Single-copy detection of somatic variants from solid and liquid biopsy

Ana-Luisa Silva¹, Paulina Klaudyna Powalowska¹, Magdalena Stolarek¹, Eleanor Ruth Gray¹, Rebecca Natalie Palmer¹, Bram Herman², Cameron Alexander Frayling¹ & Barnaby William Balmforth¹*

Accurate detection of somatic variants, against a background of wild-type molecules, is essential for clinical decision making in oncology. Existing approaches, such as allele-specific real-time PCR, are typically limited to a single target gene and lack sensitivity. Alternatively, next-generation sequencing methods suffer from slow turnaround time, high costs, and are complex to implement, typically limiting them to single-site use. Here, we report a method, which we term Allele-Specific PYrophosphorolysis Reaction (ASPYRE), for high sensitivity detection of panels of somatic variants. ASPYRE has a simple workflow and is compatible with standard molecular biology reagents and real-time PCR instruments. We show that ASPYRE has single molecule sensitivity and is tolerant of DNA extracted from plasma and formalin fixed paraffin embedded (FFPE) samples. We also demonstrate two multiplex panels, including one for detection of 47 EGFR variants. ASPYRE presents an effective and accessible method that simplifies highly sensitive and multiplexed detection of somatic variants.

Genotyping of somatic variants is standard of care for many cancer types, with results guiding the use of targeted and immune therapies. Test procedures commonly involve genetic analysis of either tumor tissue from a biopsy, or surgical resection, or circulating-tumor DNA (ctDNA) from a peripheral blood draw. Most commonly, tissue biopsies are used for initial genotyping because they directly sample the tumor. However, tissue biopsies also have limitations including associated morbidities and can be difficult to obtain, underestimate intratumor heterogeneity, have insufficient cell content, and DNA yields can be insufficient or low quality¹. In contrast, analysis of plasma-derived ctDNA from a liquid biopsy is non-invasive and can more completely sample intratumor heterogeneity and metastases²⁻⁵ but has limited sensitivity if a patient has a low tumor burden or a low-shedding tumor. This makes analysis of early-stage tumors particularly challenging⁶, since, for many patients, current assays are insufficiently sensitive.

Tissue biopsies are subject to pathology review and those with insufficient tumor content, that cannot be rescued by macrodissection, are excluded from genetic testing. This ensures that clonal somatic variants in tested samples have variant allele fractions (VAFs) of at least 5–10%. In contrast, there is no equivalent quality control step for a peripheral blood draw, and clonal somatic variants exhibit a wide range of VAFs from < 0.1 to 10%.

Methods for somatic variant detection include real-time quantitative PCR (qPCR), digital PCR (dPCR) and next-generation sequencing (NGS). Real-time and dPCR are often limited to single genes. Allele-specific qPCR has a limit of detection around 1% and dPCR < 0.1% VAF⁷⁻⁹. Sequential testing of multiple single-gene assays can exhaust available material and increase costs, particularly if a repeat biopsy or blood draw is required⁹⁻¹¹. Dividing material between multiple assays can also decrease assay sensitivity compared to using all available material in a single multiplexed assay. In this context, guidelines often support panel-based testing by NGS. Error-correction can decrease the limit of detection for NGS to <0.1% VAF¹². However, NGS workflows require significant investment in equipment, training and require complex bioinformatic analysis. In addition, turnaround times for NGS are around 13 days compared to 2–3 days for PCR assays¹³. In order to achieve clinically acceptable turnaround times, laboratories may batch less than the optimal number of samples in one sequencing run—in effect trading off cost against turnaround time.

