Validation of a Simple, Fast and Robust Novel Method for Complex Genomic Analysis of Actionable NSCLC Variants in Tissue and Plasma

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ASPYRE®

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Abstract

Introduction: Allele-Specific PYrophosphorolysis REaction (ASPYRE) is a novel method relying on enzymatic degradation of probes hybridized with perfect complementarity to target DNA strands. ASPYRE is fast, simple and inexpensive and can be performed in a platform-agnostic fashion using standard real-time PCR instruments. Using this technology we created ASPYRE-Lung, a targeted panel of 114 genomic variants across 11 genes with simultaneous DNA and RNA workflows. The assay covers the NCCN guideline recommended biomarkers for patients with non-small cell lung cancer (NSCLC), informing timely treatment decisions in a majority of patients.

Methods: A set of contrived samples was generated for each of the 114 DNA variants, RNA fusions or exon skipping events targeted by the assay. Target variant allele fractions (VAFs) ranged from approximately 0.1%, 0.2%, 0.5%, 1.0% to 5.0% (and 50.0% in the training dataset) for DNA variants, 6 or 30 copies for RNA fusions and 30 and 150 copies for MET exon 14 skipping events. To mimic liquid biopsy samples, we used a background of genomic DNA from peripheral blood leukocytes or cell-free RNA (cfRNA) derived from healthy individuals (n=1,212). For tissue specimens, samples had a background of genomic DNA or RNA derived from non-neoplastic formalin fixed paraffin embedded (FFPE) lung tissue (n=1,086). Seven independent operators generated three independent data sets from all samples. Data were analyzed using a support-vector machine (SVM) classifier. To evaluate assay performance we used a cross-validation approach, a resampling method that iteratively uses different portions of the data to test and train models. Variants were interpreted as detected or not-detected by the SVM.

Results: The SVM classifier made a total of 121,718 distinct variant calls. For the contrived FFPE samples, assay specificity was >99.9% and sensitivity was >96%. For the set of contrived plasma samples assay specificity was >99.9% and sensitivity was 91.6%. The typical turnaround time from extracted nucleic acids to result was approximately 6 hours. Throughput was highly scalable and we routinely processed up to 16 samples per run.

Conclusions: We describe validation of a novel method for detecting a panel of clinically actionable NSCLC-associated mutations and RNA fusions from FFPE tissue or plasma. The simple workflow includes four steps that involve only reagent transfer and incubation. The assay is robust and precise with performance characteristics approaching that of NGS,

but with faster turnaround times, lower complexity workflows, simple bioinformatic analysis, reduced cost, and with no variant of unknown significance, (VUS)-type variants, to interpret.

Introduction

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 events from RNA[2].

Here, we extend these observations in large and comprehensive training and test data sets with the goals of developing and validating a novel variant calling algorithm for use with the ASPYRE-Lung testing panel and demonstrating assay wide performance characteristics.

Materials & Methods

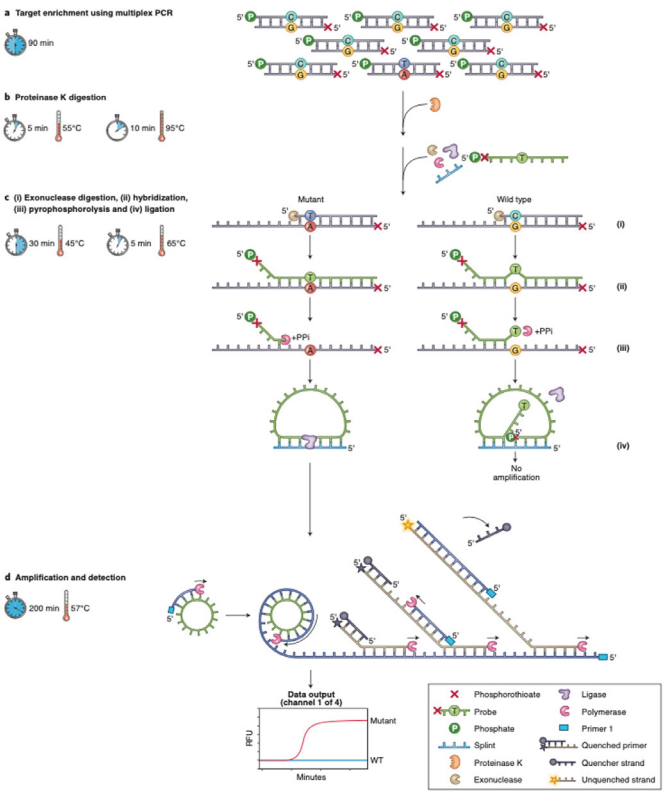
An overview of ASPYRE assay steps for both DNA and RNA variant detection is shown in **Panels A and B**. An ASPYRE-Lung panel assay general overview is shown in **Panel C**.

