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Minimizing Sample Failure Rates for Challenging Clinical Tumor Samples

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Identification of somatic variants in cancer by high-throughput sequencing has become common clinical practice, largely because many of these variants may be predictive biomarkers for targeted therapies. However, there can be high sample quality control (QC) failure rates for some tests that prevent the return of results. Stem-loop inhibition mediated amplification (SLIMamp) is a patented technology that has been incorporated into commercially available cancer next-generation sequencing testing kits. The claimed advantage is that these kits can interrogate challenging formalin-fixed, paraffin-embedded tissue samples with low tumor purity, poor-quality DNA, and/or low-input DNA, resulting in a high sample QC pass rate. The study aimed to substantiate that claim using Pillar Biosciences oncoReveal Solid Tumor Panel. Forty-eight samples that had failed one or more preanalytical QC sample parameters for whole-exome sequencing from the Australian Translational Genomics Centre's ISO15189-accredited diagnostic genomics laboratory were acquired. XING Genomic Services performed an exploratory data analysis to characterize the samples and then tested the samples in their ISO15189-accredited laboratory. Clinical reports could be generated for 37 (77%) samples, of which 29 (60%) contained clinically actionable or significant variants that would not otherwise have been identified. Eleven samples were deemed unreportable, and the sequencing data were likely dominated by artifacts. A novel postsequencing QC metric was developed that can discriminate between clinically reportable and unreportable samples. *(J Mol Diagn 2023, 25: 263—273; https://doi.org/10.1016/j.jmoldx.2023.01.008)*

Identification of somatic variants in cancer by high-throughput sequencing has become common clinical practice because many of these variants may be predictive biomarkers for targeted therapies or have diagnostic or prognostic relevance. However, there can be high sample quality control (QC) failure rates (up to approximately 45% for some tests) preventing the return of results, which may affect patient treatment decisions.[1—8] Clinical samples, especially in the case of solid tumor testing, are usually formalin-fixed, paraffin-embedded tissue (FFPET) sections, and these are known to be challenging due to the damaging effect of formalin on nucleic acids, small biopsy specimen size, and/or low tumor cell content in the tested specimen.[9—12] Although hybrid-capture—based next-generation sequencing (NGS) tests are valued as clinical tests because of their sensitivity and uniformity of coverage of targeted genomic regions, they require large amounts of high-quality DNA (usually $\geq$50 ng) as input to achieve a successful test result. These requirements result in higher sample QC failure rates for hybrid-capture methods.[13—17] Amplicon-based NGS tests are generally more successful at testing challenging clinical samples than hybrid-capture

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methods because of lower DNA input requirements. However, these tests are still vulnerable to poor-quality DNA.

Pillar Biosciences (Natick, MA) has patented stem-loop inhibition mediated amplification (SLIMamp) technology and incorporated this into commercially available amplicon-based NGS cancer testing kits specifically to overcome the input DNA challenges. The claim is that these kits can successfully interrogate FFPET samples with poor-quality DNA and/or low-input DNA amounts, resulting in a higher sample QC pass rate than either hybrid-capture methods or conventional amplicon-based sequencing methods, as discussed previously.[18–21] In addition, SLIMamp enables the enrichment of target amplicons tiled across long genomic regions, not just hotspots, to allow sequencing of multiple entire gene coding regions in an automatable, highly multiplexed single reaction tube.[22] This approach provides a promising alternative to hybrid-capture methods due to the ability to interrogate entire genes from difficult samples, a feature that conventional amplicon struggle to offer without amplicon dropout. This was originally shown by using 5 to 100 ng of input DNA from both clinical samples and reference standards for the coding sequences of *BRCA1* and *BRCA2*; however, an exploration of the effectiveness of SLIMamp tests on truly challenging clinical samples has not yet been published.

The aim of the current study was to verify the claim of Pillar Biosciences that SLIMamp technology can successfully test challenging samples using their amplicon-based NGS oncoReveal Solid Tumor Panel (STP) test. Forty-eight samples that had failed one or more preanalytical QC sample metrics for comprehensive genome profiling (CGP) by either whole-exome or Illumina TSO500 panel sequencing from the Australian Translational Genomics Centre (ATGC), an ISO15189-accredited diagnostic genomics laboratory, were identified and provided to XING Genomic Services (XGS; Sinnamon Park, Queensland, Australia). CGP testing of these patient samples had been requested by treating clinicians but was not performed due to poor quality of the samples. XGS performed an exploratory data analysis using preanalytical QC metrics specific to the STP test to further characterize the quality of samples and then tested all samples in their ISO15189-accredited laboratory using the STP test that had been previously analytically and clinically validated.

Materials and Methods

ATGC Patient Samples

Forty-eight extracted DNA samples were provided by ATGC for use in this study. Table 1 details the study population characteristics. Whole-exome sequencing testing had been requested for 44 samples, and TSO500 DNA testing had been requested for four samples. Forty-seven samples were derived from FFPET, and one sample was derived from blood. Samples were considered by ATGC to have failed CGP preanalytical QC metrics if they yielded <260 ng DNA and/or quality below DNA Integrity Number (DIN) 3.6 and average fragment size <3600 bp (as determined by using the TapeStation genomic DNA assay; Agilent Technologies, Mulgrave, VIC, Australia). Samples were only considered for inclusion in this study if the patient had previously consented to involvement in research studies.