To address these challenges, we developed Allele-Specific PYrophosphorolysis REaction (ASPYRE), a simple-to-use qualitative method for high-sensitivity detection across panels of somatic variants. The novel technology is based on pyrophosphorolysis, in which a high concentration of pyrophosphate ion is used to drive the DNA polymerisation reaction in reverse, resulting in the 3’→5’ de-polymerisation of double-stranded DNA¹⁴. This DNA...
Accurate detection of variant molecules requires a threshold that distinguishes reactions containing one, or more, variant molecules from reactions containing only wild-type molecules. To define assay thresholds, we used 20 ng of custom cell-free (cfDNA) reference standard that mimics post-extraction yield and fragment size distributions found in liquid biopsies (SeraCare Life Sciences). Three variants associated with NSCLC were amplified in a multiplex PCR; including one EBRB2 exon 17 substitution, one EBRB2 exon 20 insertion, and one EML4-ALK fusion. We assessed three VAFs (0%, 0.1% and 0.5%) using eight replicates of each (total 24 PCR reactions). Following the initial PCR, and enzymatic clean up, reactions were split into three tubes, each detecting one variant. For all variants, the resulting quantification cycle (Cq) values from reactions containing only wild-type template (0% VAF) were clearly separable from those including variant molecules (0.1% and 0.5% VAF) (Fig. 2a). We set Cq thresholds for each of the variants at the lower value of either five standard deviations below the mean of the wild-type reaction or two standard deviations above the mean of the 0.1% VAF samples.

We next assessed the selected thresholds and assay background using a range of sample types and DNA inputs. We assessed DNA inputs from 2 ng to 1 μg using wild-type human genomic DNA ultrasonicated to ~150 bp. Additional samples included FFPE from patients with NSCLC, wild-type reference standard FFPE control mates and reagent lots were within statistical noise of equal (ANOVA F-test, P = 0.5). All wild-type samples (0% VAF) presented as negative, giving a specificity of 100%. For the remaining samples, we estimated the mean number of intact variant molecules that could be amplified by PCR in each reaction ("Methods"). We then used probit regression to estimate the 95% limit of detection. LoD95 results were 5.2 variant molecules for EBRB2 exon 17, 4.0 for EBRB2 exon 20, and 2.9 for the EML4-ALK fusion. We then compared our results to an ideal assay that perfectly detects variant molecules that can be amplified by PCR, failing only when there are no such molecules in the sample ("Methods"). In this case, sensitivity is dependent only on sampling and the LoD95 is 3.0 variant.
Figure 1. Overview of the ASPYRE assay. (a) Multiplex PCR amplification of wild-type and mutant alleles. (b) Enzymatic digestion of the PCR enzyme and subsequent heat-inactivation. (c) Exonuclease digestion to create single-stranded target molecules; hybridization of oligonucleotide probes to both mutant and wild-type sequences, with a mismatch to the wild-type sequence at the site of mutation; and pyrophosphorolysis of hybridized probes. (d) Circularization of only those probes that have been digested beyond the mutation site; and isothermal amplification and detection of circularized probes. Artwork by Debbie Maizels, Zoobotanica Scientific Illustration.
molecules. These results indicate that ASPYRE was able to detect molecules with single molecule sensitivity. In further support of this hypothesis, for all three combinations of two variants, the probability that the same sample was positive for both variants was consistent with independent sampling of variant molecules (Cochran–Mantel–Haenszel test: $ERBB2$ exon 17 and $ERBB2$ exon 20 $P = 0.5$; $ERBB2$ exon 17 and $EML4$-$ALK$ $P = 0.4$; $ERBB2$ exons 17 and 20 $P = 0.4$).
exon 20 and EML4-ALK P = 0.5). The exception was one sample at 0.5% VAF with 5 ng input, which was negative for all three variants. This result is likely an assay failure, given the number of input variant molecules, and allows us to estimate a false-negative rate of 1%. In future, use of a positive control will allow identification of assay failures. Taken together, these data indicate that ASPYRE can achieve detection of a single molecule of variant DNA against a wild-type background, with high sensitivity and specificity.

EGFR panel. We next applied ASPYRE to detection of 47 EGFR variants, commonly used in treatment selection for NSCLC. These included 46 deletions in exon 19 and the L858R (COSM6224) mutation in exon 2117. Two target regions of EGFR were co-amplified by PCR and reactions were subject to enzymatic clean up. Each reaction was then split into 47 tubes, one detecting L858R and the other 46 each detecting a single deletion.