A total of 114 DNA and RNA variants were tested in a set of experiments designed to generate a dataset to develop, train and validate a SVM algorithm to perform variant calling. The study included a large number of contrived samples designed to mimic clinical samples. These were prepared in-house by spiking synthetic oligonucleotides into the described backgrounds following quantification by digital PCR (dPCR). The background materials used to construct the sample sets were representative of the 2 types of clinical samples that will be used with the panel; either DNA/RNA extracted from normal FFPE lung tissue or a liquid biopsy mimic comprising genomic DNA or cfRNA derived from healthy individuals.

Target VAFs across different experiments ranged from approximately 0.1% to 5.0% (and 50.0% for samples in the training dataset only) for the DNA variants, while RNA abnormalities were assessed at input levels of 6 or 30 copies for fusions and 30 and 150 copies for MET exon 14 skipping events. Wild type DNA and RNA samples were also included.

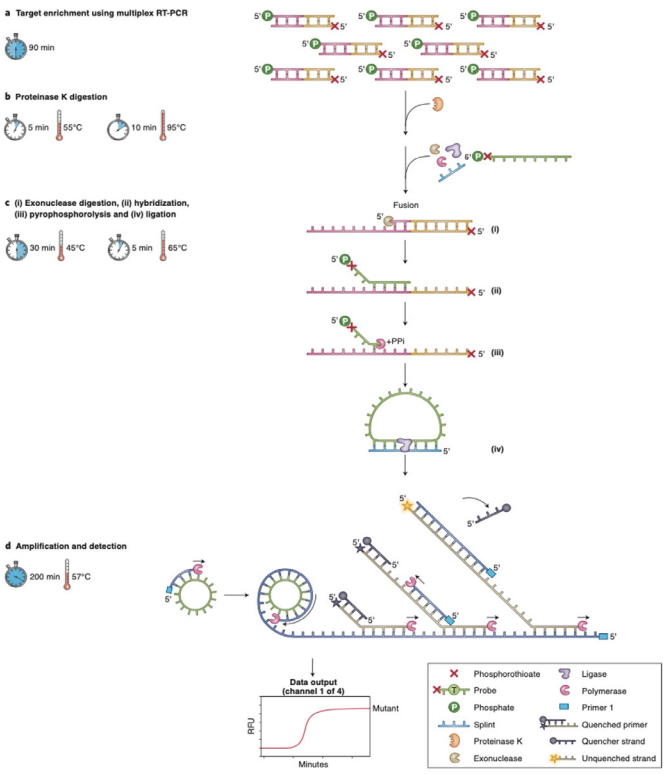
DNA samples were tested using 4 different lots of ASPYRE-Lung reagents with one sample repeat performed per lot. RNA samples were tested using 3 different lots of ASPYRE-Lung reagents with 2 sample repeats per lot. Six different 384-well ThermoFisher QuantStudio™ 5 qPCR instruments, at least 17 different thermal cyclers and 7 different operators were used in the experiments.

DNA



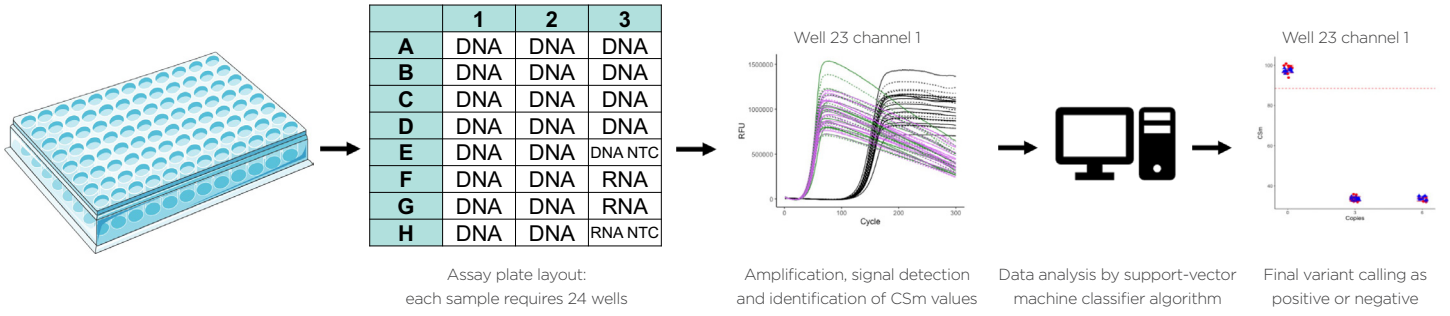
PANEL A – General overview of steps for detection of single nucleotide variants, insertions, deletions and complex variants from DNA in the following genes in the ASPYRE-Lung panel: *BRAF, EGFR, ERBB2* and *KRAS*. Figure modified from Silva et al.