DNA Extraction and QC

ATGC solid tumor samples were macrodissected from slides and deparaffinized by using xylene and ethanol. DNA lysate from FFPET was extracted, with an added uracil-N-glycosylase step from the GeneRead DNA FFPE Kit (catalog number 180134; Qiagen, Clayton, VIC, Australia) to reduce potential cytosine deamination artifacts, using an automated QIAsymphony DSP DNA Mini Kit (catalog number 937236; Qiagen) according to the TLC200 protocol. DNA was extracted from the blood sample on the automated QIAsymphony DSP DNA Mini Kit (catalog number 937236; Qiagen) according to the B200 protocol. DNA samples were then quantified by using the Qubit 1X dsDNA HS Assay Kit (catalog number Q33231, Thermo Fisher Scientific, Scoresby, VIC, Australia). FFPET-derived DNA was also qualified on the TapeStation genomic DNA assay (catalog numbers 5067-5365 and 5067-5366; Agilent) to determine DIN and average fragment size.

ATGC provided extracted DNA to XGS. If enough DNA was available for both quantification and testing, according to the concentration and amount provided by ATGC, total DNA concentration was confirmed by XGS using the Qubit dsDNA assay.

The QIAseq DNA QuantiMIZE kit (catalog number 333414; Qiagen) was used to quantify and qualify amplifiable DNA. This kit uses two qPCR assays that interrogate 40 genomic loci to determine the amounts of amplifiable DNA fragments in a sample. Briefly, samples or control genomic DNA were mixed with a qPCR master mix and QuantiMIZE primer pairs. Real-time PCR was performed according to the manufacturer's instructions, and C_T values were analyzed to determine concentration and absolute quantities of amplifiable DNA. Samples were tested in triplicate.

Library Preparation

The oncoReveal Solid Tumor Panel (STP) kit (catalog number HDA-HS-1005-24; Pillar Biosciences) is a multigene test that targets hotspot variants considered to be driving events in solid tumors; it is recommended for use with colorectal, melanoma, thyroid, non–small-cell lung, and pancreatic cancers, as well as gastrointestinal stromal tumors and gliomas. It covers 23,895 bases across regions of interests in 47 genes, including *AKT1*, *ALK*, *ARAF*, *BRAF*, *CDKN2A*, *CTNNB1*, *CYSLTR2*, *DDR2*, *EGFR*, *E1F1AX*, *ERBB2*, *ERBB4*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *GNA11*, *GNAQ*, *GNAS*, *H3F3A*, *HIST1H3B*, *HRAS*, *IDH1*,

Table 1 ATGC Study Population Characteristics

Characteristics	Samples ($N = 48$)	
	n	%
Sex		
Male	32	67
Female	16	33
Diagnosis		
Colorectal cancer	7	15
Lung cancer	7	15
Prostate cancer	6	13
Gastric cancer	3	6
Neuroendocrine carcinoma	3	6
Squamous cell carcinoma	3	6
Breast cancer	2	4
Leiomyosarcoma	2	4
Osteosarcoma	2	4
Pancreatic cancer	2	4
Thyroid cancer	2	4
Adenoid cystic carcinoma (salivary)	1	2
Adrenal cortical carcinoma	1	2
Angiosarcoma	1	2
Leukemia	1	2
Liposarcoma	1	2
Neuroblastoma	1	2
Parathyroid carcinoma	1	2
Perineuroma	1	2
Renal cancer	1	2

Diagnoses fit for the Solid Tumor Panel test are shown in bold. Even though all ATGC samples were tested with this panel, it is only designed to be relevant to a subset of samples in this study.

IDH2, *KIT*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, *NTRK1*, *PDGFRA*, *PIK3CA*, *PLCB4*, *POLD1*, *POLE*, *PTEN*, *PTPN11*, *RAC1*, *RAF1*, *RET*, *SF3B1*, *SMAD4*, *SRSF2*, *STK11*, the *TERT* promoter, *TP53*, and *TSHR*.

The STP kit was used to prepare amplicon libraries for all 48 ATGC samples. If total library input DNA was estimated to be <20 ng, libraries were prepared directly from the material in the provided tube. In two cases, a tube appeared to contain no liquid, and thus the library was prepared from 6.25 μL of water that was used to wash the inside of the tube to solubilize any DNA that may have been present. Libraries were prepared according to the manufacturer's instructions. Briefly, each region of interest was amplified by using gene-specific primers in the first round of PCR, after which excess primers were digested, and the PCR products were purified via size selection. Next, index adaptors were added to each library for sample tracking. This mix was further amplified (ie, indexing-PCR) and purified. The final libraries were then quantified by using the Qubit assay and normalized for sequencing.

Sequencing

Libraries were sequenced on a NextSeq 550 (Illumina, Melbourne, VIC, Australia). Because some of these samples were sequenced in batches with routine clinical samples, a uniform level of coverage across all samples was not achieved. However, a minimum of 3500× mean coverage was required, as per test validation, for a sample to pass coverage-based QC.

Short Read Alignment and Variant Detection

Short read alignment, coverage analysis, and variant calling were performed by using the PiVAT bioinformatics platform (version 2020.2.2) of Pillar Biosciences with default settings. Variants called by PiVAT were filtered to remove those with a variant allele frequency (VAF) of <3%. The validated limit of detection (LOD) of the STP test as implemented by XGS is 5% based on a minimum tumor content of 20%, although variants with VAFs between 3% and 5% may be reported if coverage depth and tumor content are sufficiently high. Variants with <200× coverage were also removed before variant annotation and interpretation. Samples were considered to pass postsequencing QC if they had a mean coverage of at least 3500× with a minimum 98% of targeted regions receiving at least 200× coverage.