We performed six DNA extractions on FFPE treated cell-line mixtures, including three at 0% VAF (HD141) and three at 1% VAF (HD850). No sample-to-sample variation was observed between the independent DNA extractions for FFPE cell-lines (Supplementary Fig. 1). We mixed the cell-lines to give 0.5%, 0.25% and 0.1% VAF samples. Each of the six DNA extractions, and three mixes, were PCR amplified in 5 replicates including 20 ng DNA input (total 45 PCR reactions). Each PCR reaction was then used in 3 replicate detection reactions (total 135 detection reactions). Cq thresholds for each of the variants were set at five standard deviations below the mean of the 0% VAF replicates. Results demonstrated 100% sensitivity at all VAFs including 0.1% (Fig. 3).

We next tested the performance of ASPYRE on patient samples. We analyzed six NSCLC FFPE tissue samples, with known variants, sourced from a commercial biobank; one positive control EGFR reference standard (HD850); and one negative control (DNA fragmented by ultrasonication, fgDNA). Patient DNA samples were found to be more fragmented than FFPE treated cell-line mixtures (Supplementary Fig. 1). We PCR amplified 20 ng input DNA from each sample and control using six replicates (total 48 PCR reactions). We first analyzed

![EGFR exon 21 L858R COSM6224](image_url)

![EGFR exon 19 deletion COSM6223](image_url)

**Figure 3.** Cq values for (a) EGFR exon 21 L858R (COSM6224) and (b) exon 19 (COSM6223) deletion. The red dashed line represents the threshold Cq used to determine the presence of variants. Results below the red dashed line indicate detection of the variant.
amplification products using polyacrylamide gel electrophoresis (PAGE) (Supplementary Fig. 2). No deletion product was observed for the positive control HD850, which had an EGFR exon 19 deletion with a VAF of 1%. Deletion products were visible for patient samples HBF003 and HBF005, consistent with high VAF EGFR exon 19 deletions. We next analyzed results from ASPYRE, using Cq thresholds for each of the variants that were set at five standard deviations below the mean of the negative control. Despite not being visible by PAGE, the positive control 1% EGFR exon 19 deletion was detected by ASPYRE. Further analysis of the patient samples identified three false positives, including two L858R replicates from HBF0004 and one COSM12413 replicate from HBF0001 (Supplementary Fig. 3). Despite this, Cq values for the false positive L858R replicates were visually separable from the positive control samples, suggesting that our threshold Cq values could be improved. For patient HBF0006, we also failed to detect an exon 19 deletion, both on PAGE and in all six replicates using ASPYRE, that was previously identified by a biobank using qPCR. To investigate this discrepancy, the biobank repeated qPCR analysis on their original DNA extraction, used for sample characterization, and our subsequent DNA extraction used for ASPYRE. The exon 19 deletion was weakly detected in the biobank’s original DNA extraction but not in our ASPYRE DNA extraction. This is consistent with sample contamination, a well-known issue when handling FFPE blocks18. Overall, sensitivity was 100%, specificity was 99.8% and concordance was 99.8%. Taken together, these results illustrate that ASPYRE is capable of detecting clinically relevant EGFR variants in FFPE and contrived cDNA samples with high sensitivity, specificity and concordance.

Discussion

In this study, we have demonstrated that the ASPYRE technology has high sensitivity and specificity for the detection of SNVs, indels and gene fusions, coupled with a wide tolerance to the sample type, DNA fragmentation profile, and DNA input quantity. Threshold Cq values defined in the validation study were successfully applied to all DNA sample types and DNA inputs. A consistent background signal was obtained for assays with differing sample types, differing DNA inputs and in the absence of template. This stands in contrast to PCR-based assays, which often suffer from carryover of inhibitors from FFPE samples19 and have background and signal Cq values that depend on input DNA concentration. The background signal of ASPYRE is consistent with non-specific amplification of the probe during the isothermal detection step20. Further optimization of reaction conditions, including use of molecular beacon probes, rather than an intercalating dye21, could reduce background fluorescence. Using 20 ng of input DNA, ASPYRE had 100% sensitivity and 100% specificity for the tested substitution, insertion and fusion variants at 0.5% and 0.1% VAF. We further investigated assay sensitivity by limiting dilution and used probit regression to demonstrate that the LoD95 was similar to that expected from Poisson sampling. This indicates that ASPYRE is capable of detecting single copies of variant molecules. Sensitivity and specificity values are significantly higher than those achieved by real-time PCR and match, or exceed, dPCR and NGS assays. This offers the potential to detect variants at low fractions in cfDNA (for patient stratification, monitoring and early detection) or tissue samples with low cellularity such as fine needle aspirates.

