patients are treated with chemotherapy alone (1). Advanced non-small cell lung cancer (NSCLC) treatment guidelines recommend testing patients for actionable genomic variants to select from 30 highly effective Food and Drug Administration-approved targeted therapies (2), which can prolong survival in biomarker-positive patients. Unfortunately, only ~50% of patients eligible for biomarker testing are successfully tested (3). Multiple gaps in single gene or multiplexed next-generation sequencing (NGS) genomic testing options collectively hinder patients from receiving targeted therapies including significant failure rates of samples at several quality control (QC) stages during library preparation and sequencing, high cost of testing, slow turn-around time (TAT), the need to interrogate both DNA and RNA to obtain complete actionable data sets, and insufficient tissue quantity [quantity not sufficient (QNS)]. US data showed 90% of 9,425 advanced NSCLC patients who had a documented tissue biopsy had core needle biopsies (9.9%) or fine needle aspirate (FNA; 79.8%) to obtain tissue for analysis at diagnosis (3), making tests that can utilize these smaller sample types crucial to avoid QNS outcomes. Overall assay failure rates can be high, reaching 25% of samples prepared for some high-throughput sequencing tests (3,4), causing treatment delays with 6–8% increased risk of death for every 4-week delay (5). The ASPYRE-Lung tissue assay was developed to meet the need for an affordable, simple, rapid, and easy to implement test that is designed to detect a range of clinically actionable variants associated with NSCLC included in the National Comprehensive Cancer Network (NCCN) guidelines [variants listed in (6)]. The assay identifies single nucleotide variants (SNVs) and insertions/deletions (indels) using DNA analysis for key oncogenes, including *EGFR*, *BRAF*, *KRAS*, and *ERBB2*. Additionally, the assay detects gene fusions involving *ALK*, *RET*, *ROS1*, and *NTRK1*, *2*, and *3*, as well as *MET* exon 14 skipping mutations, all through RNA analysis. The use of both DNA and RNA allows for comprehensive variant detection across different mutation types, ensuring that clinically significant alterations are identified. Each variant detected by the assay is pathogenic, as it is either targetable by existing therapies or provides prognostic information. This design alleviates the need for manual review of alterations that lack clinical relevance, streamlining the interpretation process for clinical laboratories. Both DNA and RNA are analyzed simultaneously in a simple four-step workflow using 20 ng DNA and 6 ng RNA input for one to sixteen patient samples simultaneously. Assay steps involve simple liquid transfer between strip tubes and plates with pre-aliquoted reagents. The equipment required is readily available in

Highlight box

Key findings

- The ASPYRE-Lung assay was established at two independent sites and tested using 61 formalin-fixed paraffin-embedded and 16 cytology patient samples. The reproducibility across all samples was high, and concordance with next-generation sequencing (NGS)-based methods was high. The assay was easy to establish using standard laboratory equipment, including a thermocycler and real-time polymerase chain reaction thermocycler.

What is known and what is new?

- Only ~50% of eligible patients with non-small cell lung cancer (NSCLC) successfully undergo biomarker testing, with gaps in provision and process at multiple stages including the high tissue requirement for sequential single-marker assays or highly complex and costly NGS panels, failures at quality control stages, the high cost of testing and long turn-around time, and failure to analyze both DNA and RNA.
- This study demonstrates the real-world experience of operators in two academic laboratories in establishing the ASPYRE-Lung reagents in-house and using it to characterize variants in 77 patient samples. Reproducibility across sites and concordance with orthogonal testing was high, and the assay was easy to adopt and run.

What is the implication, and what should change now?

- ASPYRE-Lung facilitates rapid analysis of a comprehensive set of biomarkers in patients with NSCLC and will enable more laboratories to perform precision genomic analysis.

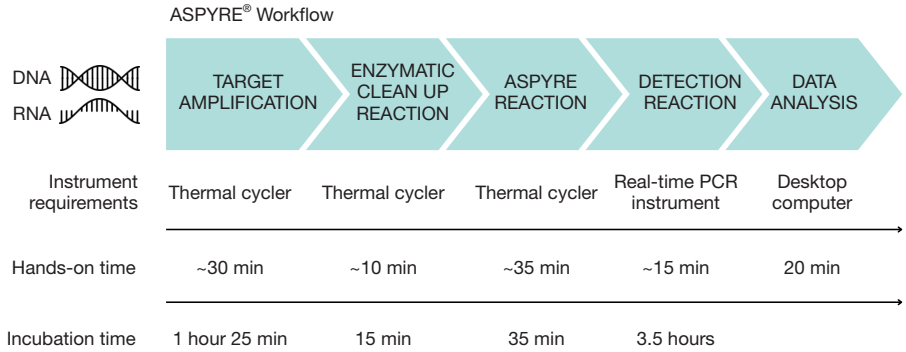

Figure 1 Schematic of ASPYRE-Lung workflow, instrument and time requirements. Analysis of the real-time PCR data takes place using a cloud-based turnkey analysis platform, and results show detection of specific variants (e.g., *EGFR* exon 19 deletion), with no need for further bioinformatic interpretation. PCR, polymerase chain reaction.

most clinical laboratories, and comprises a polymerase chain reaction (PCR) thermocycler for three preparatory steps, and a quantitative real-time PCR (qPCR) thermocycler for the detection step (*Figure 1*).

In this study, we describe the establishment of the ASPYRE-Lung assay in two College of American Pathologists (CAP)/Clinical Laboratory Improvement Amendments (CLIA) academic laboratories in the United States, and a comparison of results from samples run at these laboratories and Biofidelity Inc. (*Figure 2*). ASPYRE-Lung results were also compared to an orthogonal method. After minimal training, external laboratories achieved excellent levels of reproducibility, and all sites had a high level of concordance with orthogonal testing. Together, this demonstrates the easy adoption of ASPYRE-Lung and its utility to rapidly detect actionable variants of NSCLC in a variety of real-world samples from patients. We present this article in accordance with the MDAR reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-525/rc).