Clinical Reporting

Samples were considered to be reportable using expert interpretation of the sequencing data in the context of associated sample QC metrics and diagnostic information. Because this study was an exploratory analysis, criteria that would discriminate between a reportable sample and an unreportable sample were not specified a priori.

Additional Data

QuantiMIZE data previously generated by XGS from an additional 47 solid tumor specimens acquired during routine patient testing or clinical test validation were included with QuantiMIZE data from the ATGC samples to characterize the effect on amplifiable FFPET-derived DNA concentration on SLIMamp kit-based test results. A total of 46 of the 47 additional samples had been successfully tested with either the oncoReveal HRD or oncoReveal BRCA1 and RAD51C methylation, both of which are SLIMamp-based kits (catalog numbers HDA-HR-1003-96 and HDA-HR-1005-96; Pillar Biosciences), and one sample was considered to have failed testing. One sample was derived from a 43-year-old FFPE block, while the other FFPET samples were between 3 months and 11 years old, relative to the test date (Supplemental Figure S1). In addition, the Horizon Discovery Quantitative Multiplex Reference Standards for Formalin-Compromised DNA (mild, moderate, and severe) were tested with the QuantiMIZE assay to characterize the extent of formalin-related DNA degradation on amplifiable DNA concentration (catalog numbers HD798, HD799, and HD803; MetaGene, Pty. Ltd., Brisbane, QLD, *Australia*).

Data Analysis of QC Metrics and Variant Attributes

Data were analyzed by using R version 4.1.2. (R Foundation for Statistical Computing, *https://www.R-project.org*, last accessed November 1, 2021). Single nucleotide variant base transition and transversion analysis was performed by using the maftools R package, version 2.10.0.[23] Feature selection analysis of attributes describing the samples was performed by using the Boruta R package, version 7.0.0.[24]

Results

Sample Presequencing QC Metrics

ATGC preanalytical QC criteria were input DNA ≥260 ng, a DIN of at least 3.6, and average fragment size of >3600 bp. A total of 40 of the 48 samples that were provided for this study failed at least two of these criteria. Only two samples were considered to fail QC based on low-input DNA alone. Five samples had a DIN of at least 3.6 (passing QC) while the rest were qualified as having very fragmented DNA, likely due to either, or a combination of, formalin fixation and attributes of the specimen that make it challenging for clinical testing (eg, fibrous tissue, calcified tissue). One sample passed all QC criteria and testing proceeded with the TSO500 test, but the sample did not generate a library of sufficient quality for sequencing and was hence included in this study. Supplemental Table S1 presents all QC metrics, where known, for the 48 ATGC samples tested here.

According to the manufacturer's instructions, the STP test requires 10 to 75 ng of input DNA, although the range that was previously used to validate this kit for clinical use was 5 to 20 ng. In this study, 20 ng of input DNA was used for library preparation, when possible. Twenty-one of the 48 ATGC samples tested contained <10 ng input DNA; 13 samples contained <5 ng or did not have enough DNA to estimate the DNA concentration or amount (Supplemental Table S1). For the latter cases, 6.25 μL of the tube contents was used or 6.25 μL of water that had been added to the tube if it appeared empty was used. After library preparation with the STP kit, the concentration of the library was measured and was considered to pass QC with a concentration ≥2 ng/μL. Twelve samples had a library concentration <2 ng/μL. Five samples had both <5 ng input DNA and a library concentration <2 ng/μL.

Because there is no stated requirement for a specific level of DNA integrity for the SLIMamp kits, a study using the QuantiMIZE assay was performed in an attempt to determine a threshold of amplifiable DNA concentration that could serve as a potential preanalytical QC metric. Only 39 of the 48 ATGC samples tested here had sufficient DNA to permit QuantiMIZE testing; these were therefore supplemented with 47 additional HRD samples from XGS's routine testing service, as well as three Horizon Discovery formalin-compromised DNA reference standards, totaling 89 samples. Figure 1 shows the distribution of amplifiable DNA concentrations in the combined ATGC and XGS samples that were considered to be either unreportable ($n = 2$) or reportable ($n = 87$) based on the postsequencing QC metric developed as part of this study (described in *Postsequencing QC Metrics*). The QuantiMIZE concentrations for the Horizon Discovery standards (mild, moderate, and severe formalin-compromised DNA) are shown for reference. Notably, almost 40% of the samples tested with the QuantiMIZE assay were estimated to be poorer quality than the severe formalin-compromised reference standard.

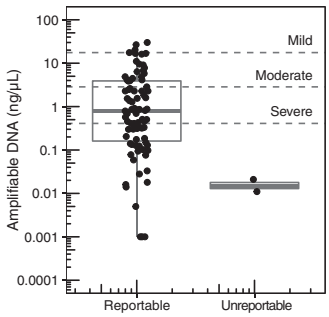

Figure 1 QuantiMIZE amplifiable DNA concentrations and sample reportability. Amplifiable DNA concentrations were determined by using the QuantiMIZE kit for 39 ATGC samples included in this study, as well as 47 additional samples that were tested in routine XGS clinical practice and three reference standards. The QuantiMIZE concentrations of the Horizon Discovery formalin-compromised reference standards (mild, moderate, and severe levels of formalin-induced DNA degradation) are shown as **horizontal gray dotted lines**. Only two samples occur in the unreportable group. The sample XMP-0092-21 is the only unreportable ATGC sample for which the QuantiMIZE assay was performed. The other unreportable sample was from routine XGS clinical testing with the oncoReveal HRD test.