We also tested NSCLC FFPE patient samples for EGFR mutations. Specificity was 99.8% but could be further improved by defining thresholds using negative control FFPE DNA samples rather than ultrasonicated DNA, which might have poor commutability22. One sample was not concordant between ASPYRE and previous qPCR results. Re-analysis of DNA extractions using qPCR confirmed the ASPYRE finding, providing 100% sensitivity and 100% concordance. Amongst our studies, we found one sample that was unexpectedly negative for all variants. This allowed us to estimate a false-negative rate of 1%. In future, including a positive control in the assay will allow straightforward identification of such assay failures.

Several assays in standard clinical usage are based on Amplification Refractory Mutation System (ARMS)23. These include Roche cobas and Qiagen therascreen single gene assays, with reported analytical LoD95 of 3% for detection of EGFR substitutions and indels (FDA Summary of Safety and Effectiveness Data cobas EGFR Mutation Test v2 and therascreen EGFR RGQ PCR Kit). In addition, enhanced allele-specific qPCR approaches have been described with improved sensitivity compared to ARMS. For example, competitive allele-specific TaqMan PCR has a reported LoD of 0.1% for EGFR L858R and 1% for T790M24. Similarly, modified blocking oligonucleotides have been applied to reduce the LoD to 0.1% for four common KRAS variants25. Compared to ASPYRE, these modified qPCR approaches have limited multiplexing. In an alternative approach, Blocker Displacement Amplification (BDA)26 has reported LoD of 0.1% and could potentially support multiplexing of multiple genes. However, BDA multiplexing is either limited by the number of independent colour channels on a qPCR instrument or requires an alternative readout, such as NGS, thereby increasing turnaround time and assay complexity.

A comparison of ASPYRE to existing assays is shown in Table 1. ASPYRE is simple, low-cost, can be implemented on real-time PCR instrumentation, and is ideally suited to multi-gene panels of clinically actionable variants. The method supports higher multiplexing than either allele-specific qPCR or dPCR, thereby avoiding dividing material between multiple assays with the associated decrease in sensitivity and increased assay costs. Our current implementation still has limitations, compared to NGS, in the number of targets that can be multiplexed and the requirement for prior knowledge of variants. Multiplexing can be addressed by parsing PCR reactions into smaller microfluidic detection reactions, although this level of multiplexing is not essential for all tumor types. For example, NCCN guidelines for NSCLC recommend testing for substitutions in EGFR, BRAF and KRAS; gene fusions in ALK, RET, ROS1, and NTRK1-3; and MET exon 14 skipping27. In this context, broader panels might not provide additional benefit, with identification of somatic variants with no known drug association having limited clinical significance. A further limitation is that we have not assessed copy number amplification: high level amplification of MET is an emerging biomarker for NSCLC27. Compared to NGS, ASPYRE has a simple laboratory workflow, requiring straightforward sequential addition of reagents without
intermediate purification steps, and does not require complex bioinformatic interpretation. The assay can be run in less than 4 h and is sufficiently cost effective to run a wide range of batch sizes from one to hundreds of samples. We anticipate that ASPYRE will help address clinical needs for highly accurate multiplexed detection of somatic variants with reductions in assay complexity allowing laboratories of all sizes to rapidly test patients.

### Methods

**Samples.** Custom cell-free DNA reference standards were supplied by SeraCare Life Sciences. Standards included either 0%, 0.1% and 0.5% VAF for ERBB2 substitution (COSM6503262), ERBB2 insertion (COSM20959), and EML4-ALK fusion (COSF463). COSM and COSF are COSMIC identifiers. Mean fragment sizes were 170 bp for the 0% VAF sample, 157 bp for the 0.1% VAF sample, and 155 bp for the 0.5% VAF sample. Samples with VAFs lower than 0.1% were prepared by dilution of the 0.1% VAF sample with the 0% VAF (wild-type) sample. The 0.1% VAF sample had two additional copies of ERBB2 compared to the EML4-ALK fusion, therefore diluted samples had VAFs of 0.07% for ERBB2 substitutions and insertions and 0.05% for EML4-ALK fusions.