Methods

Clinical samples

Formalin-fixed paraffin-embedded (FFPE) lung tissue blocks from patients with a confirmed NSCLC diagnosis were obtained prospectively from commercial biobanks (Geneticist, Tissue Solutions, Reprocell, BocaBio, Cureline, VitroVivo). Each biobank worked with affiliated clinics to recruit eligible patients. Informed consents were obtained and all clinical data were de-identified by the clinics. Data collected included any prior treatment history (negative for all biobank patients). Selected archived samples were identified from the Hospital of the University of Pennsylvania laboratory. Pathology review was performed on all tissue samples to ensure a minimum of 10% tumor. Samples from commercial biobanks were chosen based on inclusion criteria of (I) sufficient yield of DNA and RNA for multiple ASPYRE-Lung assay runs, and (II) sufficient DNA available for targeted enrichment NGS. Samples were excluded if there was significant necrosis in the sample (over 40%). Samples provided by the Hospital of the University of Pennsylvania laboratory consisted of residual clinical material and comprised FFPE lung tissue curls, macrodissected FFPE lung tissue slides, peritoneal fluid, pleural effusion, pleural fluid, fresh tissue, FNA, and FNA rinses. Numbers of samples of each type are shown in *Figure 2*. In total, there were 54 samples collected prospectively, and 23 samples from retrospectively identified clinical samples.

Sample processing, nucleic acid extraction and storage

Biobank-derived samples: lung biopsy samples from patients with NSCLC were collected prospectively between August 2020 and August 2021, and FFPE tissue blocks were prepared by collection sites, before sending to the Biofidelity Ltd. site, Cambridge, UK, for extraction. Blocks were manually sectioned with a microtome (Shandon Finesse, ThermoFisher, Waltham, MA, USA), producing three 12 µM thick curls. DNA and RNA were extracted from specimens in parallel using the Quick-DNA/RNA™ FFPE miniprep kit (Zymo Research, Irvine, CA, USA).

Residual clinical samples from the Hospital of the University of Pennsylvania: samples were retrospectively

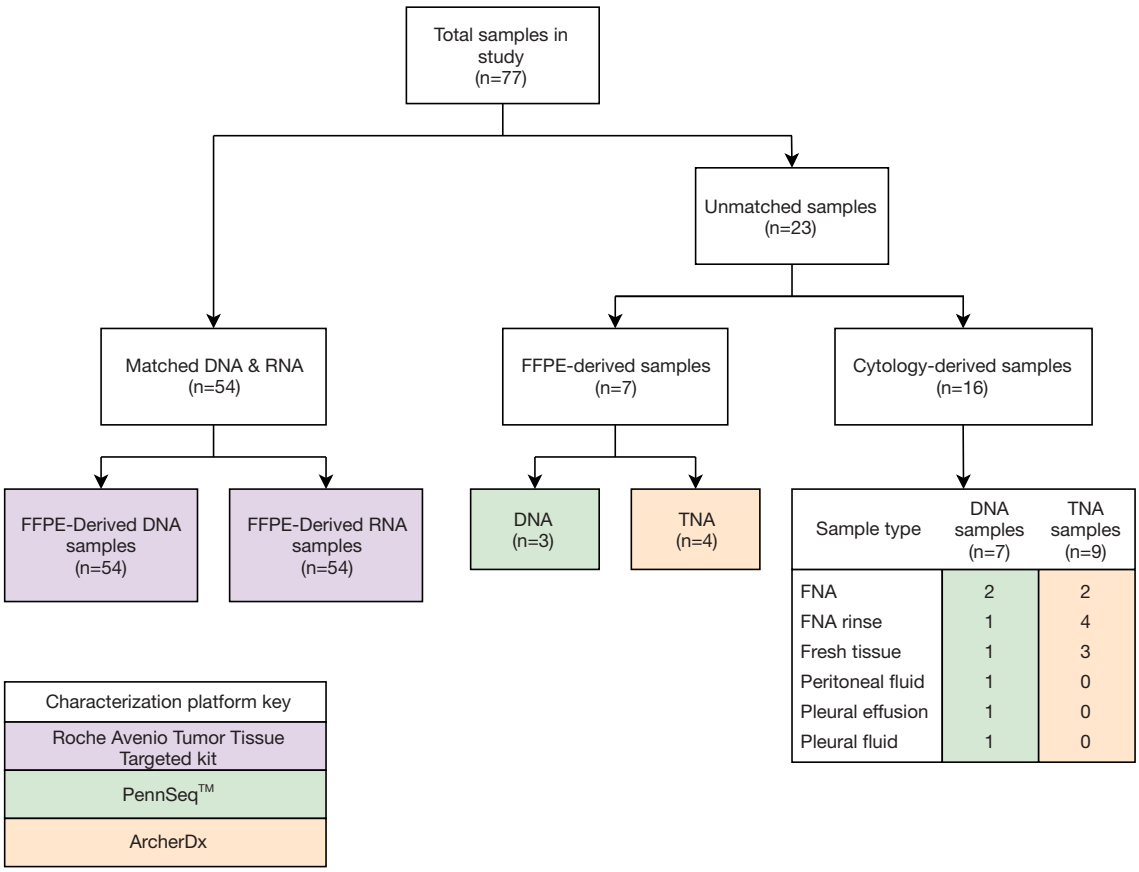

Figure 2 Samples analyzed in this study, source and orthogonal characterization method. A breakdown of sample type and the orthogonal test result is in Tables S1,S2. FFPE, formalin-fixed paraffin-embedded; TNA, total nucleic acid; FNA, fine needle aspirate.

identified from cases received between August 2017 and August 2022, sourced from patients with cancer. FFPE tissue blocks were manually sectioned with a microtome (HM235, Microm, Waltham, MA, USA) as either 10 µM curls (≥40% tumor) or 5 µM slides (<40% tumor). If slides were prepared, macrodissection was performed to enrich tumor content, prior to nucleic acid extraction. DNA and total nucleic acid (TNA) were extracted from FFPE specimens in parallel using the Agencourt FormaPure kit (Beckman, Brea, CA, USA). Fresh tissue and fluid samples were processed using the same kit but without deparaffinization and decrosslinking steps, and large fresh tissue specimens were incubated in lysis buffer overnight.

For all samples, nucleic acid concentration was determined with the Qubit™ DNA or RNA high-sensitivity kit (ThermoFisher). Extracted DNA was stored short-term (<1 week) at 4 ℃ with long-term storage at –20 ℃ and extracted RNA or TNA was stored at –80 ℃.