RNA



PANEL B – General overview of steps for detection of fusions and exon skipping events from RNA in the following genes in the ASPYRE-Lung panel: *ALK, RET, ROS1, MET, NTRK1, NTRK2* and *NTRK3*.

Assay overview



	1	2	3
A	DNA	DNA	DNA
B	DNA	DNA	DNA
C	DNA	DNA	DNA
D	DNA	DNA	DNA
E	DNA	DNA	DNA NTC
F	DNA	DNA	RNA
G	DNA	DNA	RNA
H	DNA	DNA	RNA NTC

Assay plate layout: each sample requires 24 wells

Amplification, signal detection and identification of CSm values

Data analysis by support-vector machine classifier algorithm

Final variant calling as positive or negative

PANEL C – General assay overview. Each sample requires 24 wells allowing for 4 patient samples per 96 well plate. Cycle sigmoid midpoint (CSm) values are identified from fluorescent data and used for classification of a variant as positive or negative by the support-vector machine classifier. Amplification curves and plotted CSm data are from Gray et al. Multiwell-plate-3d icon by Servier https://smart.servier.com/ is licensed under CC-BY 3.0 Unported https://creativecommons.org/licenses/by/3.0/.

Results

Summary of study datasets

Training dataset

	Contrived FFPE samples		Contrived plasma samples	
	DNA	RNA	DNA	RNA
Number of samples analyzed	660	456	1,313	234
Number of samples included*	652	447	1,306	230
Total samples included	1,099		1,536	

Test dataset

	Contrived FFPE samples		Contrived plasma samples	
	DNA	RNA	DNA	RNA
Number of samples analyzed	639	447	982	230
Number of samples included*	1,086		1,212	
Total samples included	48,882		72,836	

PANEL D – Details on sample numbers and types included in the training and test datasets. *In the training dataset, the number of samples analyzed is higher than the number of samples included in the test dataset due to exclusion of a small number that demonstrated failure of internal controls, failure of negative controls or known/suspected experimental error and were therefore unsuitable for use.

ASPYRE-Lung panel performance in test dataset

		True positive	True negative	False positive	False negative	Sensitivity (%)	Specificity (%)
Contrived FFPE samples	**Overall number**	994	47,844	3	41	96.04	99.99
	>1% VAF (DNA) or ≥ 30 copies (RNA)	517	22,349	3	5	99.04	99.99
Contrived plasma samples	**Overall number**	1,032	71,706	3	95	91.57	100
	≥0.5% VAF (DNA) or ≥ 30 copies (RNA)	601	43,437	3	8	98.69	99.99

PANEL E – Final ASPYRE-Lung panel results and performance of the SVM classifier in the test dataset, summarized.

Discussion

Our data from this large and challenging sample set demonstrates that the ASPYRE-Lung genomic testing panel used in conjunction with the variant calling algorithm we have developed based on a SVM classifier, is highly sensitive and specific with performance characteristics appropriate for use in a clinical laboratory. We believe that ASPYRE-based testing has the potential for more rapid and efficient genomic evaluation of individuals with NSCLC relative to current testing workflows.

Many patients now receive next generation sequencing (NGS) panel-based testing, yet 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 main steps after DNA/RNA extraction that require no more than basic reagent transfer 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, ease of implementation and use, 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.
- The ASPYRE-Lung panel comprises 114 actionable DNA and RNA variants in *ALK, BRAF, EGFR, ERBB2, KRAS, MET, NTRK1, NTRK2, NTRK3, RET, ROS1* and is easily performed on common laboratory equipment.
- We developed and tested an effective variant calling algorithm for use with data from ASPYRE reactions.
- The ASPYRE-Lung panel demonstrates very high sensitivity and specificity across a large number of analytically challenging contrived FFPE and plasma samples including at very low variant frequencies and copy numbers.
- The ASPYRE-Lung panel has performance characteristics well suited to clinical laboratory based genomic analysis.

References

1. Silva et al. 2021. Single-copy detection of somatic variants from solid and liquid biopsy. Sci Rep. 11(1):6068.

2. Gray et al. 2022. Ultra-sensitive molecular detection of gene fusions from RNA using ASPYRE. BMC Med Genomics. 15(1):215.

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