Human genomic DNA was supplied by Promega (G3041) and fragmented by ultrasonication to a mean size of ~150 bp to mimic post-extraction size distribution from a liquid biopsy sample. FFPE cell-lines were supplied by Horizon Discovery and included 100% EGFR wild type FFPE DNA (HD141) and 1% EGFR Quantitative Multiplex FFPE Reference Standard (HD850). Six FFPE blocks from patients with confirmed NSCLC adenocarcinoma were supplied by BioIVT (Supplementary Table 1). Cell-free cfDNA reference standards included EGFR Multiplex cfDNA Reference Standard Set (HD825) and Multiplex 1 cfDNA Reference Standard Set (HD780).

**DNA extraction and quantitation.** The ReliaPrep FFPE gDNA Miniprep System (Promega) was used to extract DNA from both FFPE cell-lines and FFPE tissue samples. DNA was quantitated using a fluorescent dye specific to double-stranded DNA (Qubit dsDNA HS assay, Thermo Fisher Scientific) and assessed by Genomic DNA ScreenTape Assay on a 4200 TapeStation (Agilent).

**Initial target amplification and proteinase K treatment.** PCR primers were designed to minimise amplicon lengths, in order to increase the probability of amplifying target molecules in fragmented DNA. We designed 10 forward and reverse primer sequences, where the primer 3' end was positioned 20–30 nucleotides from the variant, and empirically tested all possible combinations.

PCR reactions were prepared by mixing 10 µL HF buffer (F555, Thermo Fisher Scientific), 0.5 µL MgSO₄ (100 mM, B1003, New England Biolabs), 2 µL primers (10 µM each), 2 µL dNTPs mix (10 mM, Promega), 0.5 µL Phusion U Hot Start Polymerase (F555, Thermo Fisher Scientific), 0.5 µL Antarctic Thermolabile UDG (100 mM, B1003, New England Biolabs), 2 µL primers (10 µM each), 2 µL dNTPs mix (10 mM, Promega), 0.5 µL Phusion U Hot Start Polymerase (F555, Thermo Fisher Scientific), 0.5 µL Antarctic Thermolabile UDG (100 mM, B1003, New England Biolabs), 2–20 ng DNA template in a 50 µL reaction.

PCR amplification of EGR exons 19 and 20 included primers 5'-A*A*G*TTAAATCCCGTCGCTA-3', 5'-/P/AGCAAGCAGAAACTACATCG-3', 5'-A*C*A*CCGAGCAGTCAAGAT-3', and 5'-/P/GCCTCCTGTCATGATTTCT-3'. Phosphorothioate bonds are denoted by an asterisk and phosphate groups by /P/. Cycling conditions (including UDG treatment) were 37 °C 10 min; 98 °C 1 min; 50 cycles of 98 °C 10 s, 57.5 °C 20 s, 72 °C 20 s; and 72 °C 5 min.

PCR amplification of ERBB2 exons 17 and 20, and EML4-ALK included primers 5'-/P/CCTGAGTCATC-CATC-3', 5'-C*C*C*CCAGGAGGCTATG-3', and 5'-/P/CGCTCCTGTCATGATTTCT-3'. Cycling conditions (including UDG treatment) were 37 °C 10 min; 98 °C 1 min; 50 cycles of 98 °C 10 s, 57.5 °C 15 s, 72 °C 15 s; and 72 °C 5 min.

After PCR, 40 µL of PCR mix was added to 50 µL of proteinase K mix in a total volume of 90 µL. Proteinase K mix included 8 µL 5x A7 buffer (50 mM Tris–acetate pH 8.0, 125 mM potassium acetate, 25 mM magnesium acetate, 0.5% Triton X-100), 2.25 µL Proteinase K (P6107S, New England Biolabs) and 39.75 µL nuclease free water. Reactions were incubated at 55 °C for 5 min followed by heat inactivation at 95 °C for 10 min.