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

For biobank-derived samples, the study was approved by the following Ethics Committees of participating institutions: the Rajiv Gandhi Cancer Institute & Research Centre (IRB# ECR/10/Inst/DC/2013/RR-16; Protocol 672/RE/AMH-27), the National Military Medical Center "Main Military Clinical Hospital" (Protocol 200111), the FGBU National Medico-Surgical Center N.I. Pirogov (Protocol CB#1164/2016), the T.C. Ministry of Health Dr. Abdurrahman Yurtaslan Ankara Oncology Health Practice and Research Center Ethics Committee (Protocol 2019-08/368), and the Kharkiv National Medical University (Protocol GI 020114). Informed consent was obtained from all individual participants.

For samples retrospectively identified for this study, the

study was approved by the Institutional Review Board (IRB) of the University of Pennsylvania IRB #2 Protocol 854192 and individual consent for this retrospective analysis was waived.

ASPYRE-Lung assay runs and training

After the concentration of samples was measured, no further pre-analytical checks were made prior to running samples through ASPYRE-Lung. The ASPYRE reaction included DNA input of 20 ng (25 µL) per PCR reaction and RNA input of 6 ng (6 µL) per reverse transcriptase PCR (RT-PCR) reaction using ASPYRE-Lung reagents with the following PCR machines: ProFlex, SimpliAmp, and Veriti (ThermoFisher) or T100 (BioRad, Hercules, CA, USA), and a QuantStudio 5 qPCR System (ThermoFisher). The reactions were conducted as previously described (7,8). Briefly, sections of eleven genes from extracted DNA and RNA are amplified by PCR or RT-PCR respectively. Products from these reactions (and one control reaction each for DNA and RNA) undergo proteolytic digestion, hybridization, pyrophosphorolysis, and ligation before a final isothermal amplification stage with fluorescent readout. All steps from PCR/RT-PCR through to isothermal amplification are part of ASPYRE-Lung reagents. The readout is processed through a bioinformatics pipeline that includes internal cross-referencing quality checks, including DNA and RNA control reaction outputs, which must fall within expected parameters for assay validity. In this study, laboratory staff at two sites were trained in the assay workflow by a field assay scientist over 3 days, conducting one demonstration run and one supervised assay run per operator. Proficiency was confirmed with an unsupervised assay run. For all training runs a panel of contrived samples was used and results were compared to expected. Clinical samples described in this study were run unsupervised.

Data analysis

Variant calls for samples and controls were generated by processing the raw data file generated by the Design and Analysis 2 software (v2.6, Thermo Scientific, Waltham, MA, USA) through the ASPYRELab software v1.1.1. This software generates a report for each sample or batch of samples that includes fluorescent output curves and a JSON file.

Statistical analysis

Positive percent agreement (PPA) and negative percent agreement (NPA) 95% binomial confidence intervals were computed using the "exact" method [Clopper-Pearson interval (9)] in custom python scripts, using the SciPy package (10).

Sequencing

Biobank-derived samples: DNA extracted from FFPE lung tissue samples was sequenced through an orthogonal method by targeted enrichment (Roche Avenio Tumor Tissue Targeted Assay, Basel, Switzerland) and sequencing (NextSeq 500, Illumina, San Diego, CA, USA) at Glasgow Polyomics (University of Glasgow, Glasgow, UK), according to the manufacturer's guidelines. Analysis was performed by the Roche Sequencing Solutions team.

Samples from the Hospital of the University of Pennsylvania were evaluated on both the PennSeq™ assay and an Archer® Custom Fusion*Plex* Panel. PennSeq™ is a capture based NGS method sequenced on the NovaSeq (Illumina) and analyzed through an internal analysis pipeline. The Archer® Custom Fusion*Plex* Panel is an NGS assay consisting of a custom FusionPlex kit sequenced on the HiSeq (Illumina) followed by analysis of the fastq files through the Archer Virtual Machine, on the clinically validated in-use software version at the time of receipt.

Results

Deployment of ASPYRE-Lung at one internal and two external sites: training

In this study, laboratory staff at two external sites, Hospital of the University of Pennsylvania and the Precision Medicine Laboratory at the Medical College of Wisconsin (MCW), were initially trained in the assay workflow using contrived samples, and the results were checked to ensure they fell within expected parameters. Proficiency was confirmed using a second panel of contrived samples.

Deployment of ASPYRE-Lung at one internal and two external sites: clinical sample analysis

Once proficiency was achieved, clinical samples were analyzed. Various sample types, representative of clinical specimens typically received at each site, were evaluated: FFPE tissue [61], FNA [4], FNA rinse [5], peritoneal fluid [1], pleural effusion [1], pleural fluid [1] and fresh tissue [4].

After extraction, nucleic acid samples included 54 matched DNA and RNA samples derived from the same sample, and unmatched DNA [10] and TNA [13]. Aliquots from a single extraction procedure were created for parallel testing using ASPYRE-Lung across the three sites for all clinical specimens. Two operators at each external site and four Biofidelity staff in total conducted assays on samples, which were all tested once per site. DNA samples were run on ASPYRE-Lung DNA input wells, and RNA or TNA on ASPYRE-Lung RNA input wells. Data from all runs were analyzed by staff at Biofidelity blinded to sample IDs and expected variant results. The assay is designed for FFPE tissue-derived samples, therefore results from the two sample groups are considered separately as FFPE-derived and non-FFPE (cytology)-derived.

FFPE lung tissue samples

Sixty-one FFPE lung specimens were compared. Samples contained variants detectable by ASPYRE-Lung in DNA or RNA, as well as samples negative for all variants. Samples included those positive for SNVs, indels, fusions, and *MET* exon 14 skipping (Table S1). There were 62 expected variant calls (including negative calls, based on data from NGS) with one sample expected to be positive for two variants. Overall, 60 out of 61 DNA, RNA, and TNA samples derived from FFPE lung tissue gave reproducible results across all three sites (reproducibility is defined as obtaining the same results for replicate samples). The variants called at each site are shown in *Table 1* and compared to calls from targeted enrichment NGS.

Overall, four out of 61 samples gave potentially discordant results either between ASPYRE-Lung sites (1/61) or between ASPYRE-Lung and the orthogonal assays (3/61). In one sample, ASPYRE-Lung at all three sites identified a *ROS1* fusion in a sample that was not corroborated by targeted enrichment NGS. In a second, ASPYRE-Lung gave a discrepant result in all sites for a sample that was *MET* exon 14 skipping variant-positive by NGS. In the third, a sample yielded concordant calls across all sites and NGS for *EGFR* p.S768_D770dup, and an additional *EGFR* p.A767_769dup at only one of the ASPYRE-Lung sites. Finally, one sample was *ALK* fusion-positive by NGS, but negative at all sites by ASPYRE-Lung.