Postsequencing QC Metrics

After sequencing and primary and secondary bioinformatics analysis, a number of potential QC metrics were explored to determine if sample reportability could be determined after sequencing but before issuing a clinical report. Coverage metrics of the STP target regions were considered first, including mean coverage, the percentage of targeted regions covered by at least 200 reads, and the overall percentage of on-target reads. Two samples did not meet the minimum mean coverage of 3500× (although they were both >3000×); five samples did not have at least 98% of targeted regions covered by a minimum of 200 reads; and 15 samples had an on-target rate <95% (Supplemental Table S1).

The number of variants called after filtering in the ATGC samples (as described in *Materials and Methods*) (Figure 2) was also considered because it seemed there was a very broad range of variant counts across all samples. Ten of the 48 ATGC samples had noticeably more variants than the other samples. Samples with a relatively high number of variants were more difficult to interpret. The VAF distribution was also very different across all samples, and samples with a high number of variants frequently had a

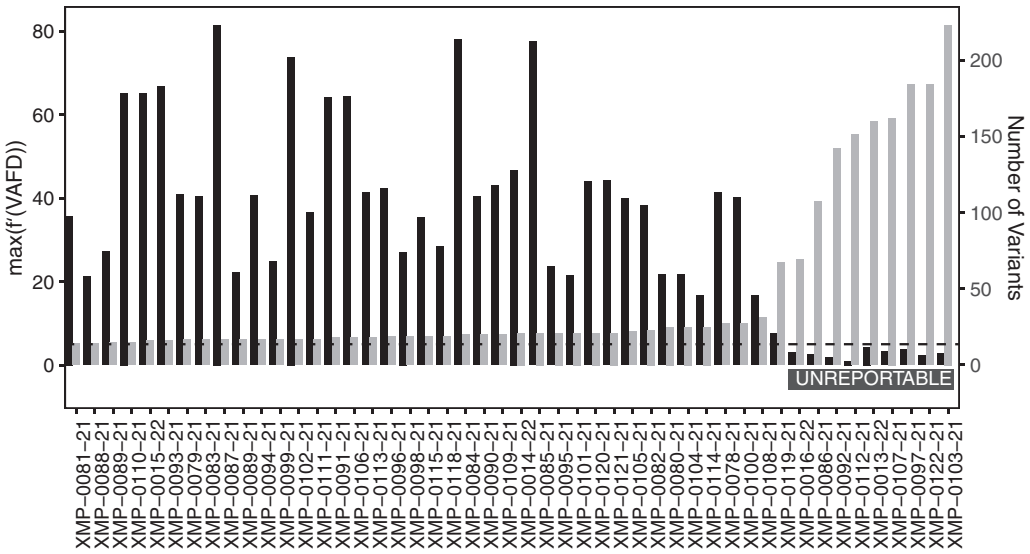

Figure 2 Number of variants called and the maximum value of the first derivative of the density curve, referred to here as max[f′(VAFD)]. The number of variants called per ATGC sample is shown in **light gray bars**. The max[f′(VAFD)] value, which is based on both the number of variants called and the allele frequencies, is shown as **dark gray bars**. Samples with max[f′(VAFD)] <5% (**horizontal black dashed line**) were considered to be unreportable.

large proportion of variants with low VAFs. The VAF distribution of the ATGC samples was quantitatively characterized by determining the kernel density estimation of all VAFs in a sample. Examples of VAF density from samples with either moderate or poor DNA quality are presented in Figure 3 as light gray areas; all ATGC samples are shown in Supplemental Figure S2, including XMP-0014-22, which represents good-quality DNA.[25,26]

When comparing the VAF densities of the reportable and unreportable groups of samples, a pattern emerged whereby the unreportable samples had a peak density at a very low VAF, and the reportable samples had densities that were more evenly spread across the entire range of VAFs. To create a single value that could capture the nature of these different VAF density patterns and potentially serve as a QC metric with a threshold value, the first derivative of the density curve (f′(VAFD)) was calculated. The maximum value of that derivative, referred to here as max(f′(VAFD)), was then identified; examples of the f′(VAFD) are shown in Figure 3 as black lines. Nine ATGC samples, all unreportable, had a max(f′(VAFD)) that was below the validated LOD for the STP test (5% VAF), which means that the vast majority (but not all) of the variants in those samples were present at VAFs below the test LOD (5%). The max(f′(VAFD)) values for the reportable ATGC samples occur at VAFs ranging from 16% to 78%; examples of the max(f′(VAFD)) are shown in Figure 3 as vertical dash-dotted gray lines.

Because these samples had been preserved in formalin, and formalin fixation induces C-to-T transitions with a specific molecular signature[27] (COSMIC Signature SBS30), the distribution of base changes in each sample was investigated to determine if this signature was closely associated with samples that were unreportable. Figure 4 presents the transition and transversion distributions for the ATGC samples, split into unreportable and reportable sample groups. There was indeed a clear difference between the groups, with the unreportable samples having a broad range of percent C-to-T transitions as well as different base change proportions across the other possible transitions and transversions.