**Pyrophosphorolysis reaction.** 7.5 µL pyrophosphorolysis (PPL) mix was prepared by mixing 0.5 µL of 20 × BFF buffer (200 mM tris–acetate pH 7.0, 600 mM potassium acetate, 342.5 mM magnesium acetate, 2% (100 mM, B1003, New England Biolabs) and 342.5 mM magnesium acetate, 2%}

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| Targeted NGS | Allele-specific qPCR | dPCR | ASPYRE |
|--------------|----------------------|------|--------|
| Sensitivity (%) | <0.1 | ~1 | <0.1 | <0.1 |
| Multiplexing | 1000+ | <20 | <6 | 50+ |
| Requires prior knowledge of variant | No | Yes | Yes | Yes |
| Instrument complexity | High | Low | Medium | Low |
| Laboratory workflow complexity | High | Low | Medium | Low |
| Data analysis complexity | High | Low | Low | Low |
| Time to result | 1–5 days | 2 h | 2 h | 4 h |
| Cost | High | Low | Medium | Low |

Table 1. Comparison of most frequently used analysis methods with ASPYRE assay.
were intact was 0.54 for fragment length) averaged over the fragment length distribution. The estimated fraction of input molecules that uniformly distributed. We therefore estimated the fraction of intact fragments using max(0, 1 - amplicon length/amplicon, and the fragmentation profile of the input DNA. We assumed that fragmentation breakpoints were fraction of input molecules, which include both PCR primer sites, was estimated using the length of the target genome. The target copy number was 2 for diploid targets but can be altered by copy number variation. The intact µ = (number of haploid genomes) × (target copy number/2) × VAF × (intact fraction of input molecules). The

Data analysis. Quantification cycle (Cq) values were identified using the CFX Maestro software (Bio-Rad). Fluorescence data and Cq values were exported from CFX Maestro software into MS Excel for subsequent analysis. Data were plotted using Prism8 software. Statistical analyses were performed using Python statsmodels and scpy.

We estimated the mean number µ of variant molecules that can be amplified by PCR in each reaction using: µ = (number of haploid genomes) × (target copy number/2) × VAF × (intact fraction of input molecules). The number of haploid genomes was estimated from the input DNA mass, assuming that 3.3 pg is one haploid genome. The target copy number was 2 for diploid targets but can be altered by copy number variation. The intact fraction of input molecules, which include both PCR primer sites, was estimated using the length of the target amplicon, and the fragmentation profile of the input DNA. We assumed that fragmentation breakpoints were uniformly distributed. We therefore estimated the fraction of intact fragments using max(0, 1 - amplicon length/fragment length) averaged over the fragment length distribution. The estimated fraction of input molecules that were intact was 0.54 for EML4-ALK, 0.61 for ERBB2 exon 17 and 0.69 for ERBB2 exon 20.

To estimate the limit of detection, we performed probit regression on log(µ), as recommended by BloodPAC28. We also compare our assay to an ideal assay that always generates a positive signal in presence of one, or more, amplifiable variant molecule(s). Under this condition, the assay sensitivity is dependent on Poisson sampling and can be estimated using the mean number of variant molecules that can be amplified by PCR, giving sensitivity = 1 - exp(- µ).

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Author contributions

B.B. & C.F. conceived the original project, designed and supervised experiments and analyzed data. A.-L.S. P.K.P. and M.S. designed and performed experiments, and analyzed data. R.N.P. analysed data. B.H. conceived original project. A.-L.S, P.K.P., M.S. & E.R.G. drafted the paper. All authors contributed to the editing and final version of the manuscript.

Competing interests

The authors are or have been employed by Biofidelity, Ltd. a privately held company and may hold stock or stock options. B.H is employed by Agilent Technologies Inc, a shareholder in Biofidelity. Biofidelity has filed provisional applications on aspects of this research (WO/2020/016590). The authors are or have been employed by Biofidelity, Ltd. a privately held company and may hold stock or stock options. B.H is employed by Agilent Technologies Inc, a shareholder in Biofidelity. Biofidelity has filed provisional applications on aspects of this research (WO/2020/016590).

Additional information

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Correspondence Correspondence and requests for materials should be addressed to B.W.B.

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