The *ROS1* fusion in one sample was found across all ASPYRE-Lung sites and was from a patient with stage IIB adenocarcinoma. In this sample, the *ROS1* fusion may be present below the limit of detection of the targeted enrichment NGS assay, which is 5% for fusions (11), and based on prediction of the fusion outcome from sequencing across intronic breakpoints. In contrast, the ASPYRE-Lung assay identifies fusions by an initial reverse transcription and then amplification of actual gene fusions that are present in RNA, with no predictive algorithmic interpretation of DNA variant sequences required. This sample was subsequently evaluated by a third assay (the Archer Custom Fusion*Plex* panel) and found to be positive for a *SLC34A2-ROS1* fusion, confirming the results from the ASPYRE-Lung assay.

An *EGFR* insertion p.A767_769dup (COSM12376 or COSM13558) was identified in one sample at a single site. This sample also had a p.S768_D770dup (COSM13428) variant identified at all three sites. The p.A767_769dup call for this sample likely represents a false positive caused by probe crosstalk. This is a known phenomenon whereby samples that are positive for one *EGFR* variant may yield a second positive call that is potentially a false-positive, due to overlapping variant sites for several insertions (12-14).

In one case, ASPYRE-Lung did not identify a *MET* exon 14 skipping event in a sample that was positive by targeted enrichment NGS. There are multiple somatic variants in DNA that can result in exon 14 skipping transcripts. The use of RNA as the target nucleic acid to detect this variant means that the outcome is detected by ASPYRE-Lung whether or not the variation in the DNA has been previously associated with exon 14 skipping. The ASPYRE-Lung assay threshold for calling *MET* exon 14 skipping events is set above intermediate signals that can arise from normal low-level exon 14 skipping events caused by alternative splicing [for example (15) and (16)], and the level of exon 14 skipping in RNA from this patient fell below this threshold.

The final discordant result compared to NGS was a sample reported as *ALK* fusion-positive by targeted enrichment NGS. On inspection of the sequencing results, this was an *EML4-ALK* fusion between exons 19 of *EML4* and only part of exon 20 of *ALK*, lacking the first 26 base pairs. *EML4* gene fusions to 5'-truncated *ALK* exon 20 have been reported in COSMIC (two out of 400 sequenced samples) but not this variant. The ASPYRE-Lung panel will detect most common variants of *EML4-ALK* fusions, however, due to the atypical and previously unreported breakpoint, this fusion is not included.

Non-FFPE (cytology) samples

Due to challenges in obtaining tissue from some patients,

Table 1 Comparison of variant profiling results of 61 FFPE samples from patients with NSCLC

Variant ID	Sample type	COSMIC ID	ASPYRE-Lung result			Targeted enrichment NGS result
			UPenn	MCW	Biofidelity	
EGFR p.E746_A750del	FFPE lung tissue, DNA	COSM6223	4	4	4	4
EGFR p.L747_S752del	FFPE lung tissue, DNA	COSM6255	1	1	1	1
EGFR p.L747_P753delinsS	FFPE lung tissue, DNA	COSM12370	1	1	1	1
EGFR p.A767_V769dup	FFPE lung tissue, DNA[†]	COSM12376	0	1	0	0
EGFR p.S768_D770dup	FFPE lung tissue, DNA	COSM13428	1	1	1	1
EGFR p.T790M	FFPE lung tissue, DNA	COSM6240	1	1	1	1
EGFR p.L858R	FFPE lung tissue, DNA	COSM6224	2	2	2	2
EGFR p.L861Q	FFPE lung tissue, DNA	COSM6213	1	1	1	1
KRAS p.G12C	FFPE lung tissue, DNA	COSM516	3	3	3	3
KRAS p.G12V	FFPE lung tissue, DNA	COSM520	1	1	1	1
KRAS p.G12A	FFPE lung tissue, DNA	COSM522	1	1	1	1
KRAS p.G13C	FFPE lung tissue, DNA	COSM527	1	1	1	1
KRAS p.Q61L	FFPE lung tissue, DNA	COSM553	1	1	1	1
BRAF p.V600E	FFPE lung tissue, DNA	COSM476	3	3	3	3
ALK fusion	FFPE lung tissue, RNA[†]/TNA	–	1 (RNA), 1 (TNA)	1 (RNA), 1 (TNA)	1 (RNA), 1 (TNA)	2 (RNA), 1 (TNA)
MET exon 14 skipping	FFPE lung tissue, RNA[†]/TNA	–	1 (RNA), 1 (TNA)	1 (RNA), 1 (TNA)	1 (RNA), 1 (TNA)	2 (RNA), 1 (TNA)
ROS1 fusion	FFPE lung tissue, RNA[†]	–	1	1	1	0
No variant detected in sample	FFPE lung tissue, DNA/RNA, DNA and TNA	–	36	36	36	35
Total positive calls	FFPE lung tissue	–	26	27	26	27

The ASPYRE-Lung assay was run independently at three sites: Biofidelity's in-house laboratory, MCW, and Hospital of the University of Pennsylvania. [†], variable results between ASPYRE-Lung runs at different sites or discordant results compared to NGS. One sample yielded two concordant variant calls, and one sample yielded one concordant and one discordant variant call, thus, total variant call number is greater than the sample number. FFPE, formalin-fixed paraffin-embedded; NSCLC, non-small cell lung cancer; UPenn, Hospital of the University of Pennsylvania; MCW, Medical College of Wisconsin; NGS, next-generation sequencing; TNA, total nucleic acid.

alternative specimen types are often collected for molecular analyses. A set of cytology samples for which NGS data were available were assessed on ASPYRE-Lung. Sample types included peritoneal fluid, pleural effusion, FNA, FNA rinses, and fresh tissue (including non-lung samples) and comprised DNA and TNA, with a single nucleic acid type from each sample analyzed (Table S2). Although these sample types and the use of TNA are formally out of validation scope for the ASPYRE-Lung FFPE tissue assay, it was of interest to see how these accessible sample types and the TNA performed. DNA and TNA sample extracts were run at the three sites as unmatched pairs, with TNA in place of RNA in the corresponding ASPYRE-Lung RNA wells (*Table 2*).