Variant Interpretation

Clinical interpretation of the variants called by using PiVAT was more difficult than usual because access to the patients' histopathology reports was not available, and the specimens could not be assessed directly. Only eight ATGC specimens had been annotated with estimated tumor cell content (10% to 60%). Two samples originated from the same specimen (XMP-0089-21) but were independent DNA extractions; one sample did not meet the minimum coverage threshold while the other one did, but both samples were found to have the same clinically significant variant, and no additional variants of significance were present only in the sample with high coverage. The STP test is intended for a specific group of solid tumors (see *Materials and Methods*), and 21 ATGC samples were received that normally would not be tested with this kit as the targeted regions were not designed to inform those diagnoses (Table 1). For the ATGC patients whose diagnoses were not fit for the STP test, 14 of 21 were reportable; 6 of 14 had no clinically significant variants; 4 of 14 had one or more pathogenic *TP53* variants; 3 of 14 had one or more pathogenic *TP53* variants as well as other clinically relevant variants; and 1 of 14, a neuroendocrine tumor of unspecified origin, had only an *EIF1AX* variant that was considered to be pathogenic and has been observed in a number of cancers, although it was

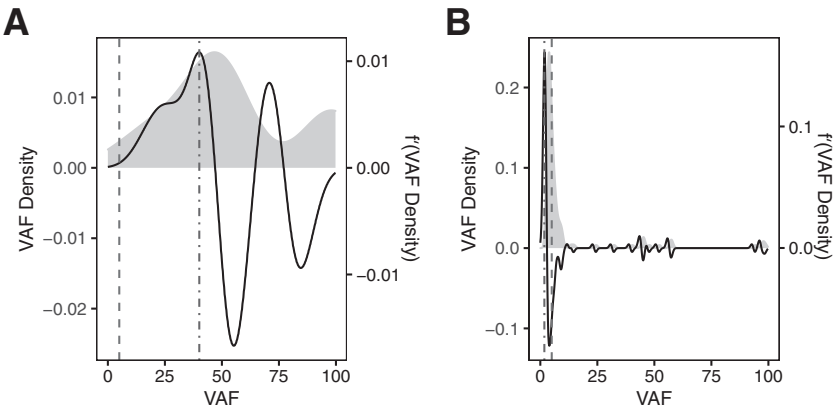

Figure 3 Variant allele frequency (VAF) density and the first derivative of the density curve [f′(VAF Density)]. Single ATGC samples representing moderate- and poor-quality DNA are shown here. The *x* axis is the percent VAF. The estimated VAF kernel density is shown in the **light gray area** with the range of *y*-values shown on the first *y* axis (left side). The f′(VAFD) is shown by the **black line** with the range of values on the second *y* axis (right side). The VAF limit of detection (5%) is shown by the **vertical dotted gray line**, which is also the threshold for sample reportability. The maximum value of that derivative, referred to here as max[f′(VAFD)], is shown by the **vertical dash-dotted gray line**. **A:** Moderate-quality DNA, represented by XMP-0100-21. This sample has 27 variants with 48% C>T. **B:** Poor-quality DNA, represented by XMP-0092-21. This sample has 142 variants with 80% C>T.

not previously established as a known driver of carcinogenesis in this tumor type (Supplemental Table S1).[28]

Resequencing of Unreportable Samples

To determine if the variants found by the STP test in the unreportable ATGC samples were sequencing artifacts or true variants, new sequencing libraries were generated from extracted DNA for the six samples that had sufficient remaining DNA (XMP-0092-21, XMP-0103-21, XMP-0112-21, XMP-0119-21, XMP-0122-21, and XMP-0016-22). The new libraries were re-sequenced from the same amount of input DNA as the original test, and the variants called in both the original data and re-sequenced data were compared. In all cases, the original and re-sequenced libraries had more variants that were unique to each library than variants that they shared (Supplemental Figure S3). For all samples except one, both the original and re-sequenced libraries were considered to be unreportable (Supplemental Figure S4). Sample XMP-0119-21 was the only originally unreportable sample that was determined to be reportable from a re-sequenced library. This sample, from a lung adenocarcinoma, had a pathogenic *KRAS* p.(Gln61His) in both the original and re-sequenced data at 10% and 7%, respectively. The reportable re-sequenced data also had a pathogenic *BRAF* p.(Val600Glu) at 5% and a likely pathogenic *PTEN* p.(Pro96Arg) at 5.3% that were not present in the unreportable sample.

Clinical Reporting

Clinical reports were confidently generated for 39 (81%) of 48 samples, of which 29 (60%) contained clinically actionable or clinically significant variants. Of these 29, the presence of mutated *EGFR*, established by other methods, was stated on the original whole-exome sequencing test request form for two samples (XMP-0095-21: "*EGFR*-positive lung adenocarcinoma"; and XMP-0101-21: "*EGFR* mutant lung cancer"). These observations were concordant with variants found by using the STP test [XMP-0095-21: *EGFR* p.(Glu746_Ala750del); and XMP-0101-21: *EGFR* p.(Glu746_Ser752delinsVal)]. For the other 27 (56%) patients, the STP test found clinically actionable or clinically significant variants that would not have otherwise been identified.