This data set represents common tissue sample preparation types that are routinely taken during the patient's diagnostic pathway when complete resections are not clinically indicated; samples were also selected based on availability of NGS data. All biomarkers that were identified by NGS were also identified by ASPYRE-Lung with 100% PPA. In this sample set, there were no biomarkers identified by ASPYRE-Lung that were not identified by NGS.

Table 2 Comparison of variant profiling results of 16 unmatched cytology samples (7 DNA and 9 TNA)

Variant ID	Sample type	COSMIC ID	ASPYRE-Lung result			Targeted enrichment NGS result
			UPenn	MCW	Biofidelity	
EGFR p.E746_S752delinsV	Peritoneal fluid, DNA	COSM12384	1	1	1	1
EGFR p.H773dup	FNA, DNA†	COSM12377	0	1	0	0
EGFR p.T790M	Pleural effusion, DNA	COSM6240	1	1	1	1
EGFR p.L858R	Pleural fluid, DNA	COSM6224	1	1	1	1
EGFR p.L858R	Pleural effusion, DNA	COSM6224	1	1	1	1
ERBB2 p.G776delinsVC	FNA rinse, DNA	COSM12553	1	1	1	1
ROS1 fusion	FNA rinse, TNA	–	2	2	2	2
RET fusion	FNA rinse, TNA	–	2	2	2	2
MET exon 14 skipping	Fresh tissue, TNA	–	1	1	1	1
No variant detected in sample	FNA, DNA†, TNA	–	2 (DNA), 2 (TNA)	1 (DNA), 2 (TNA)	2 (DNA), 2 (TNA)	2 (DNA), 2 (TNA)
No variant detected in sample	Fresh tissue, DNA, TNA	–	1 (DNA), 2 (TNA)	1 (DNA), 2 (TNA)	1 (DNA), 2 (TNA)	1 (DNA), 2 (TNA)
Total positive calls	–	–	10	11	10	10

†, samples that gave discrepant results between ASPYRE-Lung sites or compared to NGS. One sample yielded two confirmed variant calls, thus, total calls are greater than the number of samples. TNA, total nucleic acid; UPenn, Hospital of the University of Pennsylvania; MCW, Medical College of Wisconsin; NGS, next-generation sequencing; FNA, fine needle aspirate.

Notably, a single pleural effusion sample was positive for two variants: *EGFR* p.T790M (COSM6240) and p.L858R (COSM6224) at all three sites and by NGS.

ASPYRE-Lung has been developed for tissue samples prepared as FFPE blocks. Cellular material in pleural effusion and peritoneal fluid samples may not be as affected by chemical damage as material from FFPE tissue but can sometimes be limited in quantity. ASPYRE-Lung requires just 20 ng DNA and 6 ng RNA, which can be the difference between being able to run a diagnostic assay and obtain results, having to subject a patient to a second biopsy, or embark on standard of care without assessing biomarkers. ASPYRE-Lung is designed for FFPE and not cytology samples, and for RNA not TNA. Nevertheless, the successful runs are an indication that the RNA-dependent part of the assay is not inhibited by the presence of co-purified DNA.

There were four FNA rinse samples with gene fusions that were analyzed in this sample set: two *RET* and two *ROS1* fusions. Gene fusions constitute important driver variants of NSCLC as specific therapies exist to target each class with compelling response rates. FNA rinse samples can be an important source of diagnostic material when opportunities for curative surgery are otherwise limited, and these results show that ASPYRE-Lung can be successfully used to detect gene fusions with these limited tissue types (17).

One FNA sample yielded negative calls for all variants at two sites and by targeted enrichment NGS, and at the third site yielded an *EGFR* exon 20 p.H773dup variant (COSM12377) call. As this variant was not found by any other site or by the orthogonal method, it remains unconfirmed, and could be below the limit of detection of both ASPYRE-Lung and the orthogonal assay, giving stochastic results.

Discussion

For cancer patients to benefit from highly active targeted therapeutics, access to cost-effective, timely, and comprehensive testing for actionable variants is crucial. Single-gene PCR tissue testing requires sequential assays to identify all actionable variants with the potential for sample exhaustion (18). Current comprehensive genomic

Table 3 Summary comparison of variant profiling results between sites

Sample group	ASPYRE-Lung reproducibility (inter-run precision) between three sites	ASPYRE-Lung reproducibility and concordance with NGS
FFPE lung tissue samples, % (95% CI)		
PPA	100.0 (95.4–100.0)	96.2 (89.2–99.2)
NPA	99.99 (99.95–100.0)	99.97 (99.92–99.99)
Non-FFPE samples, % (95% CI)		
PPA	100.0 (88.4–100.0)	100.0 (88.4–100.0)
NPA	99.93 (99.63–100.0)	99.93 (99.63–100.0)

Data are presented as % (95% confidence interval). The denominator result was taken from the ASPYRE-Lung assay variants that were also captured by the NGS assay. FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing; PPA, positive percent agreement; NPA, negative percent agreement.

testing options via NGS are limited by high cost, long TATs, complicated analysis, and high assay QNS failure rates (3,19).

We designed the ASPYRE-Lung FFPE tissue assay to address these limitations in genomic testing. Advantages of the ASPYRE-Lung assay include the ability to detect all NCCN-recommended genes for NSCLC using a single, simple assay workflow for DNA and RNA with no specialist or large equipment required (just standard PCR and qPCR thermocyclers) and therefore minimal startup costs. Samples with a minimum tumor content of 10% can be used with just 20 ng DNA and 6 ng RNA required per sample and low QC and QNS failure rates.

The assay detects amplification of synthetic probes rather than target nucleic acid, meaning that high-level multiplexing using the same conditions for all 114 variants is possible without sacrificing sensitivity. Operationally, there are four simple steps that involve liquid transfer, resulting in a rapid 2-day TAT from sample receipt to result or 8 hours from extracted sample in to result out, with 2 hours of hands-on time. An automated cloud-based algorithm performs analysis, requiring no user interpretation. Variants detected within the ASPYRE-Lung assay are clinically actionable or prognostic according to current clinical practice guidelines for advanced NSCLC treatment, enabling effective use as a rapid first-line screening tool with minimal tissue usage before reflex to NGS-based assays for samples yielding negative results if required.

There were two components of this study: (I) concordance of ASPYRE-Lung using clinical specimens performed across two academic sites by experienced laboratory staff new to the ASPYRE-Lung assay; and (II) concordance of ASPYRE-Lung with orthogonal, NGS-based assays. Clinical samples included DNA and TNA from both FFPE tissue, fresh tissue, and cytology samples, including pleural effusion, peritoneal fluid, FNA, and FNA rinses, that were previously characterized by NGS. We found high reproducibility between sites, with 75/77 (97%) samples returning the same result across all three sites, or 108/110 (98%) identical positive variant calls (*Table 3*).

The ASPYRE-Lung assay was established at two external CLIA academic laboratories which then ran clinical samples without supervision. The clinical samples were successfully assayed after minimal training, with no invalid sample results during runs. The assay was cost-efficient and simple to adopt, utilizing existing PCR and qPCR thermocyclers, with no need for specialized bioinformatics to inform actionable results.

For 72/77 samples, results were entirely concordant both between ASPYRE sites and with orthogonal testing methods. This includes samples that are technically out of scope for the assay including peritoneal fluid, pleural effusion, fresh tissue, FNA and FNA rinse, and input of TNA rather than RNA.

Most samples that returned positive calls had a single result, however, two samples were positive for two variants by both NGS and ASPYRE-Lung run at all three sites. Samples containing multiple variants are not uncommon and can arise through several mechanisms including tumor heterogeneity and acquired resistance. In these two cases, both samples were positive for p.T790M as well as another *EGFR* driver variant (p.L861Q for a FFPE lung tissue sample and p.L858R for a pleural effusion sample). If multiple variants are present, it is important to detect them all, especially if the variants confer resistance to classes of

inhibitors. Interestingly, the p.T790M/p.L861Q sample was from a patient with stage III squamous cell carcinoma (Table S1), taken prior to treatment. T790M typically arises after treatment with first-line tyrosine kinase inhibitors (20) and increases the affinity of the oncogenic L858R driver variant for ATP (21). Hereditary T790M germline variants are known, although more commonly identified in adenocarcinoma, though cases of advanced adenocarcinoma with squamous cell transformation have also been reported (22-25). It is critical that assays can detect multiple actionable variants when they co-exist in a patient sample to establish appropriate clinical treatment plans.

One sample was positive for a *ROS1* gene fusion by ASPYRE at all three sites. This call was not made by the targeted enrichment NGS assay but was confirmed through the Archer Custom Fusion*Plex* panel. Gene fusions are identified by the ASPYRE-Lung assay directly through analysis of the RNA. Conversely, some target enrichment assays, such as the Roche Avenio assay used in this study, detect fusions through analysis of DNA breakpoints. This requires tiling probes across long intronic regions that include repetitive sequences, which can reduce sensitivity (26). The level of the gene fusion variant in the DNA extracted from this sample remains unknown but could be below the Roche Avenio limit of detection (given as 5%). Analysis via the Archer Custom Fusion*Plex* panel which uses a TNA input indicated that 93% of reads spanning either side of the breakpoint supported the fusion call and the ASPYRE-Lung result.

Across all the samples in this study, there were three that were positive for an *ALK* fusion by NGS. One of these was not detected by ASPYRE-Lung at any site, and it had a variant that is not in the ASPYRE-Lung panel. On inspection of the sequencing data, this *ALK* fusion had an unusual breakpoint in the middle of exon 20. Breakpoints in fusions generally occur in an intronic region, leaving intact exons, and this mid-exon 20 fusion has not been reported previously to the COSMIC database (https://cancer.sanger.ac.uk/cosmic). The addition of new targets to the ASPYRE-Lung panel can be simply achieved by adding new oligonucleotides and probes to the assay to amplify and detect additional variants. As new therapies increase the variants recommended for testing by guidelines, the ASPYRE-Lung panel can be easily adapted to increase numbers of different fusion partners and include different exons of any partners.

Within the entire sample set there were four samples that were predicted positive for *MET* exon 14 skipping variants by NGS, and ASPYRE-Lung yielded positive calls for three of them. *MET* exon 14 skipping is a known driver mutation of cancer (27), however, exon 14 skipping transcripts are also present during development and as part of normal cell processes (15,16). ASPYRE-Lung is therefore tuned to a threshold for this variant that avoids potentially misleading results from high but physiological levels, and thus yielded a negative result for one sample that was deemed positive by NGS.

One FFPE lung tissue sample yielded positive calls by ASPYRE-Lung at all three sites and NGS for an *EGFR* exon 20 c.2303_2311dup variant, and at a single site for an *EGFR* exon 20 c.2300_2308dup variant. These two variants include partial crossover of the duplicated site (2300_2308dupCCAGCGTGG and 2303_2311dupGCGTGGACA), and this result likely indicates probe crosstalk. Current treatment guidelines indicate that there is no evidence to support different treatment recommendations for these two variants.

An FNA sample was negative at two sites and by NGS for all variants tested, and positive at one site for *EGFR* exon 20 c.2317_2319dup. This could be a false positive result or could be a stochastic positive call which can arise from samples with variant allele fractions that are below the limit of detection.

Conclusions

The ASPYRE-Lung assay was established at two external academic laboratories, and nucleic acid derived from 77 samples was run at each site. Results were compared between sites and to orthogonal NGS-based methods. The overall PPA was 100.0% and NPA 99.99% for all 77 samples across the three sites, and PPA 97.2% and NPA 99.96% compared to NGS. The ASPYRE-Lung assay was easy to adopt and run and provides an option for users who require a simple, cost-effective, and rapid way to assess 114 actionable variants of NSCLC, enabling more patients with NSCLC to benefit from highly active and well-tolerated targeted therapeutics.

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Footnote

Reporting Checklist: The authors have completed the MDAR

reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-525/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-525/dss