Discussion

A common limitation of genetic testing of clinical FFPET samples to identify predictive biomarkers for targeted therapies is the high preanalytical QC failure rate of tests requiring large quantities and high-quality input DNA, resulting in many patients having no test result or requiring a repeat biopsy. Moreover, sample failure affects clinical trial recruitment, leading to more patients being screened and fewer patients with successful paired biopsy specimens required for predictive biomarker studies. Sample failures, therefore, add considerably to the cost and time required to complete trials and affect their probability of success.

The SLIMamp technology of Pillar Biosciences promises to reduce sample failure rates by generating reportable and clinically useful results from samples with low amounts of DNA and/or poor-quality DNA. This approach is an effective compromise between no testing and comprehensive testing and could be used in clinical practice and clinical trials as a reflex test when a sample proves to be unsuitable for, or fails, CGP. Although CGP testing could still be performed, regardless of whether the sample passes QC, the cost of a failed test may not be considered an acceptable risk. CGP test costs tend to be at least AU$1000, and usually significantly more. In addition, the workflow is longer than amplicon testing, and thus the time from sample receipt to test result is usually at least 2 weeks. If the sample fails testing, and a different test is performed, even more time is lost. SLIMamp tests may provide an attractive alternative to

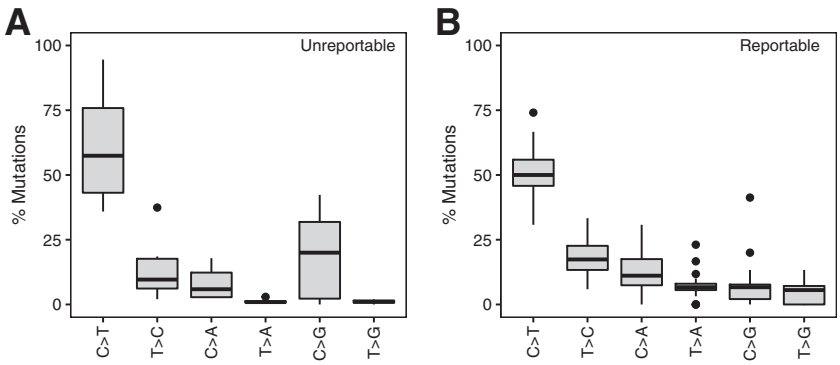

Figure 4 Single nucleotide variant base change distributions. The percentage of mutations of each type of base change is shown for the two sample groups of the ATGC samples. **A:** Base change distributions for unreportable samples. **B:** Reportable samples.

manage cost and turnaround time risks when a sample is of questionable quality. However, because specific QC criteria describing samples that are suitable for SLIMamp-based testing and clinical reporting have not been rigorously characterized, the potential reduction level in sample failure rate is undefined. A clear definition of the characteristics of a reportable sample is desirable to determine which samples, and how many, fail CGP testing but could potentially be tested successfully with a SLIMamp-based test.

Sample Characterization and Classification

Forty-eight samples were acquired that were not tested with CGP due to failure of one or more preanalytical QC criteria, although one sample was included that passed all criteria but failed TSO500 post-library QC. Of these, 90% were considered untestable because of poor DNA integrity (DIN < 3.6). DIN is not a metric that XGS can measure in-house, and thus DNA quality was characterized by using the QuantiMIZE assay when possible. Before this study, the QuantiMIZE assay was performed on 50 routine XGS clinical samples, and the amplifiable DNA concentration was noted in an attempt to determine a threshold below which samples consistently failed testing, similar to that observed by Sekita-Hatakeyama et al[29] when performing amplicon-based NGS testing of pancreatic masses, as well as by others.[30–33] Thirty-nine of the samples tested as part of this study were also assayed with QuantiMIZE. Unlike the pancreatic mass study, in which samples clearly failed testing when the amplifiable DNA concentration fell below 1 ng/μL, samples tested with SLIMamp tests were successful even at concentrations of 0.001 ng/μL, which is the lower limit of the assay (Figure 1, Supplemental Figure S5). Notably, almost 40% of the samples tested with the QuantiMIZE assay were estimated to be poorer quality than the severe formalin-compromised reference standard. It seems plausible that the SLIMamp technology is indeed increasing testing suitability of incredibly poor-quality DNA as measured by either DIN or QuantiMIZE.

It is evident that DNA input amount is also not associated with testing success for the SLIMamp STP test (Supplemental Table S1). Pillar Biosciences recommends using a minimum of 10 ng of DNA for the STP kit, although the minimum amount that was used to clinically validate this test was 5 ng, and Barua et al[34] used a minimum of 2.5 ng. The lowest input DNA amount that was used for a reportable sample in this study was 1.25 ng (XMP-0093-21). No association was found between DNA input amount and sample reportability, although there is presumably a minimum amount below which not enough material is available for amplification.

After STP library preparation of the ATGC samples, the DNA concentration of the library was measured by using the Qubit assay. A concentration of at least 2 ng/μL was found to be closely associated with reportable samples; with a lower concentration (1 to 2 ng/μL), these tended to be unreportable. However, this threshold did not provide perfect discrimination, as three samples with concentrations below 2 ng/μL were still found to be reportable. Although preanalytical QC metrics, such as DNA integrity and input amount, did not predict reportability, the library concentration could potentially be used as a reasonable post-library QC metric to determine which samples should proceed to sequencing, noting that some reportable samples might be untested with this strategy.