Peer Review File: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-525/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-525/coif). All authors report that this work was supported by Biofidelity Ltd., UK. S.E.H. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. She served as technical consultant for potential investors at Biofidelity (Oct 2023 to Jan 2024). She received honoraria and travel expenses from ThermoFisher to present on NSCLC at a corporate workshop at AMP 2024. C.G. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. S.J.S. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. B.A.S. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. K.A.S. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. 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E.S. is an employee of Biofidelity Inc. and has financial interest including salary, stock, and stock options. E.S. is a former employee of Adaptive Biotechnologies, which have no interests in the work presented herein. W.J.L. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. WJL. is a consultant of Biofidelity Inc. and has financial interest including consultancy fees and work-related travel to AACR 2024 where data from this work was presented. A. Cooke reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. A. Cooke was an employee of Biofidelity Ltd. and has financial interest including salary and stock. B.W.B. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. B.W.B. reports that Biofidelity Ltd. has provided travel expenses for work-related travel to AACR 2024 where data from this work was presented. B.W.B. is an employee of Biofidelity Ltd. and has financial interest including salary, intellectual property, stock, and stock options. R.J.O. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. R.J.O. was an employee of Biofidelity Ltd., and has financial interest including salary, stock, and stock options. H.V.R. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. H.V.R. was a consultant of Biofidelity Inc. and had financial interest including consultancy fees. H.V.R. is a current employee of Belay Diagnostics, which have no interests in the work presented herein. V.M.V.D. reports that Biofidelity provided all materials (reagents, consumables, etc.) to complete the work. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). For biobank-derived samples, the study was approved by the following Ethics Committees of participating institutions: the Rajiv Gandhi Cancer Institute & Research Centre (IRB# ECR/10/Inst/DC/2013/RR-16; Protocol 672/RE/AMH-27), the National Military Medical Center "Main Military Clinical Hospital" (Protocol 200111), the FGBU National Medico-Surgical Center N.I. Pirogov (Protocol CB#1164/2016), the T.C. Ministry of Health Dr. Abdurrahman Yurtaslan Ankara Oncology Health Practice and Research Center Ethics Committee (Protocol 2019-08/368), and the Kharkiv National Medical University (Protocol GI 020114). Informed consent was obtained from all individual participants. For samples retrospectively identified for this study, the study was approved by the Institutional Review Board (IRB) of the University of Pennsylvania IRB #2 Protocol 854192 and individual consent for this retrospective analysis was waived.

References

1. Reck M, Rodríguez-Abreu D, Robinson AG, et al. Five-Year Outcomes With Pembrolizumab Versus Chemotherapy for Metastatic Non-Small-Cell Lung Cancer With PD-L1 Tumor Proportion Score ≥ 50. J Clin Oncol 2021;39:2339-49.
2. NCI. List of Targeted Therapy Drugs Approved for Specific Types of Cancer. [Cited 2023 Sep 21]. Available online: https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/approved-drug-list#targeted-therapy-approved-for-lung-cancer
3. Sadik H, Pritchard D, Keeling DM, et al. Impact of Clinical Practice Gaps on the Implementation of Personalized Medicine in Advanced Non-Small-Cell Lung Cancer. JCO Precis Oncol 2022;6:e2200246.
4. Hagemann IS, Devarakonda S, Lockwood CM, et al. Clinical next-generation sequencing in patients with non-small cell lung cancer. Cancer 2015;121:631-9.
5. Hanna TP, King WD, Thibodeau S, et al. Mortality due to cancer treatment delay: systematic review and meta-analysis. BMJ 2020;371:m4087.
6. Evans RT, Gillon-Zhang E, Brown JN, et al. ASPYRE-Lung: validation of a simple, fast, robust and novel method for multi-variant genomic analysis of actionable NSCLC variants in FFPE tissue. Front Oncol 2024;14:1420162.
7. Silva AL, Powalowska PK, Stolarek M, et al. Single-copy detection of somatic variants from solid and liquid biopsy. Sci Rep 2021;11:6068.
8. Gray ER, Mordaka JM, Christoforou ER, et al. Ultra-

sensitive molecular detection of gene fusions from RNA using ASPYRE. BMC Med Genomics 2022;15:215.
9. Clopper CJ, Pearson ES. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika 1934;26:404-13.
10. Jones E, Oliphant T, Peterson P. SciPy: Open Source Scientific Tools for Python. 2001. Available online: http://www.scipy.org/
11. Roche. AVENIO Tumor Tissue Targeted Kit V2. [Cited 2024 Apr 3]. Available online: https://sequencing.roche.com/global/en/products/group/avenio-tumor-tissue-targeted-kit.html
12. Kanaoka K, Tamiya A, Inagaki Y, et al. Possible False Results With cobas(®) EGFR Mutation Test v2 and Oncomine Dx Target Test for EGFR Mutation. Anticancer Res 2023;43:2771-6.
13. Shepherd P, Sheath KL, Tin ST, et al. Lung cancer mutation testing: a clinical retesting study of agreement between a real-time PCR and a mass spectrometry test. Oncotarget 2017;8:101437-51.
14. Keppens C, Palma JF, Das PM, et al. Detection of EGFR Variants in Plasma: A Multilaboratory Comparison of a Real-Time PCR EGFR Mutation Test in Europe. J Mol Diagn 2018;20:483-94.
15. Mitiushkina NV, Kholmatov MM, Tiurin VI, et al. Comparative analysis of expression of mutant and wild-type alleles is essential for reliable PCR-based detection of MET exon 14 skipping. Biochimie 2019;165:267-74.
16. Gray MJ, Kannu P, Sharma S, et al. Mutations Preventing Regulated Exon Skipping in MET Cause Osteofibrous Dysplasia. Am J Hum Genet 2015;97:837-47.
17. Wei S, Lieberman D, Morrissette JJ, et al. Using "residual" FNA rinse and body fluid specimens for next-generation sequencing: An institutional experience. Cancer Cytopathol 2016;124:324-9.
18. Nesline MK, Subbiah V, Previs RA, et al. The Impact of Prior Single-Gene Testing on Comprehensive Genomic Profiling Results for Patients with Non-Small Cell Lung Cancer. Oncol Ther 2024;12:329-43.
19. Tsimberidou AM, Sireci A, Dumanois R, et al. Strategies to Address the Clinical Practice Gaps Affecting the Implementation of Personalized Medicine in Cancer Care. JCO Oncol Pract 2024;20:761-6.
20. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.
21. Yun CH, Mengwasser KE, Toms AV, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc Natl Acad Sci U S A 2008;105:2070-5.
22. Yamaguchi F, Kato E, Wakabayashi A, et al. Effect of osimertinib treatment on lung adenocarcinoma with squamous cell transformation harboring the T790M mutation: A case report and literature review. Mol Clin Oncol 2019;11:127-31.
23. Izumi H, Yamasaki A, Ueda Y, et al. Squamous Cell Carcinoma Transformation from EGFR-mutated Lung Adenocarcinoma: A Case Report and Literature Review. Clin Lung Cancer 2018;19:e63-6.
24. Jukna A, Montanari G, Mengoli MC, et al. Squamous Cell Carcinoma "Transformation" Concurrent with Secondary T790M Mutation in Resistant EGFR-Mutated Adenocarcinomas. J Thorac Oncol 2016;11:e49-51.
25. Maeda C, Shinada K, Murakami S, et al. Efficacy of osimertinib for lung squamous cell carcinoma with de novo EGFR T790M-positive: Case report and literature review. Thorac Cancer 2023;14:2886-9.
26. Benayed R, Offin M, Mullaney K, et al. High Yield of RNA Sequencing for Targetable Kinase Fusions in Lung Adenocarcinomas with No Mitogenic Driver Alteration Detected by DNA Sequencing and Low Tumor Mutation Burden. Clin Cancer Res 2019;25:4712-22.
27. My Cancer Genome. MET Exon 14 Skipping. [Cited 2024 Apr 3]. Available online: https://www.mycancergenome.org/content/alteration/met-exon-14-skipping/