Characterization of postsequencing attributes yielded more informative QC metrics than either presequencing or post-library metrics. The percentage of reads covering the regions targeted by the STP kit (on-target rate) is closely associated with reportable samples, with most of those samples having a rate <95%. However, several reportable samples were also below this threshold, showing that this metric is not perfectly predictive of reportability. The on-target rate is not necessarily the best attribute to describe sample reportability, however, because in many cases, higher coverage sequencing can compensate for lower on-target sequencing and a reportable result could still be generated, as was observed in four samples [XMP-0089-21 (1), XMP-0096-21, XMP-0104-21, and XMP-0108-21].

Because FFPET samples are known to harbor DNA with formalin-induced artifacts, the association between the percentage of C-to-T transitions and sample reportability was investigated. Both reportable and unreportable samples did indeed have a high percentage of variants that were C-to-T transitions, consistent with their exposure to formalin (Figure 4). However, the mean C-to-T transition percentage was similar in both groups, with a slightly higher mean value in the unreportable samples (51% and 59%, respectively). Reportable samples had less variability in this metric, whereas unreportable samples had a broader range of percent C-to-T transitions (31% to 74% and 36% to 95%). However, if this metric was examined for a single sample, it would not be possible to use it to distinguish between reportable and unreportable samples except at the extreme values because the range of observed values overlaps substantially in both sample groups. Interestingly, unreportable samples were associated with an increased percentage of C-to-G transversions (likely sequencing errors or PCR artifacts). T-to-A transitions and T-to-G transversions were almost entirely absent in the unreportable samples. Similar to the C-to-T transitions, the observed ranges of these values in both sample groups overlap, and thus they cannot be used to clearly discriminate between them.

One characteristic of unreportable samples that was readily observable was the increased number of total variants called relative to reportable samples. By simply counting the number of variants called in a sample, it seemed that reportable samples tended to have <50 total variants (Figure 3). By including the allele frequencies of the variants, a clearer pattern emerged. Samples with more than approximately 50 total variants tended to have more variants with very low allele frequencies. These were almost exclusively single nucleotide variants and not small insertions/deletions. As a result of this observation, the VAFs were investigated in more detail. To enable a comparison of the aggregated data (number and allele frequencies of variants), the kernel density of VAFs was used. This density estimate, which visually looks like a smoothed histogram, was estimated for all samples, and the density plots of reportable and unreportable samples were compared (Supplemental Figure S2). Unreportable samples displayed a marked difference from reportable samples in that the densities had a peak at a very low VAF with a long right tail. Reportable samples showed VAF peaks at allele frequencies >20% and, in most cases, >40%. The peak value is likely related to the tumor cell content present in the sample, although tumor cell content estimations were available for only eight samples, and therefore this relationship could not be explored more comprehensively. Sample XMP-0014-22, which passed all ATGC's preanalytical QC metrics but failed TSO500 library preparation, was estimated to contain <20% tumor cells. Indeed, it appears to have a VAF density that is reflective of a sample with mostly germline DNA content and little to no tumor DNA content (VAF peaks near 50% and 100% representing heterozygous and homozygous single nucleotide polymorphisms, respectively) (Supplemental Figure S2). Considered as a whole, these observations suggest that unreportable samples are characterized by a preponderance of variants with low allele frequencies; very few variants with high frequencies; and essentially no minor peaks of variants with VAFs >5%.

max(f′(VAFD)) as a QC Metric

To be able to use the aggregate VAF attributes as a simple QC metric, they needed to be translated into a binary classifier ("reportable sample" or "unreportable sample"). By applying a simple peak detection method, the VAF density could be characterized by a single value: the maximum value of the first derivative of the VAF kernel density curve, or max(f′(VAFD)) (Supplemental Figure S6). Furthermore, by setting a threshold for this value, the VAF LOD of the STP test (5%), perfect discrimination between reportable and unreportable samples was achieved. This metric can be calculated from a variant call format (VCF) file, or even just a list of VAFs, and therefore can be easily applied to NGS data.

The high number of variants and the preponderance of low VAFs in the unreportable samples were likely due to the quality of the DNA samples, as described by Wong et al.[35] Good-quality DNA is not highly fragmented and can be easily amplified by PCR. Sufficient copies of good-quality DNA fragments enable the creation of a complex sequencing library so that all regions of interest are well represented in the sequencing data. In addition, these DNA fragments likely contain fewer formalin-induced artifacts. Moderate-quality DNA is fragmented and contains some formalin-induced artifacts but is of sufficient quality for amplification, library creation, and sequencing. Very-poor-quality DNA is highly fragmented with more artifacts and, especially when DNA input amount is very limited, contains an insufficient number of amplifiable fragments such that the regions of interest are not adequately represented in the sequencing data. The samples tested in this study can thus be grouped into good/moderate-quality DNA (reportable samples) and poor-quality DNA (unreportable samples) by the max(f′(VAFD)), rather than by more intuitive attributes such as DIN or amplifiable DNA concentration. For example, the only ATGC sample in this study to pass all preanalytical QC metrics, XMP-0014-22, which was also considered to be reportable (Supplemental Figure S6), likely contained good-quality DNA as evidenced by every metric that was used to characterize it. It is still unclear why it failed TSO500 library preparation, but it did produce a good STP library and good sequencing data based on all post-analytical metrics, including the max(f′(VAFD)). Sample XMP-0100-21 failed all three of ATGC's preanalytical QC metrics but passed the XGS metrics, suggesting that this sample is likely representative of moderate-quality DNA.