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Supplementary

Table S1 FFPE lung tissue samples from patients with a confirmed NSCLC diagnosis

Sample ID	Sample form	Nucleic acid analyzed	Pathology or clinical diagnosis	Result from NGS-based assays
NSCLC_005	FFPE lung tissue	DNA/RNA	Neuroendocrine	No variants identified
NSCLC_009	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_010	FFPE lung tissue	DNA/RNA	Neuroendocrine/large cell carcinoma	COSM520
				KRAS p.G12V
NSCLC_016	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_018	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM6223
				EGFR p.E746_A750del
NSCLC_019	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_020	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_022	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_023	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_025	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM29312
				MET exon 14 skipping
NSCLC_028	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_033	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_037	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_038	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_039	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_040	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_041	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_042	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_044	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_046	FFPE lung tissue	DNA/RNA	Large cell carcinoma	No variants identified
NSCLC_048	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM6223
				EGFR p.E746_A750del
NSCLC_049	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM516
				KRAS p.G12C
NSCLC_051	FFPE lung tissue	DNA/RNA	Large cell carcinoma	No variants identified
NSCLC_052	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	COSM6240; COSM6213
				EGFR p.T790M; *EGFR* p.L861Q
NSCLC_055	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_057	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM476
				BRAF p.V600E
NSCLC_060	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_061	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM6224
				EGFR p.L858R
NSCLC_069	FFPE lung tissue	DNA/RNA	Adenocarcinoma	*EML4*/*ALK* fusion[†]
NSCLC_070	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_073	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_077	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_081	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM13428
				EGFR p.S768_D770dup
NSCLC_088	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM553
				KRAS p.Q61L
NSCLC_094	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM6223
				EGFR p.E746_A750del
NSCLC_098	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM476
				BRAF p.V600E
NSCLC_103	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM6224
				EGFR p.L858R
NSCLC_105	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM12370
				EGFR p.L747_P753delinsS
NSCLC_109	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_111	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM476
				BRAF p.V600E
NSCLC_112	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM6223
				EGFR p.E746_A750del
NSCLC_119	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM516
				KRAS p.G12C
NSCLC_120	FFPE lung tissue	DNA/RNA	Pleomorphic cell carcinoma	No variants identified
NSCLC_123	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_125	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_127	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_128	FFPE lung tissue	DNA/RNA	Adenocarcinoma	No variants identified
NSCLC_130	FFPE lung tissue	DNA/RNA	Large cell carcinoma	COSM522
				KRAS p.G12A
NSCLC_131	FFPE lung tissue	DNA/RNA	Adenocarcinoma	COSM29312
				MET exon 14 skipping
NSCLC_140	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_143	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_144	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_148	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
NSCLC_149	FFPE lung tissue	DNA/RNA	Squamous cell carcinoma	No variants identified
UP03	FFPE slide	DNA	NSCLC	COSM6255
				EGFR p.L747_Ser752del
UP07	FFPE lung tissue	DNA	NSCLC	COSM516
				KRAS p.G12C
UP08	FFPE slide	DNA	NSCLC	COSM527
				KRAS p.G13C
UP17	FFPE lung tissue	TNA	NSCLC	*EML4*/*ALK* fusion
UP18	FFPE lung tissue	TNA	NSCLC	*EML4*/*ALK* fusion
UP23	FFPE slide	TNA	NSCLC	COSM29312
				MET exon 14 skipping
UP28	FFPE lung tissue	TNA	NSCLC	No variants identified

The table shows the clinical characteristics associated with each sample. [†], not an *ALK* fusion that is detected by the ASPYRE panel. FFPE, formalin-fixed paraffin-embedded; NSCLC, non-small cell lung cancer; NGS, next-generation sequencing; TNA, total nucleic acid.

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Table S2 Cytology sample types and derived nucleic acid used in this study

Sample ID	Sample form	Nucleic acid analyzed	Pathology or clinical diagnosis	Result from NGS-based assays
UP02	Peritoneal fluid	DNA	NSCLC	COSM12384
				EGFR p.E746_S752delinsV
UP04	Pleural fluid	DNA	NSCLC	COSM6224
				EGFR p.L858R
UP05	Pleural effusion	DNA	NSCLC	COSM6224; COSM6240
				EGFR p.L858R; *EGFR* p.T790M
UP10	FNA rinse	DNA	NSCLC	COSM12553
				ERBB2 p.G776delinsVC
UP12	Fresh tissue, liver	DNA	None	No variants identified
UP15	FNA	DNA	NSCLC	No variants identified
UP16	FNA	DNA	NSCLC	No variants identified
UP19	FNA rinse	TNA	NSCLC	*RET* fusion
UP20	FNA rinse	TNA	NSCLC	*RET* fusion
UP21	FNA rinse	TNA	NSCLC	*ROS1* fusion
UP22	FNA rinse	TNA	NSCLC	*ROS1* fusion
UP24	Fresh tissue, brain	TNA	Lung cancer	COSM29312
				MET exon 14 skipping
UP26	FNA	TNA	NSCLC	No variants identified
UP27	FNA	TNA	Lung cancer	No variants identified
UP31	Fresh tissue, lung	TNA	NSCLC	No variants identified
UP32	Fresh tissue, brain	TNA	Lung cancer	No variants identified

Fresh tissue, FNA, FNA rinse, pleural fluid and effusion samples were sourced from patients with the indicated clinical diagnoses, extracted and the nucleic acid shown used in this study. Tissue source is lung, unless stated otherwise. Also shown are the biomarkers identified by NGS-based panels for each sample. NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; FNA, fine needle aspirate; TNA, total nucleic acid.

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