The tumor cell content was estimated to be 30%, and indeed the VAF density reflects the presence of both germline and somatic variants (Figure 3A). It is helpful to compare both of these samples to XMP-0092-21, which failed every QC metric [including max(f′(VAFD))], except for minimum coverage (Figure 3B), in order to understand how the sample attributes differ. This sample likely contained very-poor-quality DNA with a low number of amplifiable fragments as well as a large proportion of formalin-induced artifacts, as the base changes in the called variants were 80% C-to-T transitions. Variants called in this sample are most likely all formalin- or sequencing-related artifacts and not true somatic or germline variants. This sample, and other samples in this study with a max(f′(VAFD)) <5%, can therefore be considered samples that are true failures based on samples of very poor quality. Libraries and sequencing data can be generated in these cases, but the data cannot be reliably interpreted to generate a safe result for the patient.

Sample XMP-0119-21 is particularly interesting as this is the only sample that was originally considered to be unreportable but was considered to be reportable upon resequencing from a new library with the same amount of input DNA (Supplemental Figure S4). Clinically significant variants called above the LOD in the reportable library include *KRAS* p.(Gln61His) at 6.8% VAF, *PTEN* p.(Pro96Arg) at 5.3%, and *BRAF* p.(Val600Glu) at 5% VAF. The *KRAS* p.(Gln61His) was called at 9.9% in the unreportable library, but the other two variants were not. Although *BRAF* and *KRAS* variants are usually mutually exclusive, there is evidence that they can co-occur and that co-occurrence can affect treatment outcomes.[36] The low VAFs suggest that the tumor content of this sample was very low. The difference in reportability between the two libraries emphasizes the stochastic nature of the DNA present in the sample. Only 1 ng DNA was used to create each library. With such a low amount, it is possible that not enough informative DNA fragments were selected for amplification from the first attempt and, by chance, more informative fragments were present in the second attempt, which resulted in a better sequencing library, although the on-target rate and post-library concentration were both low (Supplemental Table S1).

SLIMamp Test QC Metric Recommendations

None of the preanalytical QC metrics that were explored in this study could reliably predict sample reportability for SLIMamp-based tests. The post-library preparation QC metric, library DNA concentration, was the earliest point in the STP testing process that any indication of sample reportability became evident. However, this metric was still not a perfect indication and, if used to classify samples as reportable or unreportable for testing, many reportable samples could be missed. The postsequencing max(f′(VAFD)) metric, however, did provide perfect discrimination and could be easily calculated, and, compared with all other potential metrics evaluated in this study (for those without missing values), this single metric had the most importance in correctly classifying sample reportability (Supplemental Figure S7). This finding suggests that application of the max(f′(VAFD)) metric after sample testing is the most useful QC metric for identifying safely reportable samples using SLIMamp-based tests.

The notion of ignoring all preanalytical QC metrics and testing every sample regardless of how poor its quality intrinsically directly contradicts regulatory preexamination guidelines for accredited diagnostic laboratories. These guidelines exist specifically to ensure that a quality standard is in place to prevent patient harm that could occur as a result of erroneous or overinterpreted test results. However, if the QC step is shifted from preexamination to post-examination, the quality standard is maintained, but the possibility of returning clinical reports for, as observed here, almost 80% of the samples that would otherwise not be tested is created. This can allow more patients to receive a timely test result and potentially avoid an additional invasive biopsy. Because of the evident robust and sensitive nature of the SLIMamp kits, this metric may not apply to other amplicon-based NGS tests or, if it does apply, the threshold may need to be adjusted based on empirical observations. Applying this metric to tests outside of those used in routine practice at XGS is prohibitively difficult because failed samples are intentionally excluded from shared data, and acquiring privately held failed sample data is a sensitive and difficult issue.

In summary, high sequencing coverage (>3000×) was ultimately achieved for all 48 samples tested here, and a QC metric that characterizes a successfully tested and clinically reportable sample was established. Clinical reports were generated for 37 (77%) samples, of which 29 (60%) contained clinically actionable or significant variants that would not otherwise have been identified. Sequencing reads for nine unreportable samples likely comprised stochastically amplified DNA with formalin-induced or sequencing-related artifacts. It was shown that the SLIMamp technology was able to successfully test samples that could not be tested by hybrid-capture sequencing or potentially, at least in some if not all cases, conventional amplicon-based NGS methods, noting that other methods are less robust to poor-quality DNA. SLIMamp kits, in combination with a post-sequencing QC metric based on the VAF distribution, enable clinical testing for predictive biomarkers on a large proportion of the most challenging clinical samples. Although amplicon-based NGS testing is not a replacement for CGP, it is clearly a valuable tool for reflex testing, particularly when pathology assessment of a sample suggests that it may not pass preanalytical QC metrics for CGP tests. It is worth noting that although the STP test used in this study is designed to enable calling of only single nucleotide variants and small insertions/deletions, amplicon-based tests that can infer copy number changes or fusion events do exist; however, these amplicon-based tests are

designed to identify specific events and cannot enable discovery of more complex types of genetic variation.

Acknowledgments

ATGC patients signed research consent forms before sample testing. The ethics application was approved by the Queensland Health Metro South Human Research Ethics Committee (HREC/17/QPAH/732).

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2023.01.008*.

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